Pathways of Acetylcholine Synthesis, Transport and Release as Targets for Treatment of Adult-Onset Cognitive Dysfunction

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Abstract: Acetylcholine (ACh) is a neurotransmitter widely diffused in central, peripheral, autonomic and enteric nervous system. This paper has reviewed the main mechanisms of ACh synthesis, storage, and release. Presynaptic choline transport supports ACh production and release, and cholinergic terminals express a unique transporter critical for neurotransmitter release. Neurons cannot synthesize choline, which is ultimately derived from the diet and is delivered through the blood stream. ACh released from cholinergic synapses is hydrolyzed by acetylcholinesterase into choline and acetyl coenzyme A and almost 50% of choline derived from ACh hydrolysis is recovered by a high-affinity choline transporter. Parallel with the development of cholinergic hypothesis of geriatric memory dysfunction, cholinergic precursor loading strategy was tried for treating cognitive impairment occurring in Alzheimer's disease. Controlled clinical studies denied clinical usefulness of choline and lecithin (phosphatidylcholine), whereas for other phospholipids involved in choline biosynthetic pathways such as cytidine 5′-diphosphocholine (CDP-choline) or alpha-glyceryl-phosphorylcholine (choline alphoscerate) a modest improvement of cognitive dysfunction in adult-onset dementia disorders is documented. These inconsistencies have probably a metabolic explanation. Free choline administration increases brain choline availability but it does not increase ACh synthesis/or release. Cholinergic precursors to serve for ACh biosynthesis should be incorporate and stored into phospholipids in brain. It is probable that appropriate ACh precursors and other correlated molecules (natural or synthesized) could represent a tool for developing therapeutic strategies by revisiting and updating treatments/supplementations coming out from this therapeutic stalemate.

1. INTRODUCTION

Acetylcholine (ACh) is largely diffused in mammals and plays an important role of neurotransmitter in central, peripheral, autonomic and enteric nervous system. It is involved in the endothelium-dependent vasodilatation in several vascular beds and is present in different cell systems, such as immune cells where it plays a role not clearly identified yet [1]. It has been discovered in 1920 and therefore it represents the first neurotransmitter of neuroscience history [1].

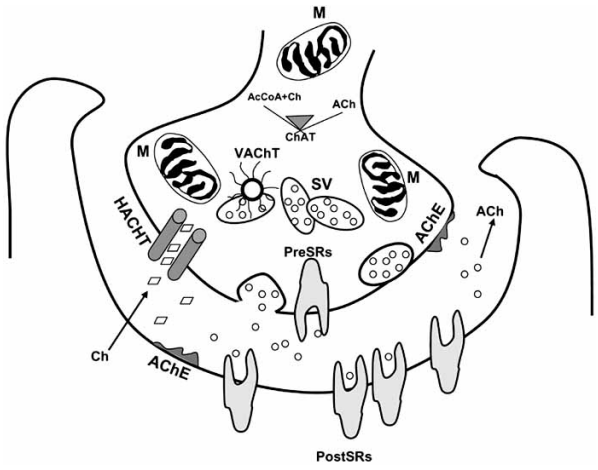

Fig. (1). Schematic representation of a cholinergic synapse summarizing mechanisms of neurotransmitter synthesis, storage, and release.

ACh: acetylcholine; AChE: acetylcholinesterase; Acetyl-CoA: acetyl coenzyme A; Ch: choline; ChAT: choline acetyltransferase; HACHT: high affinity choline transporter; M: mitochondria; PreSRs: presynaptic receptors; PostSRs: postsynaptic receptors; SV: synaptic vesicle; VAChT: vesicular acetylcholine transporter.

The process of ACh synthesis, storage, and release requires the expression of several specialized enzymatic systems (Fig. **1**). In the initial step of ACh synthesis, choline is taken up from the extracellular space by a high affinity Na^+-dependent uptake system (defined at the dime of discovery as SDHACU) located predominantly in terminals of cholinergic neurons [2]. Transfer of choline, by SDHACU into the cholinergic terminals is considered to be the rate-determining step for the synthesis of ACh. This Na^+-dependent choline transport is correlated to the release and on-demand synthesis of ACh and likely expresses the status of cholinergic neuronal activity [3-5]. Mechanism(s) underlying the modulation of choline transporter and hence the availability of choline for ACh synthesis have been extensively investigated.

The majority of "classic" neurotransmitters are synthesised in the cytosol of nerve terminals and are then stored into synaptic vesicles prior to exocytotic release. The enzyme choline acetyltransferase (ChAT) is responsible for ACh synthesis, and it uses choline taken up by SDHACU [6]. The neurotransmitter is accumulated in synaptic vesicles by the activity of a 12-transmembrane domain protein, the vesicular ACh transporter (VAChT) (Fig. **2**). It exchanges two protons generated by a proton ATPase with one ACh molecule by electrochemical gradient [7].

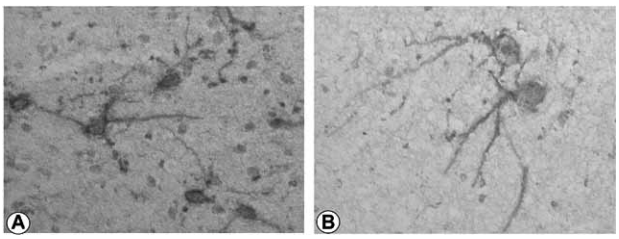

Fig. (2). VAChT immunoreactivity in neurons of rat neostriatum (**A**), and nucleus basalis magnocellularis (**B**). 200X.

2. BRAIN CHOLINERGIC SYSTEMS

The main data on the anatomical organization of brain cholinergic systems were obtained by immunohistochemical and more recently *in situ* hybridization techniques for identifying the ACh biosynthetic enzyme ChAT. ChAT is considered the most specific marker for identifying cholinergic neurons in the central and peripheral nervous systems. The organization of brain cholinergic systems in several mammals including humans has been extensively reviewed [8-11]. Brain cholinergic neurons form a global network characterized by nerve cell bodies located in selected areas from which ascending and to a lesser extent descending projections originate (Fig. **3**). Cholinergic nerve cell bodies were identified in

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the basal forebrain, striatum (caudate nucleus (Fig. **4A**), putamen and nucleus accumbens), mesopontine tegmental nuclei (pedunculopontine tegmental, laterodorsal tegmental and parabigeminal nuclei), cranial motor nuclei and spinal motor neurons [8, 9].

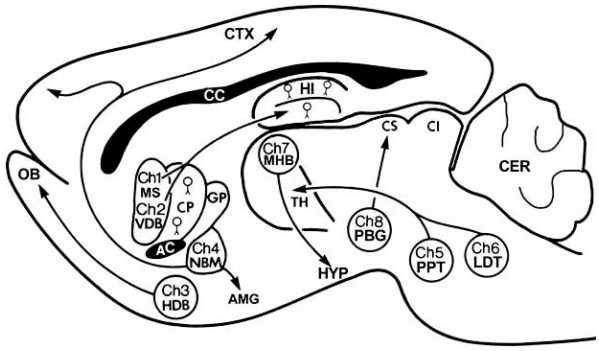

Fig. (3). Main cholinergic nuclei and their projections represented in rat brain.

