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Choline and Cholinergic Neurons

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Summary. Mammalian neurons can synthesize choline by methylating phosphatidylethanolamine and hydrolyzing the resulting phosphatidylcholine. This process is stimulated by catecholamines. The phosphatidylethanolamine is synthesized in part from phosphatidylserine; hence the amino acids methionine (acting after conversion to *S*-adenosylmethionine) and serine can be the ultimate precursors of choline. Brain choline concentrations are generally higher than plasma concentrations, but depend on plasma concentrations because of the kinetic characteristics of the blood-brain-barrier transport system. When cholinergic neurons are activated, acetylcholine release can be enhanced by treatments that increase plasma choline (for example, consumption of certain foods).

Choline is present in membranes of all cells, where it constitutes the polar subunit of lecithin [phosphatidylcholine (PC)], sphingomyelin, and plasmalogens. Within cholinergic neurons, free choline is also the immediate precursor of the neurotransmitter acetylcholine (ACh), and its concentration can influence the rates at which the ACh is synthesized (*1*) and released (*2–4*).

Until a few years ago, the free choline molecules in the brain and the bloodstream were generally believed to constitute separate metabolic pools, the positive charge of choline's quaternary nitrogen precluding its passage across the blood-brain barrier (BBB) (*5, 6*). Since it was also thought that brain cells were incapable of synthesizing choline (*5–7*), the fact that neurons contained sufficient choline to maintain ACh synthesis was attributed to either an extraordinarily efficient reuptake mechanism (*8*)—which enabled them to reutilize almost all of the choline formed intrasynaptically from ACh hydrolysis (Fig. 1)—or to the uptake and subsequent hydrolysis of circulating PC or lysolecithin (*5, 9*). The discoveries that choline passes easily between blood and brain [its facilitated diffusion mediated by a specific system located within capillary endothelia (*10*)] and that treatments which raise plasma choline concentrations [like eating certain foods (*1, 11*)] also increase brain choline (*12*) provided a way of explaining how neurons sustain the synthesis of ACh and of membrane PC. However, it remained unclear why brain choline concentrations tend to be higher than those in the plasma (*1*), or why jugular venous choline concentrations can be higher than those of the arterial blood entering the brain (*13, 14*).

This paradox may be resolved by the more recent observations that brain neurons can indeed synthesize choline (*15*) by the sequential methylation of phosphatidylethanolamine (PE) (*16–18*) or ethanolamine plasmalogens (*19*), and the hydrolysis of the resulting phospholipids. While many questions remain unanswered concerning the compartmentation and fates of brain choline molecules, it is now possible to propose models delineating choline's sources and sinks and to predict the circumstances in which an increase in choline's availability will enhance ACh synthesis within particular cholinergic neurons.

This article describes the mechanisms that allow neuronal choline concentrations to affect ACh synthesis and summarizes the factors that affect these concentrations.

Choline Concentration and Acetylcholine Synthesis

Characteristics of choline acetyltransferase. Choline acetyltransferase (CAT) catalyzes the reaction: choline + acetyl-CoA → acetylcholine + CoA (where CoA refers to coenzyme A). The enzyme apparently exists as a monomeric protein of molecular weight 58,000 to 70,000 (*20*) or as aggregates whose existence may reflect procedures used for their purification (*21, 22*). Microheterogeneity of CAT has also been claimed on the basis of differences in isoelectric points (*22, 23*). It is not known whether these isoenzymes—which also reportedly differ in kinetic properties (*21*)—are products of different genes or posttranslational modifications of the same peptide; they might also reflect aggregates composed of different numbers of subunits (*21*). Soluble forms of CAT can bind reversibly to membranes (*24*); moreover, a membrane-bound form of CAT, not dissociated from synaptosomal membranes by salt washes, may exist (*25, 26*), and may be associated with synaptic vesicles or with the presynaptic plasma membranes of cholinergic neurons (*27*). The binding of CAT to membranes could represent a regulatory mechanism in ACh synthesis: ACh formed by the bound form might be protected against hydrolysis by acetylcholinesterase (AChE) (*25*). Membrane-bound CAT might also be located near choline-supplying systems, for example, choline-uptake mechanisms or enzymes that liberate choline from phospholipids (Fig. 1).