Ch1-Ch8: main cholinergic brain nuclei; AC: nucleus accumbens; AMG: amigdala; CC: corpus callosum; CTX: cerebral cortex; CER: cerebellum; CS: superior colliculus; CI: inferior colliculus; CP: caudate-putamen; GP: globus pallidus; HDB: horizontal diagonal band; HI: hippocampus; HYP: hypothalamus; MS: medial septum; NBM: nucleus basalis magnocellularis; OB: olfactory bulb; PBG: parabigeminal nucleus; TH: thalamus; VDB: ventral diagonal band; MHB: mesopontine tegmental nuclei; PPT: pedunculopontine tegmental nuclei; LTD: laterodorsal tegmental nuclei Modified from [205].

The human cholinergic basal forebrain includes large (magnocellular) hyper chromic neurons localized in the septal/diagonal band complex (of Broca) and in the nucleus basalis magnocelluaris (of Meynert). More recent classifications have defined cholinergic neurons of basal forebrain as Ch1-Ch4 neurons. Cholinergic neurons of the medial septum (Ch1) compose 10% of this region. The diagonal band includes the Ch2 (vertical limb) and Ch3 (horizontal limb) subgroups. These neurons are fusiform in shape. Cholinergic neurons of these areas account approximately the 70% of all neurons of the Ch2 group and the 1–2% of neurons of the Ch3 group. Neurons of the *nucleus basalis magnocelluaris* defined as Ch4 cells are fusiform to multipolar in shape (Fig. **4B**) and are cholinergic by approximately the 90%. Their number in humans is estimated in approximately 210,000 per hemisphere. These neurons were separated topographically into anterior (Ch4a), intermediate (Ch4i) and posterior (Ch4p) subfields. The anterior portion can be further divided into medial (Ch4am) and lateral (Ch4al) sectors, the intermediate subfield is further divided into dorsal (Ch4id) and ventral (Ch4iv) portions [10]. Ch-1 and Ch-2 neurons provide the major cholinergic innervation to the hippocampus. Cholinergic neurons of the Ch3 subfield supply the olfactory system, whereas Ch4 neurons innervate the amygdala and cerebral neocortex [8-10]. Ascending cholinergic pathways that rely upon the cerebral cortex (Fig. **4C**) are involved in important higher brain functions such as attention, arousal, motivation, memory and consciousness [8, 12].

The basal forebrain cholinergic complex degenerates in several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Down's syndrome, the Parkinsonism–dementia complex, progressive supranuclear palsy, Korsakoff's syndrome, olivo-ponto-cerebellar atrophy and pugilistic dementia. A cholinergic loss is also probable in Pick's disease or after chronic ethanol intake and traumatic brain injury [11].

Several possible causes for the basal forebrain neurodegeneration were considered such as (1) excitotoxic injury; (2) growth factor deprivation; (3) oxidative stress; (4) inflammation; (5) mitochondrial dysfunction; and (6) beta-amyloid toxicity. Of course, each hypothesis can involve several complex sub-mechanisms, each potentially coexisting with one or more of others [11]. However, in spite of the extensive studies performed in the last years, the mechanisms underlying brain cholinergic vulnerability in human neurodegenerative diseases characterized by cognitive impairment is still unclear [11].

Evidence of loss of basal forebrain cholinergic neurotransmission and the development of the "cholinergic hypothesis" of memory dysfunction in Alzheimer's disease resulted in the proposal and clinical evaluation of three categories of cholinomimetic drugs, the ACh precursors, acetylcholinesterase/cholinesterase (AChE/ChE) inhibitors and M1 muscarinic cholinergic receptor (mAChR) agonists and M2 mAChR antagonists [13] [14].

Biological actions of ACh are mediated through the interaction with two major subtypes of cholinergic receptors based on differential binding, the muscarinic and the nicotinic receptors. DNA cloning has identified five subtypes of muscarinic receptors. All of these muscarinic receptors are G-protein linked. Muscarinic receptor subtypes have a differential distribution in mammalian brain [15]. The nicotinic receptor is a ligand-gated channel composed of five subunits. It is responsible for the effects of ACh in autonomic ganglia and the neuromuscular junction. There are some nicotinic receptors expressed in the central nervous system, the classification and functional role of which was recently reviewed [16].

3. ACh SYNTHESIS

3.1. The Biosynthetic Pathway

The ACh synthesis involves acetyl coenzyme-A (ACo-A), choline and ACh biosynthetic enzyme ChAT. ChAT is a key marker required for cholinergic neurotransmission. Molecular genetics has evidenced the genomic structure of ChAT which was found to be unique compared with other enzymes involved in neurotransmitter biosynthesis. This protein and that relative to VAChT are both encoded by two embedded genes, the VAChT gene lying within the first intron of the ChAT gene [17-20]. The ChAT and VAChT co-

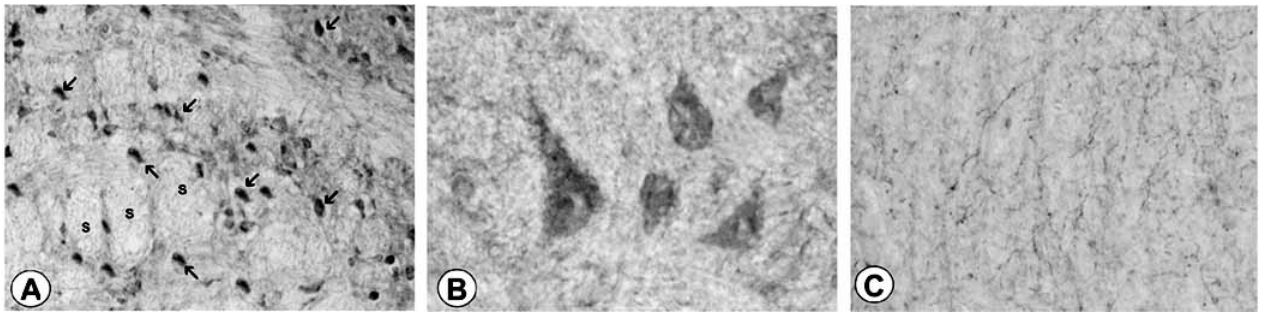

Fig. (4). Microanatomy of cholinergic interneurons of rat neostriatum (**A**) 100X, nucleus basalis magnocellularis multipolar nuclei (**B**) 400X, and cholinergic fibres supplying cerebral cortex (**C**) 200X identified by acetylcholine immunohistochemistry. Note in neostriatum that acetylcholine-immunoreactive neurons (arrows) are located in matrix and not in striosomes (s).

expression was detailed by some studies [21-24], although it is not univocally admitted [25, 26].

Catalysis of ACh was described in detail more than sixty years ago [27], but mechanisms of physiological regulation of ChAT activity are still unknown. ChAT is a single-strand globular protein with a molecular weight of 82 or 69 kDa in humans and is expressed in two main forms in cholinergic nerve terminals: a) soluble (80-90%), b) membrane-bound (10-20%) form [28, 29]. ChAT activity and expression have been used as hallmarks for cholinergic neurons. They can be modified by a variety of extracellular signals, such as growth factors and pharmacological compounds [21, 30]. Furthermore, several agents known as differentiation factors (retinoids and dexamethasone) or compounds increasing levels of cAMP were used to amplify ChAT activity [26, 31].

Short- and long-term regulation of ChAT activity has been described. Short-term regulation may be related to sub cellular distribution of the enzyme, ionic modifications in the pre-synaptic terminal [32] and phosphorylation induced by protein kinase [33]. Phosphorylation of cellular proteins by kinases is a common and critical regulatory mechanism in intracellular signalling. Phosphorylation of proteins involved in ACh synthesis may produce multiple effects. Short-term exposure to phosphatase inhibitors (calyculin A or okadaic acid) reduced ACh synthesis and ChAT activity in brain slices [34]. More recently, it has been shown that in NS-20y cells okadaic acid induces an increase of ChAT activity after a 24 h treatment, possibly due to an accumulation of ChAT mRNA [35]. Phosphorylation by casein kinase II and protein kinase C (PKC) increased enzyme activity, whereas alpha-calcium/calmodulin-dependent kinase phosphorylated ChAT, but did not change it [36]. Serine 440 was recently shown to be one of the PKC phosphorylation sites, and mutation analysis suggests that phosphorylation may change sub cellular distribution and activity of the enzyme [33]. Therefore, there is a possibility that phosphorylation modulates the partitioning of the enzyme between cytoplasm and membrane compartments [33,37], alters catalytic activity and substrate specificity or stabilizes functional enzyme complexes [36] which will in turn regulate ACh biosynthesis [38].

Neurotrophins are a family of small polypeptides regulating survival, differentiation and plasticity of neurons. Nerve growth factor (NGF) is the prototype molecule in this family. Exposure (for days) of basal forebrain cholinergic neurons to NGF increased ChAT activity by 300% above control, as well as the release of ACh, which depends upon a vesicular pool [39]. NGF administrated intracerebroventricularly by osmotic minipump to Fischer-344 rats aged 4 and 24 months for 2 weeks promotes the survival and enhances the neurotransmitter phenotype of basal forebrain and striatal cholinergic neurons [40]. In young adult rats, increased ChAT and choline uptake activities were accompanied by enhanced ACh synthesis, basal and depolarization-induced release of both endogenous and newly synthesized neurotransmitter, being the largest effect observed in striatum. In aged animals, the responses to NGF were less uniform and choline uptake was increased only in frontal cortex and striatum. ACh content of synaptosomes was not affected by age or NGF treatment, but deficits in both basal and evoked release of newly synthesized ACh were observed in aged rats. NGF treatment had no significant effect on the basal release of newly synthesized ACh in aged rats [40].

3.2. Choline and ACh Synthesis

In cholinergic neurons the hydrolysis of phosphatidylcholine by a phospholipase D (PLD) –type enzyme generates in part the precursor choline used for the synthesis of the neurotransmitter ACh. The molecular identity of the relevant PLD was determined using, as a model, murine basal forebrain cholinergic cells (SN56) [41]. ACh levels were examined in cells incubated in a choline-free medium, to ensure that ACh was synthesised entirely from intracellular choline stores. In SN56 cells, PLD2 but not PLD1 were identified. This observation suggests that the PLD2 might be preferentially expressed in cholinergic neurons. These data are consistent with the report that a variant of PC12 cells express exclusively PLD2 [42]. This expression is stimulated by NGF [42], a treatment known to up regulate the cholinergic phenotype of PC12 cells [43, 44]. On the other hand, PLD1 is also highly expressed in brain tissue [45-47], notably in neurons of medial septum, cranial motor nuclei and ventral spinal cord [48], which are likely cholinergic. No data are currently available on the possible co-expression of PLD1 and/orPLD2 and cholinergic markers (e.g. ChAT) *in vivo*.

Stimulation of PLD activity by phorbol 12-myristate 13-acetate (PMA) and the resulting increase of ACh synthesis from PLD-generated choline indicates that this pathway is regulated by PMA-responsive PKC isozymes [41]. *In vivo*, this form of regulation of ACh synthesis is probably controlled by neurotransmitters expressed by neurons innervating cholinergic areas. There are different neurotransmitters whose receptors are coupled to PKC-dependent activation of PLD [49]. These mechanisms regulating choline availability for ACh synthesis are likely widespread [41].

Among the five muscarinic acetylcholine receptors, M1 and M3 receptors efficiently activate PLD (and PKC) in various cell types [50, 51], whereas activation of PLD by M2 receptor is less pronounced [50]. This difference is probably due to the M2 and M4 coupling system of G protein second messengers, because M1, M3 and M5 receptors couple to $G_{q/11}$ whereas M2 and M4 couple to $G_{i/o}$. Theses observations suggest that in the cholinergic synapse, ACh released into the synaptic cleft might stimulate PLD activity leading to a release of choline that could be used for ACh synthesis.

Although it is clear that the PLD-generated choline is efficiently utilized by cholinergic cells (SN56) as a precursor of ACh, the quantitative significance of this pathway and mechanisms of its regulation were not fully determined.

3.3. Sodium-Dependent High-Affinity Choline Uptake Modifications and ACh Synthesis

It has been reported that whereas acute administration of choline does not alter the steady-state concentration of ACh in brain in physiological conditions [52, 53], choline attenuates pharmacologically-induced alterations both in the rate of high-affinity choline uptake (HACU) and steady-state concentration of ACh [52-54]. The acronym HACU refers to the ACh uptake system irrespectively of it ion sensitivity. Acute administration of choline can antagonize alterations in the steady-state concentration of ACh and in the rate of HACU induced by a variety of pharmacological agents increasing cholinergic neuronal activity *via* different mechanisms. These findings support the hypothesis that despite acute choline administration does not alter the steady-state concentration of ACh under normal conditions, it plays a significant role in regulating the synthesis of ACh during periods of increased neuronal demand [55].

HACU is a critical element in the synthetic pathway for ACh, and is known to display activity-dependent regulation *in vivo* and *in vitro*. A large body of evidence indicates that HACU is coupled to cholinergic neuronal activity [55,56].

An analysis of the effect of hemicholinum mustard (HCM) on HACU activity and ACh synthesis did not revealed an increased synthesis of unlabelled ACh in HCM-treated tissues compared to controls as expected if an intraterminal pool is capable of making a physiologically relevant contribution to ACh synthesis [56]. An alternative explanation for the increase of unlabelled ACh across time is an up-regulation of HACU, which is documented after depolarization, and continued acetylation of newly transported choline [55].