The soluble form of CAT has higher affinities for both of its substrates (choline and acetyl-CoA) when assayed at low ionic strength [Michaelis constant (K_m) for choline, 350 μM; for acetyl-CoA, 2.5 μM] than when assayed at higher ionic strength (K_m for choline, 6700 μM; for acetyl-CoA, 77 μM) (*28, 29*). The activity of CAT in crude synaptosomal preparations (presumably containing both its soluble and membrane-bound forms) also varies with ionic strength; the affinity for choline of synaptosomal CAT appears to be greater than that of soluble CAT (K_m = 22 μM at low ionic strength and 540 μM at high ionic strength) (*28, 29*). Both products of choline's acetylation are competitive CAT inhibitors, ACh exhibiting an inhibition constant (K_i) of 45 mM when the synaptosomal form is assayed at physiologic ionic strengths. This inhibition might influence ACh's rate of synthesis, since ACh concentrations inside nerve terminals may reach 20 to 50 mM (*30*). Synaptosomal CAT has a K_i for the free form of CoA (CoASH) of 5.5 to 20 μM (*21*), and the CoASH concentration in rat brain is estimated to be 18 μM (*31*). If the K_i is similar in vivo, some mechanism must exist for removing this product from the vicinity of the enzyme, thus allowing CoA's recycling and ACh's synthesis to continue when neuronal activity is increased. Rossier and Benda

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(*28, 29*) noted that the kinetic properties of CAT were affected not by ionic strength per se, but by chloride (Cl^-) concentration. They proposed a model in which chloride, entering presynaptic terminals during the action potential, activates CAT, increasing its maximum velocity (V_{max}). Concurrently the affinities of the enzyme for both of its substrates are decreased, perhaps rendering its actual activity more dependent on intraneuronal choline and acetyl-CoA concentrations. At the same time, the enzyme's K_i for ACh is increased, further accelerating ACh synthesis. After the action potential has passed, Cl^- concentrations in nerve terminals decrease, presumably slowing ACh synthesis. This model becomes more attractive if CAT is actually bound to presynaptic plasma membranes (*27*), since Cl^- levels in its vicinity might then be expected to vary considerably with action potentials.

Apparently no information is available concerning the possibility that CAT is subject to allosteric changes—for example, in response to neuronal firing. The activities of two other rate-limiting enzymes in neurotransmitter production, tyrosine hydroxylase and tryptophan hydroxylase, are so affected (*32*). When catecholaminergic cells fire, a protein kinase phosphorylates tyrosine hydroxylase molecules, thereby increasing its affinity for its cofactor and diminishing its sensitivity to end-product inhibition; this increases the dependence of hydroxylase on substrate (tyrosine) concentrations (*33*). Similarly when serotonergic neurons fire, a calmodulin-dependent protein kinase apparently phosphorylates a subunit of tryptophan hydroxylase, thereby activating the enzyme.

Effects of choline administration. No matter which of the above estimates are taken for CAT's affinities for its two substrates, the concentrations of these compounds normally found in rat brain [30 μM for choline (*1*) and 2 to 20 μM for acetyl-CoA (*31*)] generally fall below those needed to saturate the enzyme; hence treatments that alter brain choline or acetyl-CoA concentrations affect the rates at which neurons accumulate ACh. Choline concentrations can be increased by giving animals the base itself (*1*) or a choline-containing compound [for example, PC (*34*)], alone or as a dietary component (*11*). Choline-deficient diets have not, in general, been found to depress brain choline levels markedly [although they do diminish brain phosphocholine (PCh) (*35*)], possibly because the body is able to liberate choline from membrane phospholipids. Brain acetyl-CoA concentrations have been diminished by the induction of thiamine deficiency (*36*).

Evidence that particular treatments that change choline or acetyl-CoA con-

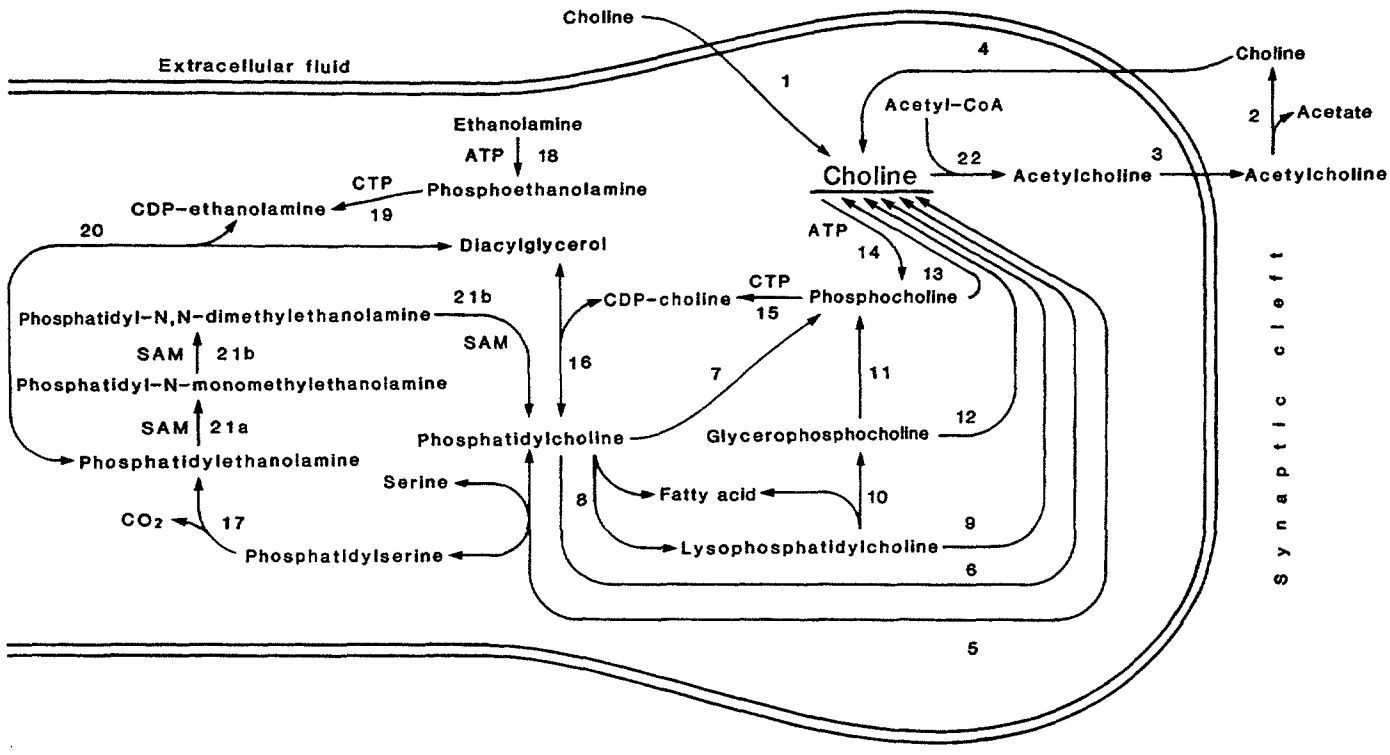

Fig. 1. Sources of choline for brain neurons. The choline in cholinergic neurons is shown as deriving from three main sources: (i) Choline in extracellular fluid, which is in equilibrium with circulating choline and the choline present in other brain cells and which presumably enters the neuron by a low-affinity transport mechanism (1); (ii) intrasynaptic choline formed by AChE (2) from the ACh released by the neuron (3), and then taken up into it by a high-affinity transport mechanism (4); (iii) neuronal PC, from which the choline is liberated by base exchange (5)—the enzymatic substitution of a serine or an ethanolamine for the choline—or phospholipase-mediated hydrolyses (6 to 13). (Other potential sources not shown include membrane plasmalogens and sphingomyelin.) The PC in neurons is shown as being formed either by the incorporation of preexisting choline [by way of base-exchange (5) or CDP-Ch (14 to 16) pathways] or by its de novo synthesis. The latter process can be initiated by the base-exchange substitution of a serine molecule for the choline in PC (5) yielding PS, which is then decarboxylated to PE (17). The PE, which can also be formed from ethanolamine through the base-exchange or CDP-ethanolamine pathway (18 to 20) is converted to PC by PEMT, which catalyzes its stepwise methylation (21, a and b), with SAM as methyl donor. Choline is acetylated to ACh by CAT (22). The numbers on the figure and in parentheses in this legend refer to the following enzymes and transport processes. (1) Low-affinity uptake of choline; (2) acetylcholinesterase (E.C. 3.1.1.7); (3) mechanism of ACh release into the synapse; (4) high-affinity uptake of choline; (5) base exchange enzyme; (6) phospholipase D (E.C. 3.1.4.4); (7) phospholipase C (E.C. 3.1.4.3); (8) phospholipases A_1 (E.C. 3.1.1.32) or A_2 (E.C. 3.1.1.4); (9) lysophospholipase D (E.C. 3.1.4.39); (10) lysophospholipase (E.C. 3.1.1.5); (11) glycerophosphocholine cholinephosphodiesterase (E.C. 3.1.4.38); (12) glycerophosphocholine phosphodiesterase (E.C. 3.1.4.2); (13) alkaline phosphatase (E.C. 3.1.3.1); (14) choline kinase (E.C. 2.7.1.32); (15) phosphocholine cytidylyltransferase (E.C. 2.7.7.15); (16) cholinephosphotransferase (E.C. 2.7.8.2); (17) phosphatidylserine decarboxylase (E.C. 4.1.1.65); (18) ethanolamine kinase (E.C. 2.7.1.82); (19) phosphoethanolamine cytidylyltransferase (E.C. 2.7.7.14); (20) ethanolaminephosphotransferase (E.C. 2.7.8.1); (21) phosphatidylethanolamine *N*-methyltransferase (forms 1 and 2) (E.C. 2.1.1.17); (22) choline acetyltransferase (E.C. 2.3.1.6). Reactions that are believed to be reversible are indicated with two-way arrows.