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Fig. **5**), an agent causing irreversible covalent modification of protein

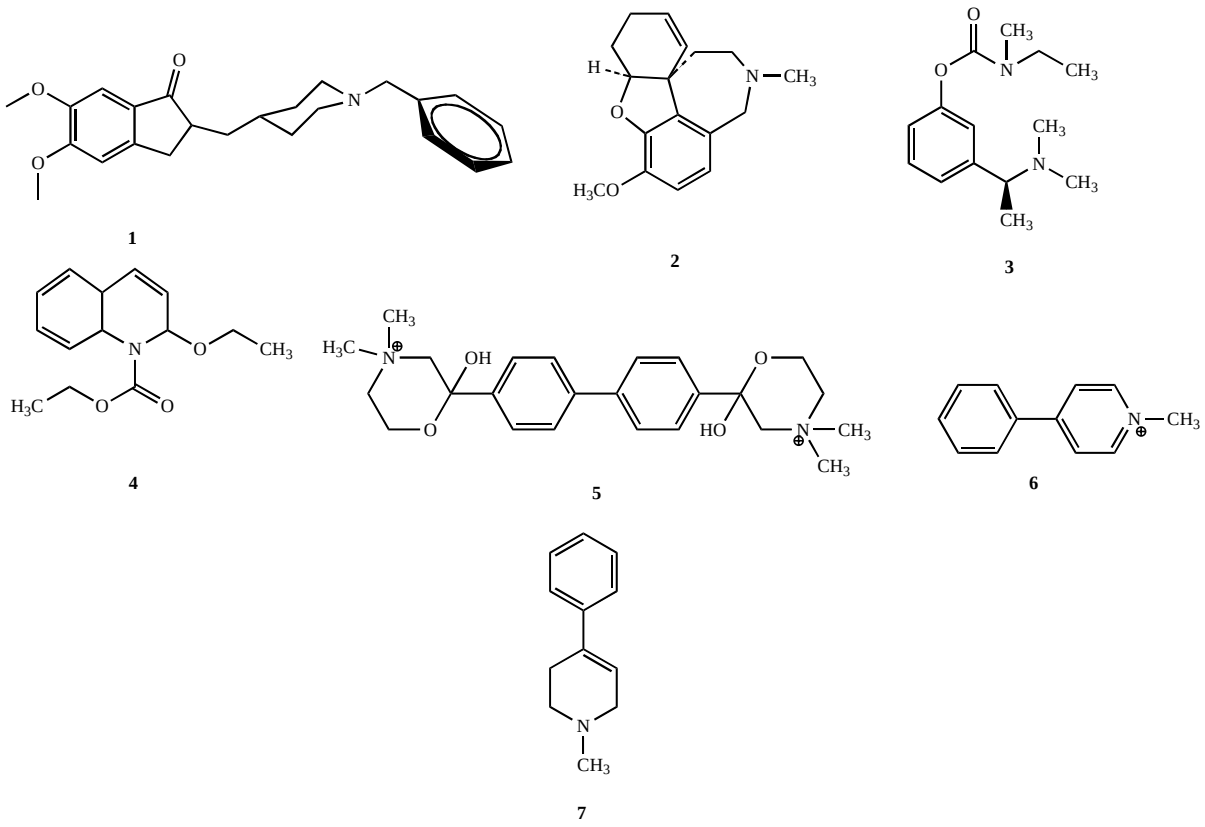

Fig. (5). Chemical structures of AChE/ChE inhibitors (**1**: Donepezil; **2**: Galantamine; **3**: Rivastigmine), compounds interfering with choline uptake mechanisms (**4**: EEDQ and **5**: HC-3) and dopaminergic neurotoxins (**6**: MPP and **7**: MPTP).

carboxyl residues, was used to inhibit HACU [57] in a model in which pre-treatment with EEDQ *in vitro* caused a time- and concentration-dependent inhibition of HACU [57]. EEDQ-induced inhibition of HACU was not reversed by repeated tissue washing. Co-incubation with hemicholinium-3 (HC-3), a highly specific and reversible inhibitor of HACU, protected against EEDQ –induced inhibition of HACU. These findings indicate that EEDQ causes a direct and irreversible inhibition of high-affinity choline transporter (CHT) in cholinergic nerve terminals. Therefore the compound may represent an investigational tool for characterization of the turnover and regulation of this transporter protein *in vivo*.

4. CHOLINE/ACETYLCHOLINE TRANSPORTERS

The role of presynaptic choline transport in support to ACh production and release first emerged from studies on cholinergic signalling in preparations such as perfused cat sympathetic superior cervical ganglia [58] and vertebrate neuromuscular junction (NMJ) [59,60]. The effect of external choline in sustaining ACh synthesis is saturable in the low micromolar range and can be competitively inhibited by HC-3 [61]. HC-3 is a bicyclic, constrained choline analogue, initially thought to represent a lethal, respiratory paralytic agent [62,63]. HC-3 lethal effects on breathing were described to be similar to those of tetanus or botulinum toxins. However, unlike these toxins, HC-3 toxicity can be relieved by artificial respiration until the drug is metabolized indicating that its effects are reversible. It is also possible to block HC-3 toxicity by choline administration. This suggests that the two molecules likely compete for a common target [61]. Toxic effects of HC-3 treatment are consistent with cessation of ACh synthesis, breakdown of neurotransmission at NMJ and subsequent respiratory arrest. This further indicates that at the NMJ HC-3 suppresses stimulation evokes ACh release [59,64]. The availability of radiolabeled choline of high specific activity has allowed the direct demonstration of HC-3 as a selective blocker of presynaptic choline uptake [65,66]. Sensitivity to low, nanomolar concentrations of HC-3 is now accepted as a major defining characteristic of presynaptic choline uptake mechanisms [67].

Extracellular choline is required to sustain ACh release and choline is efficiently recaptured in a saturable, HC-3-sensitive manner. This indicates that cholinergic terminals express an unique transporter critical for neurotransmitter release. However, the identification of CHT as a molecular entity was thwarted by the low density of mammalian cholinergic terminals in many preparations, relative to more numerous, low-affinity (but high-capacity) choline uptake mechanisms found in surrounding cells [61].

Different studies have evidenced the presence of two types of CHT (Na^+- dependent and independent) [66,68-73]. These investigations have shown that most of choline transported by high affinity, Na^+- dependent, HC3-sensitive mechanism is efficiently converted to ACh [66,74,and 75]. This suggests that choline is metabolically linked proximally to sites of ACh synthesis [61]. These findings also indicate that CHT must be localized near synaptic ACh release sites to facilitate recapture of choline compared to other sites on presynaptic plasma membrane [61].

CHT mechanisms are apparently shared by all cholinergic vertebrate or invertebrate neurons analyzed [58,68,69,76-80]. Studies involving selective destruction (i.e. lesioning) of cholinergic axons such as those performed at the level of septal–hippocampal pathway support this contention. This destruction abolishes the ability of the target region to support Na^+- dependent, HC3-sensitive, high –affinity choline uptake [81-84].

Low-affinity choline uptake has not been clearly defined at a molecular level, but might actually be supported by multiple trans-

porters. Among candidates, the organic cation transporter type 2 (OCT2) expressed in neurons and/or other substances that can transport choline, probably exerts a relevant role [85,86].

Transport capacity is coupled to neuronal activity and it can be altered by pharmacological agents [87-90], depolarization [91], and electrical stimulation [92], which are factors affecting ACh release and synthesis. Mechanism(s) underlying the activation of Na^+- dependent, high affinity CHT are controversial and not well defined. Ionic fluxes [92,93], ACh release [94,95] and the concentration of cytoplasmatic ACh [96] may be involved.

The definition of a cholinergic neuron–specific choline transport process supporting ACh synthesis leads reasonably to questions concerning how the regulation of CHT parallels changes in ACh synthesis. In general, when the CHT is functional, ACh synthesis and release are sustained, even during persistent and prolonged stimulation [58,97]. Since ACh itself is not recycled and the transport of choline is rate limiting in the ACh synthesis, choline supply must increase during periods of enhanced ACh release, to avoid depletion of the neurotransmitter at the presynaptic vesicle stores. Extracellular concentrations (5-10 μM) of choline saturate CHT even in the absence of ACh release and hydrolysis. This indicates that the only way to enhance CHT-mediated choline uptake is to increase the number of functional CHTs at presynaptic plasma membrane. Indeed, the maximal rate of CHT-mediated choline uptake (V_{max}) is modulated in response to chemical or electrical manipulation of cholinergic firing rates [61,98-102]. This may have important implications in the design/development of cholinergic neurotransmission enhancers.

Despite its physiological importance, complementary DNAs for high-affinity choline transporter were identified only in 2000 by Okuda and co-workers [6] in a systematic study aimed at identifying cDNAs encoding for several neurotransmitter transporters including GABA, noradrenaline, dopamine, serotonin, glycine and glutamate transporters [6]. In these studies, a cDNA encoding the high-affinity CHT was identified and characterized in nematode *Caenorhabditis elegans*. In the same study, a rat homologue of high-affinity CHT was isolated and characterized [6]. This investigation has evidenced the same characteristics for high-affinity CHT in cholinergic nerve endings and non neural cells [6]. These findings could explain why the high affinity CHT was not previously cloned. High-affinity CHT has no significant homology with members of neurotransmitter transporter family, precluding the use of homology-based cloning to obtain high-affinity CHT cDNA. This CHT is not expected to belong to the Na^+-dependent glucose transporter family because it depends by Na^+ but not by Cl^- ions [103], whereas the high-affinity choline uptake in synaptosomes as well as other neurotransmitter transporters depend on both Na^+ and Cl^- ions [71]. The transport rate by high-affinity CHT is not efficient compared to high-capacity, low-affinity transporters present in Xenopus oocytes or cultured cells. This probably explains failures to detect in the past transporter by expression cloning.

4.1. Functional Significance of High-Affinity CHT

The predominant localization of high-affinity CHT in organelles of endocytic origin suggests that trafficking of the transporter between intracellular membrane compartments and plasma membrane may limit the abundance of transporter at cell surface [104]. Molecular dissection of the mechanisms of high-affinity CHT internalization is of particular importance, as fine-tuning of this internalization mechanism may regulate choline transport activity, intracellular choline levels and, consequently, ACh synthesis. Trafficking of high affinity CHT may also underscore important characteristic of cholinergic neurons. For example, it is striking that choline supplementation therapy presents limited benefit in cholinergic dysfunction [105], which is in sharp contrast with the well-established dopaminergic enhancement by the administration of the dopamine precursor L-DOPA [106]. The topic of choline precursors and their use to relief cognitive dysfunction in pathologies characterized by deficits in basal forebrain cholinergic transmission will be analyzed below.

Distinct features of cholinergic and monoaminergic neurons that underlie different effects of precursors are poorly understood, but the possibility that trafficking of plasma membrane neurotransmitter transporters has a role in these differences is worthy of further examination [104]. In cholinergic neurons, the presynaptic CHT mediates HACU as the rate-limiting step in ACh synthesis. It has been demonstrated that HACU is increased by behaviourally and pharmacologically-induced activity of cholinergic neurons *in vivo*. Molecular mechanisms of these changes in CHT function and regulation have been partly clarified only recently. Cloning of high affinity CHT has led to the generation of new tools, including monoclonal, specific anti-CHT antibodies and CHT knockout mice [6,107]. This has allowed to investigate the possibility of a presynaptic, CHT-mediated, molecular plasticity mechanism, regulated by and necessary for sustaining *in vivo* cholinergic activity [108]. Studies in various mouse models of cholinergic dysfunction, including AChE transgenic and knockout mice, ChAT heterozygote mice, muscarinic and nicotinic receptor knockout mice, as well as CHT knockout and heterozygote mice, provided new information on the role of CHT expression and regulation in response to long-term alterations in cholinergic neurotransmission [108]. These models highlight the capacity of CHT to provide for functional compensation in states of cholinergic dysfunction.

In a relatively recent study the transport of choline and its relationships to the organic cation transporters expression was evaluated in a rat brain microvessel endothelial cell line (RBE4) [109]. The main characteristic of choline uptake in RBE4 cells is summarized below: (1) RBE4 expresses a saturable transport system for the uptake of choline; (2) The transport system is Na^+ -independent and exhibits high affinity for choline; (3) The choline analogue hemicholinium-3 competes with choline; (4) The transport system recognize several other organic cations including 1-methyl-4-phenylpyridinium (MPP), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); (5) Monoamines (e.g., dopamine, serotonin, and noradrenaline) exhibit little or no affinity for this transport system; (6) Tetraethyl ammonium (TEA), a prototypical organic cation widely used in studies involving the transport of organic cation, interacts with low affinity with this transport system [109] (Fig. **5**).

Among the three different organic cation transporters so far identified in mammalian cells at the molecular level, OCT1 and OCT2 transport choline in a Na^+-independent manner [110], whereas OCT3 does not recognize choline as a substrate [111-113]. A similar transport system was described not only in brain but also in various epithelial cells derived from non-neuronal tissues [6,114-116]. Therefore, it seems that the expression of the Na^+-independent high-affinity choline transport system may not be restricted to nerve cells. This system is likely to play a critical role in the delivery of choline in various cell types of neuronal and non-neuronal origin [117-120].

Neurotransmitter transporters located at the cell surface regulate synaptic signalling and homeostasis of several neurotransmitter systems. Cholinergic neurons function differs from that of other neurons (monoaminergic, GABAergic and glutamatergic), as the enzyme AChE is the major limiting mechanism for controlling ACh at the synapse. The widespread presence of high-affinity CHT in intracellular organelles makes this transport protein different from other plasma membrane neurotransmitter transporters, which are localized predominantly at the cell surface.

It has been suggested that the high-affinity CHT belongs to a Na^+-dependent glucose transporter (SLC5) family, whereas other neurotransmitter transporters belong to Na^+ - and Cl^- -dependent solute transporter family (SLC6) [121]. This hypothesis is inconsis-

tent with evidence of a Cl^- dependence of high-affinity CHT [104,106]. On the other hand, there is only a slight degree of homology between high-affinity CHT and other members of SLC5 family, and there is no evidence of similarity in trafficking motifs with other SLC5 family components.

A feature common to high-affinity CHT and some other plasma membrane neurotransmitter transporters is their clathrin-dependent internalization [122-126]. However, despite this similarity, most of the members of the SLC6 gene family of neurotransmitter transporters are mainly expressed at the cell surface in neurons and cultured cells [104].