centrations also affect ACh synthesis and release can be obtained by (i) direct measurements of tissue ACh levels, (ii) estimates of the amounts of ACh released into perfusion fluids in vivo or in vitro, or (iii) examination of postsynaptic processes thought to be controlled by the release of presynaptic ACh. All of these approaches have been used with variable success to study the effects of changing brain choline concentrations. All have characteristic pitfalls: for example, the concentration of ACh in tissue reflects not only its synthesis but also its rate of release; hence a treatment that accelerates both processes might fail to alter its concentration, whereas one that selectively slows ACh release might elevate tissue ACh without changing its synthesis. Unfortunately, the metabolism of ACh yields no product (like 5-hydroxyindoleacetic acid for serotinin or homovanillic acid for dopamine) whose concentration reflects its rate of release, nor is there an intermediate of ACh synthesis (like 5-hydroxytryptophan for serotonin or dihydroxyphenylalanine for dopamine) whose accumulation can provide an index of its synthesis. The precursor of ACh—choline—is the same as the product of its metabolism. Isotopic techniques using labeled choline or PCh to assess ACh turnover have been of relatively little use, since this approach is subject to error when applied in conjunction with treatments that change absolute ACh concentration (*37*).

The initial descriptions of the increases in brain ACh concentration after choline administration to rats (*1*, *38*) were followed by publications suggesting that this treatment also could affect cholinergic neurotransmission: it could cause chemical changes in neurons that were not themselves cholinergic but which received inputs from those cholinergic neurons with elevated ACh. Thus, choline administered to rats rapidly activated the enzyme tyrosine hydroxylase within the caudate nucleus and increased the apparent release of dopamine from these neurons (*39*). When rats were given large oral doses of choline, the tyrosine hydroxylase activity of the adrenal medulla increased after 24 hours (*40*); this change, which could be blocked by cycloheximide and which was similar to the increases produced by giving animals drugs that enhanced ACh release by accelerating splanchnic nerve firing (*41*), was thought to reflect increased synthesis of the enzyme in chromaffin cells. Such increases did not occur in unilaterally denervated adrenals, even though these glands received as much of the administered choline as intact organs did; thus the effect of the choline was indirect and mediated by enhancing ACh release from splanchnic nerve terminals. [Choline at very high concentrations can apparently interact directly with cholinergic receptors (*42*).] Similarly, choline enhanced epinephrine release from intact adrenals but not from denervated organs, even though the latter retained their capacity to respond to nicotine (*43*).

Neuronal activity and choline-dependence. If rats received not only choline but also a second, concurrent treatment (for example, exposure to cold, hypoglycemic doses of insulin, or hypotensive agents) thought to accelerate splanchnic firing, the resulting increase in tyrosine hydroxylase activity was always at least twice as great as the sum of the responses caused by either treatment alone (*44*). This observation gave rise to the hypothesis that the extent to which a given cholinergic neuron makes more ACh when provided with additional choline is related to its physiological activity. Presumably, the splanchnic activation caused by the other treatments increased the choline responsiveness of the nerve terminals either by altering some constituent of the ACh synthetic apparatus (for example, CAT's kinetic properties or intracellular localization or the concentration of acetyl-CoA) or because intraneuronal choline concentrations, already limiting, would have declined as more was utilized for ACh synthesis. Although the biochemical mechanism underlying the relationship between neuronal activity and choline responsiveness remains obscure, the relationship itself has been demonstrated in a variety of experimental preparations, for example, ACh release from ganglia, brain slices, or minces depolarized electrically or by potassium (*45*, *46*); ACh release from the electrically stimulated vagus or phrenic nerves (*2–4*); the increased effect of exogenous choline (or PC) on brain ACh concentrations among animals treated with atropine, oxotremorine, fluphenazine, or pentylenetetrazole (*47*); or with $ACTH_{(1-24)}$ (adrenocorticotropic hormone) (*48*) or atropine plus glucose (*49*); the restoration of ACh concentrations after kainic acid lesions (*50*); and the development of muscle action potentials in the myasthenic syndrome (*51*).