4.2. High Affinity-Choline Transporter, Cholinergic Transmission and Cognition

One line of research on the possible relationship between age/neurodegenerative disease-related memory and behavioural changes and neurochemical systems was focused on ACh (e.g.,[127]). It is documented that several neurotransmitters are involved in regulating complex activities such as cognitive functions and that a variety of systems may take a part in the pathophysiology of age-related dementia disorders such as Alzheimer's disease (e.g., [128-131]). There is, however, as above mentioned in the section "brain cholinergic systems", a strong rationale for considering the cerebrocortical/hippocampal cholinergic system related to spatial learning and memory. Lesions of cholinergic projections to the hippocampus produce profound deficits in spatial behaviour [132] and cholinergic deficits have been found to occur during aging and in Alzheimer's disease (e.g., [133-136]).

There is substantial evidence to indicate that a wide range of attention processes are mediated by frontal and parietal cortical networks [137,138] and that forebrain cholinergic systems (mainly cerebrocortical cholinergic projections) represent an integral and necessary component of these networks. The pivotal role of cholinergic system in the cognitive functions was initially demonstrated by studies showing that specific muscarinic receptors antagonists (scopolamine or atropine), induce severe although transitory memory impairment [139,140].

Attentive abilities are generally considered to be an important cluster of variables determining the efficacy of learning process [141]. Cognitive impairment associated with aging, adult-onset dementia and schizophrenia probably depends from abnormalities in the integrity and regulation of cortical cholinergic inputs [142,143]. Cellular mechanisms contributing to acute or sustained changes in cholinergic transmission are not well known. A better knowledge of these mechanisms is important for understanding neuronal mechanisms underlying different levels of attentive performance and dysfunction [144]. Identification of signalling pathways mediating acute and long term plastic changes in the regulation and capacity of the high-affinity CHT is probably an important step for the development of these hypotheses.

Although the role of choline uptake in the synthesis and release of ACh and the ability of other neurotransmitter systems to influence CHT functions have been studied for more than 30 years, molecular mechanisms contributing to the plasticity of CHT functions were clarified only recently. From a technical point of view, only more recent studies have used new methodologies to measure extracellular choline concentrations and have therefore emphasized the impressive role of CHT in intact brain. These investigations have also indicated that regional activation of CHT functions might be associated with attention (see below). There is limited, yet intriguing, evidence that dysregulation in the capacity of the CHT to transport choline is associated with the decline in cholinergic transmission in Alzheimer's disease. Consequently, the signalling pathways regulating CHT represent a target for the development of pharmacological treatments for cognitive impairment related to basal cholinergic forebrain injury. In a recent review, regulation of CHT capacity, plasticity of CHT capacity, clearance of ACh-derived choline, CHT activity and cognitive functions and possible CHT regulation in cognitive disorders were analyzed in detail [145].

High-affinity CHT is expressed mainly by cholinergic neurons and represents an important anatomical marker of cholinergic systems [146,147]. The ACh synthesis rate is affected by the capacity of the high-affinity CHT to transport choline. This process is Na^+-dependent [76] and choline is transported into presynaptic terminals. On the other hand, it is well known that the presynaptic cholinergic activity represents an important factor in CHT capacity regulation. High-affinity CHT capacity has been considered as a marker for the cholinergic transmission status [98,148,149]. There is a relationship between the number of high-affinity CHTs in neuronal membrane and/or their affinity for choline and ACh synthesis or release alterations [145]. Another mechanism of high-affinity CHTs regulation has been indicated by presynaptic activity-dependent translocation of CHTs from cytoplasmic pools to the terminal membrane [150-152].

A quantification of the co-localization of high-affinity CHT and VAChT has revealed that, although all high-affinity CHT-positive vesicles are VAChT-positive, only one-half of these possess high-affinity CHT [151]. On the basis of these findings has been hypothesized that two major subpopulations of cholinergic synaptic vesicles exist and that they are distinguishable by their CHT content. Moreover, it has been speculated that the "VAChT only" pool preferentially supports low rates of ACh release, whereas "CHT/VAChT" pool might be recruited in response to higher firing rates [151]. One question arising from these findings is why levels of high-affinity CHT protein at the plasma membrane are not constitutively at maximal levels to ensure efficient recycling of choline for ACh biosynthesis, regardless of ACh release rates. One possibility is for a role for choline itself as a neurotransmitter, with its extracellular levels modulated *via* the high-affinity CHT activity [151]. The large reserve of high-affinity CHTs on cholinergic synaptic vesicles and the Ca^{2+}-dependent, botulinum neurotoxin C-sensitive regulation of choline uptake indicates a role for trafficking in coupling CHT regulation to ACh release [151].

Previous investigations on CHT regulation have defined models of high-affinity CHT regulation based on the activation of previously silent plasma membrane CHTs [153]. It would be important to re-evaluate these earlier conclusions on the light of more recent data above reviewed to integrate results into a coherent model that can guide future developments of this promising research area.

Several lines of evidence suggest that high-affinity CHTs is regulated by various signalling pathways. Anomalous high-affinity CHT capacity regulation in neurological disorders could reflect pathological processes influencing mainly cholinergic neurons. Dysregulation of high-affinity CHT function might be secondary to diseases altering neuronal circuits afferent to cholinergic neurons. As the cognitive functions of cortical cholinergic inputs are relatively well understood, determination of the regulation of CHT function in disease processes, irrespective of aetiological theories, is probably central to the development of conclusive hypotheses about neuronal mechanisms mediating cognitive symptoms of these disorders [145].

Evidence that supports the involvement of atypical high-affinity CHT regulation in impaired cholinergic transmission and the cognitive symptoms of neuropsychiatric disorders has remained circumstantial. In fact, chronic ethanol exposure suppresses HACU in rat cerebral cortex [154]. This observation could support the hypothesis that inability of non-amnesic alcohol-addicted patients to activate the cholinergic system is partly due to dysfunctional high-affinity CHT capacity [155]. Different studies on schizophrenia, [156,157] support the hypothesis that abnormally reactive cortical cholinergic inputs contribute to the cognitive symptoms of this pathology

probably through interactions with other dysregulated neurotransmitter systems.