Not all investigators have observed increases in brain ACh after choline or PC administration (*52*). In some cases this may be explained by the failure to increase brain choline concentrations adequately. In others it may reflect the operation of negative feedback mechanisms, activated soon after choline administration, which keep the total ACh output from certain neurons more or less constant. Such mechanisms could utilize multisynaptic arcs that modulate the firing frequency of their cholinergic component when ACh release per firing changes, or alternatively, inhibitory cholinergic autoreceptors. In either case ACh turnover would be decreased. The neurons most likely to retain the ability to respond to additional choline would be those that are not constituents of multisynaptic negative feedback loops (such as peripheral autonomic neurons), those lacking presynaptic cholinergic receptors, or those in which frequent firing is sustained because of a pressing physiologic need for their output of ACh. [This last formulation underlies attempts to use choline or PC to treat neurodegenerative diseases associated with the loss of cholinergic brain neurons (*53*): perhaps the surviving neurons in the involved tract fire more frequently and thus retain the ability to respond to additional choline.]

The existence of feedback mechanisms for keeping ACh output more or less constant is consistent with clinical findings on the use of choline or lecithin to treat disorders like tardive dyskinesia (*54*). A patient who is responsive to choline or to physostigmine and who develops severe cholinergic side effects after physostigmine (because of inappropriate increases in cholinergic transmission at synapses not affected by the disease process) will usually fail to manifest them after choline.

Factors Supplying Choline to the Brain Neurons

Circulating choline: Transport across the blood-brain barrier. Because circulating choline easily crosses the BBB, its passage mediated by an unsaturated bidirectional transport system (*10*), plasma choline concentrations constitute an important determinant of brain choline. At all times these concentrations are influenced by the fluxes of choline to and from peripheral organs (where the base is utilized for PC synthesis, among other things). Postprandially, while choline-containing food is being digested and absorbed, the plasma receives additional quantities of choline sufficient to elevate its choline concentration by several times (*55*). For such elevations to affect the amounts of choline actually available for acetylation in brain neurons, they must first increase the choline concentration in the brain's extracellular fluid. This increase, which has not yet been directly demonstrated, must then en-

hance the net flux of choline into cholinergic neurons. The kinetic characteristics of the choline transport systems in the BBB and cholinergic neurons are compatible with the view that these processes do occur.

A BBB transport mechanism for choline, presumably located within capillary endothelia, has been characterized through the use of the single-injection technique of Oldendorf (*10*). [Studies showing that radioactive choline injected systemically could be found in the brain indicated that a BBB transport system for choline might exist (*6*).] The carrier is bidirectional and normally unsaturated with choline (K_t = 0.44 m*M*) (*56*); it does not require metabolic energy and cannot maintain a concentration gradient. Hence, the direction of net choline flux is normally from brain to blood (*13, 14*), except when the concentration in blood has been elevated substantially by exogenous choline (*10*). When blood choline rises, brain choline does also (*1*). Initially, this increase may reflect a slowing of choline's efflux, secondary to a decrease in its brain-to-blood concentration gradient. However, since blood choline concentrations approach or exceed those in brain, the continuing rise in brain choline probably reflects an influx of circulating choline molecules.

Single-injection techniques have not made it possible to demonstrate a significant influx of circulating lecithin or lysolecithin into the brain (*10*).