5. CHOLINERGIC PRECURSOR LOADING AS A COGNITION ENHANCING STRATEGY

Compounds designed for treatment of age-related dementia disorders including Alzheimer's disease studied using rigorous methodology, act by reinforcement of cholinergic neurotransmission in the central nervous system [13,14]. The above mentioned "cholinergic hypothesis" of age-related memory dysfunction has resulted in the clinical evaluation of three types of cholinomimetic drugs, namely ACh precursors, AChE inhibitors and cholinergic receptor agonists [158]. The use of cholinergic precursors in this therapeutic indication will be discussed below. Based on the findings that after being released at the synapse, ACh is inactivated by AChE, compounds such as physostigmine, acting by slowing down degradation of ACh were developed as well (AChE/ChE inhibitors). The purpose of drugs of this class was to obtain a durable and sustained activation of the postsynaptic receptors by ACh. Injections of physostigmine in healthy subjects and in Alzheimer's disease patients improved memory function. Oral administration of physostigmine produced modest results, coherent with those found after injection of the compound. The AChE inhibitor, tacrine hydrochloride (tetrahydroaminoacridine) was approved by the U.S. Food and Drug Administration in 1990 for relieving cognitive dysfunction of Alzheimer's disease. Tacrine, which is characterized by a pronounced hepatotoxicity demonstrated a moderate improvement in objective cognitive tests with 15 to 20% of all patients treated presenting a therapeutic response greater than that obtained in the placebo groups. Approximately half of all patients receiving tacrine withdrew from the clinical trials for peripheral cholinergic adverse effects or hepatotoxicity. On the whole, treatment with tacrine resulted in little improvement in daily activity scales. ChE inhibitors developed subsequently and now in the pharmaceutical market (donepezil, galantamine and rivastigmine) (Fig. **5**) have a better tolerability and safety profile compared to tacrine and are widely used for countering cognitive dysfunction of Alzheimer's disease [159]. These compounds improve at 6 months the Mini Mental Scale Evaluation (MMSE) from 2 to 4 points and the ADAS-cog scale from 4 to 5 points. Moreover, they affect positively the clinical global impression of caregivers. ChE inhibitors are currently approved for the symptomatic treatment of mild-moderate Alzheimer's disease and were investigated in numerous clinical trials. Critical analysis of the results of the main clinical studies suggests that beneficial long-term effects of these compounds on the cognitive, functional, and behavioural symptoms of Alzheimer's disease are small and not always apparent in practice [160-162]. The progressive loss of cholinergic neurons and synapses, reducing the substrate through which these compounds exert their effect or the development of AChE/ChE isoforms resistant to inhibition (tolerance) are the most probable reasons for the short lasting effects of this class of drugs.

5.1. Choline and High-Affinity CHT

In 1998, choline was identified as an essential nutrient for humans by the National Academy of Sciences, United State [163]. Choline is a quaternary amine (trimethyl-β-hydroxy-ethylammonium) [164] and its concentration in plasma has physiological range of 10-50 μM [86]. Choline is predominantly employed for the synthesis of essential lipid components of the cell membranes, phosphatidylcholine (PC) and sphingomyelin, and for the production of potent lipid mediators such as platelet-activating factor and lysophosphatidylcholine [165]. Quantitatively, PC is the most important metabolite of choline and accounts for approximately one-half of the total membrane lipid content [166]. Finally, choline is best known for its key role in neurons as precursor of the neurotransmitter acetylcholine [167,168]. Adequate intake level for choline is 550mg/day for men and 425 mg/day for women [169]. Choline content varies considerably among common foods [170] and dietary choline supplementation could improve multiple body function, including cognition [171], learning, and memory [172,173]. Therefore, choline is an essential dietary component for the normal functioning of organisms.

Because it is predominantly utilized for membrane PC, choline has a significant impact on liver function and systemic lipid metabolism. In experimental models as well as in humans [174-177], choline deficiency results in the formation of "non-alcoholic fatty liver", associated with accumulation of hepatic lipids and can increase sensitivity to inflammation [178]. The majority of cellular choline is phosphorylated by choline kinase (CK) to phosphocholine, to which CTP: choline-phosphate cytidylyltransferase (Pcyt1) can then be added by Pcyt1 to yield CDP-choline. The formation of PC results from the reaction of CDP-choline with diacylglycerol (DAG), catalyzed by CDP-choline: DAG choline phosphotransferase. This pathway is known as the Kennedy's (CDP-choline) pathway for *de novo* synthesis of PC and is essential for the formation of membrane PC in all nucleated cells.

The synthesis of the neurotransmitter ACh in cholinergic neurons depends on the availability of its precursor choline, most of which is derived from the circulation (reviewed in [167]) and enters cholinergic neurons *via* a process catalyzed by a specific transporter [6,179]. Part of choline used for ACh synthesis is stored in membrane PC. Previous studies have shown that this choline is liberated from PC in a one-step reaction. This indicates that it is produced by phospholipase D an enzyme previously mentioned. This enzyme hydrolyzes phospholipids into choline and phosphatidic acid (PA) [180]. The molecular identity of PLDs (1 and 2) was largely investigated and extensively reviewed [41,181].

A compartmentalization of choline and ACh metabolism in cultured sympathetic neurons was detected. This investigation has demonstrated that choline uptake in cell bodies of proximal and distal axons is different both in terms of rate and mechanism of incorporation [182]. These differences could modify ACh biosynthesis and influence cholinergic system functioning.

5.2. Choline Precursors and High-Affinity CHT

As the role of choline in neurobiology has been largely established, here we will discuss the possible role of choline precursors in treatment of adult-onset cognitive dysfunction [13,183].

Nervous tissue cannot synthesize choline, which is ultimately derived from diet and is delivered to neurons through the blood stream. ACh released from cholinergic synapses is hydrolyzed by AChE into choline and acetyl coenzyme A. Almost 50% of choline derived ACh hydrolysis is recovered by a high-affinity CHT. As a consequence, neurons require a further supply of choline for the new synthesis of ACh [184]. Cholinergic neurons have apparently a particular avidity for choline. If choline is not provided in enough amounts, neuron can obtain it by hydrolyzing membrane phospholipids. It was hypothesized that this auto-cannibalism mechanism may have a role in cholinergic neuron degeneration occurring in Alzheimer's disease [185].

Parallel with the development of cholinergic approach of geriatric memory dysfunction [127] and on the basis of encouraging results obtained with L-DOPA in the treatment of Parkinson's disease, choline precursors were the first pharmacological approach tried in the treatment of Alzheimer's disease [186]. Despite cheering up early results [187,188], controlled clinical trials did not confirm a clinical usefulness of choline and lecithin (phosphatidylcholine) in the treatment of cognitive dysfunction in Alzheimer's disease. These negative clinical results did not reject the hypothesis that choline supplementation could contribute to prevent membrane

damage due to intense cholinergic activity [186]. The causes of the lack of success of this therapeutic strategy are unclear. In spite of many studies, several aspects of choline deposition, transport, metabolism and utilization for ACh synthesizing were not clarified yet. On the other hand, recent critical reviews of the main clinical studies assessing the activity of cholinergic precursors in the treatment of adult-onset dementia disorders suggested that the lack of clinical benefits obtained with choline or lecithin are not shared by other phospholipids involved in choline biosynthetic pathways such as cytidine 5′-diphosphocholine (CDP-choline) and alpha-glyceryl-phosphorylcholine (choline alphoscerate). For these two compounds and particularly for choline alphoscerate, a modest improvement of cognitive dysfunction in dementia of neurodegenerative and vascular origin is documented [13,189].