Neuronal uptake of choline. The transport of choline across plasma membranes has been examined through the use of synaptosomes, isolated ganglia, tissue slices (brain and peripheral), and cells in culture (*57–62*). When these preparations were incubated with various concentrations of isotopically labeled choline, two kinetically distinct uptake mechanisms could be resolved: (i) a saturable, high-affinity process, with an apparent K_t in the range of 0.1 to 10 μ*M* (*57, 63–65*), and (ii) a high-capacity, low-affinity process with an apparent K_t of 30 to 200 μ*M* (*57, 63–65*), perhaps reflecting diffusion. The kinetic constants of the high-affinity mechanism depend on the ionic composition of the assay medium, Na^+ increasing choline's V_{max} (*56, 66*) and diminishing its K_t (*65*). Ca^{2+} may also stimulate choline uptake directly (*59*) or indirectly by promoting ACh release (*58, 67*). The high-affinity process is apparently associated with terminals (*59, 62, 68*) but not perikarya (*61*) of cholinergic neurons. Recognition of this distribution led to the hypothesis that cholinergic terminals use high-affinity uptake to sequester the choline derived from the hydrolysis of ACh released into the synaptic cleft. High-affinity choline uptake has also been observed in noncholinergic cells such as glia in culture (*69, 70*), noncholinergic neuroblastoma (*69, 71*), cultured cortical neurons from fetal rats (*72, 73*), and noncholinergic photoreceptor cells of rabbit retina (*74*).

Choline transported across plasma membranes is rapidly metabolized. When synaptosomes (*50, 63, 75*) or preparations of chick ciliary muscle (*62*) were incubated in the presence of 0.5 to 5.0 μ*M* labeled choline, a major fraction of the transported label could be recovered as ACh. In neuronal cultures (*72, 76*) and in photoreceptor cells (*74*), the choline label is recovered predominantly as PCh and phospholipids, and not as ACh. The association between choline's transport and its acetylation observed in some preparations has been interpreted as reflecting direct (*77*) or kinetic (*62, 78*) coupling; other observers, noting that the ACh synthesized intrasynaptosomally derives primarily from a preexisting choline pool (*79*) and that ACh synthesis can be inhibited without affecting choline transport, have concluded that high-affinity choline uptake need not be a selective source for the ACh precursor (*80*) and that choline's uptake is independent of its acetylation.

Estimates of the choline transport rate must take into account the fact that all experimental preparations studied both contain endogenous free choline and generate more of the precursor as phospholipids break down; this material mixes with both the extracellular and intracellular choline pools. For example, synaptosomes were estimated to contain 1.5 nmole of choline per milligram of protein and release 0.3 nmole/mg into the medium during a 4-minute incubation (*80*). Over this incubation period the resulting increase in the extrasynaptosomal choline concentration would be about 0.5 μ*M*, a substantial value when compared with the K_t of the high-affinity uptake mechanism (0.9 μ*M*). Thus the release of intracellular choline into the medium will artifactually reduce observed K_t values. Similarly, intracellular choline concentrations must be considered: not all of the synaptosomes prepared from mammalian brain are cholinergic, so that 1.5 nmole of choline per milligram of protein may be an over- or underestimation of the real choline concentration in cholinergic synaptosomes. Loading synaptosomes with exogenous choline stimulates their choline influx (*76, 81*). Such transactivation can be explained by a mechanism in which attachment of its ligand to the carrier molecule facilitates the carrier's passage across the membrane. Indeed, the higher the extrasynaptosomal concentration of choline, the greater the efflux of synaptosomal choline into the medium (*76, 81*). Preparations of choline carrier incorporated into liposomes also exhibit transactivation (*82*). Inhibition of influx or efflux by a competing substrate present on the same side of the membrane by ACh, which by itself is very poorly transported, has also been observed (*83*). Thus, cytoplasmic ACh may immobilize the choline carrier on the cytoplasmic side of the plasma membrane such that only after ACh concentrations fall (that is, during release or as a postmortem artifact) is it free to cross the membrane and transport the choline (*83, 84*). Obviously this model suggests that the carrier is least mobile while it is binding ACh, of intermediate mobility when it is free, and most mobile when it binds choline. Similar conclusions were drawn in studies (*85*) of a nonmetabolizable choline analog.

Since the apparent affinity of the carrier for choline can be influenced by extra- and intracellular choline concentrations, it seems unlikely that simple Michaelis-Menten kinetics will be adequate to characterize the parameters of choline transport. Indeed, when synaptosomes or neurons in culture were incubated with 50 μ*M* choline (to increase intracellular levels) and then subjected to various concentrations of radioactive choline, only a single uptake system could be observed, with an apparent K_t of 14 to 26 μ*M* (*76*). The same study showed that treatment of synaptosomes with neuraminidase increased the K_t of choline transport from 5 to 20 μ*M* without affecting ACh synthesis. Choline concentrations in plasma are in the range [10 to 30 μ*M*; (*11*)] expected to affect choline transport into peripheral neurons, and thus to affect ACh synthesis.

The high-affinity uptake process is probably saturated at choline concentrations normally occurring in plasma and in total brain tissue (*1*). Hence, the low-affinity mechanism is most likely to mediate the increase in tissue choline occurring when plasma choline rises and the resulting enhancement of ACh release from activated neurons (*4*).

Choline synthesis by neurons. Despite indirect evidence that brain could synthesize choline molecules (*13, 86*), most authors until recently maintained that cerebral tissue lacked this property (*87, 88*). In the last few years an enzymatic system that forms PC and thus choline has been well characterized in mammali-

an brain. This system catalyzes the stepwise methylation of PE using *S*-adenosylmethionine (SAM) as its methyl donor; it is thus a phosphatidylethanolamine *N*-methyltransferase (PEMT) (Fig. 1). PEMT activity was present in membranous fractions from homogenates of rat and bovine brain (*16–18*) with highest specific activities in nerve endings (synaptosomes) (*16, 18*). The PEMT activity of brain neurons apparently reflects at least two distinct enzymes, which can be distinguished by their kinetic properties. The first methyltransferase catalyzes the conversion of PE to phosphatidyl *N*-monomethylethanolamine (PME) and the second methyltransferase catalyzes the conversion of PME to phosphatidyl *N*,*N*-dimethylethanolamine (PDE) and of PDE to PC (*18, 89*). The first enzyme is located mainly on the cytoplasmic side of the synaptosomal plasma membrane, whereas the active site of the second transferase faces the outside (extracellular space) (*90*). The first methyltransferase has a high affinity for SAM in preparations from adult animals (apparent K_m, 2 to 4 μM) (*18, 91, 92*), whereas the membrane-associated second enzyme has a lower affinity for the cofactor (apparent K_m, 20 to 110 μM) (*18, 89–92*). The solubilized enzyme has an apparent K_m of 1 to 2 μM (*89*). During the first postnatal week, the affinity of the first enzyme for SAM is lower than that of the enzyme from the adult rat's brain (*91, 92*). It is not clear whether the apparent age-dependence of PEMT's affinities for SAM reflects different enzyme molecules or development-related differences in the physicochemical properties of the membranes containing the enzymes. The activity of the first enzyme in rat brain is highest neonatally (15 pmole of methyl groups incorporated into PME per milligram of protein per hour) (*91*); the second enzyme is highest approximately 2 weeks after birth (40 to 90 pmole of methyl groups incorporated into PC per milligram of protein per hour) (*91, 92*). Assuming that the enzyme is operating at its maximal velocity in vivo, about 10 percent of choline's efflux from brain to plasma can be accounted for by PEMT activity. All such estimates are subject to errors, however.

Choline plasmalogen can also be synthesized by the methylation pathway (*19*). It is not known whether ethanolamine plasmalogen *N*-methyltransferase is identical with PEMT.

The activity of PEMT may be regulated by various neurotransmitters. When synaptosomes were incubated with dopamine (100 μM), the incorporations of methyl groups into PME, PDE, and PC were increased by factors of 1.8, 1.6, and 2.1, respectively. The effect of dopamine, which was observed at concentrations as low as 1 μM, could be blocked by haloperidol (a dopamine receptor antagonist) (*93*). The question thus arises whether neuronal choline synthesis in situ, and choline's availability for ACh synthesis, are normally regulated by other neurotransmitters, such as catecholamines. Changing of PEMT activity may constitute one of the mechanisms through which neurons (not necessarily cholinergic) respond to catecholamines. Stimulation of PE methylation in reticulocyte plasma membranes by the β-adrenergic agonist isoproterenol is associated with the change of the physicochemical properties of the membranes and the activation of their adenylate cyclase (*94*); possibly similar mechanisms exist in neuronal tissue. This activation of PEMT has been proposed as a necessary step in evoking cellular response to β-adrenergic stimulation (*94*).