Fig. (**6**) summarizes choline anabolic pathways. As shown, the enzyme glyceryl-phosphorylcholine diesterase transforms alpha-glyceryl-phosphorylcholine into a molecule of choline and another of glycerol-1-phosphate. Choline can be used to synthesize ACh, whereas glycerol-1-phosphate after being phosphorylated can enter in the pool of phospholipids [190]. These pathways provide both free choline and phospholipids for preparing ACh and to reorganize nerve cell membrane components. Free choline administration increases brain choline availability but it does not increase ACh synthesis/or release [191,192]. This could represent the reason of its ineffectiveness in relieving cognitive symptoms of Alzheimer's disease [13,189]. Probably, cholinergic precursors to serve for ACh biosynthesis should be incorporate and stored into phospholipids in brain [193].

Putative ACh precursors proposed to increase ACh brain levels were lecithin, phosphatidylserine, CDP-choline and choline alphoscerate. Preclinical investigations have demonstrated that lecithin increased brain choline and ACh concentrations [193,194], although lecithin effect on ACh synthesis was not confirmed [195]. This lecithin behaviour was justified by suggesting a mechanism involving ACh release stimulation [196]. ACh content and its release were increased by treatment with both phosphatidylserine [197] and CDP-choline [198,199]. The same results were obtained with choline alphoscerate [200]. Choline alphoscerate induced a rise in plasma choline level higher than that elicited by CDP-choline [201]. The reason of the different effects of the above compounds on ACh synthesis and release are unknown. Comparatively, lecithin provided the least effects on ACh biosynthesis and was ineffective on cognitive dysfunction, whereas other choline precursors (CDP-choline, choline alphoscerate) investigated in clinical trials, provided more consistent effects [186,202].

The pharmacological and clinical role of CDP-choline was recently reviewed [183]. In an experimental rat model of acute induced ischemia [203] the integrity of blood-brain barrier (BBB) was investigated by labelled iodinate albumin, and brain metabolism by enzyme histochemistry. In this model, administration of CDP-choline reduced vasogenic cerebral edema and restored BBB integrity. The size of brain infarctions was smaller in the CDP-choline-treated group and the compound decreased the activity of lactate dehydrogenase, succinyl dehydrogenase, monoamine oxidase, and acid phosphatase, emphasizing its protective role through a direct action at cell membrane level [203]. The CDP-choline effect on cognitive performance was analyzed using a model of traumatic brain lesion in rats, induced by a controlled lateral impact [199]. The results demonstrated that animals treated with CDP-choline had significantly less cognitive deficits than saline treated ones [199]. The ACh release also was increased after a single CDP-choline administration in some cerebral areas involved in cognitive function (e.g. hippocampus) [199]. The pharmacological profile and clinical activity of choline alphoscerate was also recently reviewed [202]. It has been hypothesized that the activity of these cholinergic precursors on cognitive function in adult-onset dementia disorders is related to their influence on ACh biosynthesis and availability [13,189].

Both CDP-choline and choline alphoscerate probably interfere with membrane phospholipid reconstruction. The enhanced ACh release induced by both CDP-choline and choline alphoscerate [13] treatment suggests an important role for the high-affinity CHT in restoring choline availability leading to a more effective ACh synthesis. Further work on the influence of the above cholinergic precursors on high-affinity CHT could provide new insights on their mechanisms of action and role on cholinergic synaptic machinery.

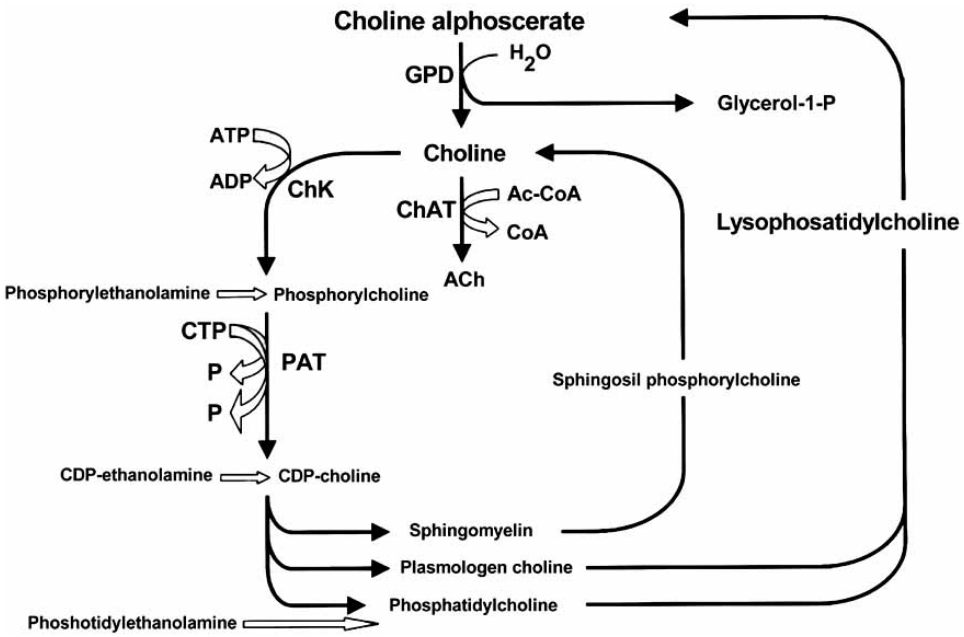

Fig. (6). Acetylcholine synthetic pathways showing the steps in which CDP-choline and choline alphoscerate can influence neurotransmitter biosynthesis. Cythidin diphosphate (CDP); Cythidin triphosphate (CTP); Glyceryl-phosphorylcholine diesterase (GPD); Choline acetyltransferase (ChAT); Choline kinase (ChK); Phosphocholine cytidyl transferase (PCT).

6. ACETYLCHOLINE RELEASING AGENTS

Another strategy proposed years ago to counter impaired cholinergic neurotransmission in adult-onset dementia disorders was the synthesis of different families of therapeutic agents enhancing ACh release (ACh releasers) [203,204]. Different from the ACh degradation inhibition and cholinergic precursor loading approach, this strategy did not provide drugs in clinical use.

CONCLUSIONS

Unfortunately, at the moment, adult-onset dementia disorders characterized by cognitive impairment and brain cholinergic hypofunction have no adequate therapy. Considering preclinical and clinical evidence available, it is conceivable to hypothesize that appropriate ACh precursors and other correlated molecules (natural or synthesized) could represent a tool for developing newer therapeutic strategies by revisiting and updating treatments/supplementations coming out from this therapeutic stalemate. In this respect, CDP-choline and choline alphoscerate, besides acting as compounds increasing ACh availability, probably interfere with membrane phospholipid reconstruction as well.

The positive effects observed with these compounds, being those elicited by choline alphoscerate treatment the most relevant [13,189], suggest that an interference with high-affinity CHT could contribute to restore choline availability bringing to a more effective ACh synthesis. CHT and VAChT regulate synaptic availability of choline and ACh and facilitate ACh release. Compounds affecting CHT and VAChT expression such as selected cholinergic precursors (personal unpublished data) may possess an additional property beyond those already known worthwhile of being deepened in future studies.

Although the role of cholinergic precursors on high-affinity CHT was not assessed in detail, it could represent a challenge for revisiting already available and developing new cholinergic precursor strategies aimed at enhancing impaired cholinergic neurotransmission in adult-onset dementia disorders.

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