The PC formed by PEMT's must be hydrolyzed to free choline if the pathway is to provide choline molecules for ACh synthesis. Estimates of the turnover rate of PC in synaptosomes indicated that there were two pools in nerve endings: one with a half-life of 2 days, the other with a half-life of 52.5 days (*95*). Such turnover rates would probably be too slow to provide much of the free choline needed for ACh synthesis. We incubated synaptosomes from rat brain with 4.5 μM [*Me*-^{3}H]SAM in the presence of PDE for 30 minutes at 37°C, extracted the [^{3}H]choline and purified it by paper electrophoresis and thin-layer chromatography (*15*). We found that radioactive free choline constituted 23 percent of the total [^{3}H]choline present in the incubation mixture (total [^{3}H]choline = free [^{3}H]choline + phosphatidyl-[^{3}H]choline). Free choline, however, constituted less than 1 percent of the total synaptosomal choline pool. Indeed, the enrichment of the free choline pool with [^{3}H]choline was about 50-fold as great as the enrichment of the PC pool with [^{3}H]PC. We conclude that PC synthesized by synaptosomal PEMT's has a relatively high turnover rate: it is rapidly degraded to free choline and may provide a considerable portion of the choline for ACh synthesis.

The ability of the PEMT pathway (i) to generate a PC pool that is quickly degraded to free choline and (ii) to be stimulated by catecholamines suggests that in vitro assays of PEMT activity (which measure only phospholipid products) cannot be used to estimate the rates at which intact brain neurons form choline. The accuracy of estimates might be improved if all the choline-containing intermediates (of PC degradation) (Fig. 1) were measured and if the tissues were exposed in vitro to neurotransmitters likely to occur in vivo.

Degradation of PC to choline in neurons can occur by several pathways. The PC can be hydrolyzed directly to choline by phospholipase D (PLD) (*96*). This enzyme is stimulated by free fatty acids (*97*), which, in turn, may arise from an action of phospholipases A_1 (PLA_1) and A_2 (PLA_2) on the PC. The activity of PLA_2 is increased when various plasma membrane receptors are activated (*98*) and may be a rate-limiting step in the synthesis of prostaglandins (*99*). That the stimulation of PLA_2 may, by enhancing fatty acid accumulation, activate PLD and thus accelerate the liberation of choline from PC is suggested by the presence of elevated fatty acid and choline concentrations in the hypoxic brain (*100*). Lysophosphatidylcholine (LPC) formed by PLA_1 and PLA_2 can be further metabolized to free choline by lysophospholipase D (LPLD) (*101*) or hydrolyzed to glycerophosphocholine (GPCh) by a lysophospholipase (LPL) (*102*); GPCh is then converted to PCh by GPCh cholinephospho-hydrolase (*103*) or to free choline by GPCh diesterase (*104*), and the PCh is hydrolyzed to free choline by alkaline phosphatase (*87, 105*).

Synthesis of PC by PEMT constitutes only one of three ways by which this phosphatide can be formed. In the cytidine 5′-diphosphocholine (CDP-Ch) pathway (Fig. 1), preexisting free choline molecules are phosphorylated to PCh by choline kinase in a reaction requiring adenosine triphosphate (ATP) (*106*). The PCh is then coupled with CTP by phosphocholine cytidyltransferase to form CDP-Ch (*107*), which reacts with diacylglycerol in the presence of diacylglycerol cholinephosphotransferase to generate PC (*108*). Phosphatidylcholine can also be formed by a base exchange enzyme that catalyzes a substitution of one phospholipid base (serine, ethanolamine, or choline) for another (*109*). The dynamics of PC synthesis and degradation by these three pathways may influence the concentration of free choline available for ACh synthesis. Synthesis of PC through the methylation pathway ultimately depends on the supply of PE. PE can be synthesized from preexisting ethanolamine by a CDP-ethanolamine pathway (*106–108*) or base exchange or made de novo by the decarboxylation of phosphatidylserine (PS).

We have investigated PE synthesis in rat brain synaptosomes incubated with

[U-^{14}C]serine. Synaptosomes incorporated [^{14}C]serine into phospholipids to form PS, which was decarboxylated sufficiently rapidly that after 15 minutes of incubation the radioactivity in the ethanolamine moiety of PE exceeded that in the serine moiety of PS (*91*). The process of serine incorporation was stimulated by Ca^{2+}, which suggests that it might have been mediated by the base-exchange pathway (*109*). Thus neuronal tissue may synthesize choline using the amino acids serine and methionine as its ultimate precursors: serine is incorporated to PS and then decarboxylated to PE, providing the ethanolamine portion of the choline molecule, and methionine is converted to SAM, which acts as a cofactor of PEMT, supplying the *N*-methyl groups of PC. PC is degraded to free choline, some of which may be used for ACh synthesis. It remains to be determined whether—as suggested in Fig. 1—a PC cycle exists through which the phosphatide backbone can, after releasing choline, be reutilized for PC and choline synthesis.

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