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REVIEW

The Transport of Choline

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ABSTRACT

Choline has many physiological functions throughout the body that are dependent on its available local supply. However, since choline is a charged hydrophilic cation, transport mechanisms are required for it to cross biological membranes. Choline transport is required for cellular membrane construction and is the rate-limiting step for acetylcholine production. Transport mechanisms include: (1) sodium-dependent high-affinity uptake mechanism in synaptosomes, (2) sodium-independent low-affinity mechanism on cellular membranes, and (3) unique choline uptake mechanisms (e.g., blood–brain barrier choline transport). A comprehensive overview of choline transport studies is provided. This review article examines landmark and current choline transport studies, molecular mapping, and molecular identification of these carriers. Information regarding the choline-binding site is presented by reviewing choline structural analog (hemicholinium-3 and 15, and other nitrogen/methyl-hydroxyl compounds) inhibition studies. Choline transport in Alzheimer's disease, brain ischemic events, and aging is also discussed. Emphasis throughout the article is placed on targeting the choline transporter in disease and use of this carrier as a drug delivery vector.

Key Words: *Choline; Hemicholinium; High-affinity; Low-affinity; Transport*

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BACKGROUND

Physiological Roles of Choline

Choline was first discovered by Strecker[1] in 1862 and has since been studied extensively as a nutrient, a component of cell membranes, and the precursor to the neurotransmitter acetylcholine.

Though diet is the major source of physiological choline,[2] it was nearly a century after its discovery that the National Academy of Sciences recognized the cation as a required nutrient, and suggested minimum recommended daily intake amounts.[3] While humans can synthesize choline *de novo*, dietary choline is considered essential for prenatal memory development,[3] liver function,[2,4] and as the principal source for methyl groups in the body.[3,4] Removal of choline from the diet reduces the synthesis of acetylcholine[5] differentially in cholinergic brain regions.[6] Fortunately, choline plasma levels are highly regulated and remain relatively invariable at ~10 μM in animals and man.[7] However, variability of plasma levels occurs with changes in choline intake. Choline-deficient diets can reduce plasma levels by approximately 50%,[2] and ingestion of choline-rich foods can increase plasma levels to 20 μM.[8]

Stabilization of plasma choline concentrations is a result of *de novo* choline synthesis from the catabolism of phosphatidylcholine (the major lipid found in eukaryotic cell membranes). Catabolism is completed by phospholipases (PL) hydrolyzing their respective bonds (i.e., PLA1 and PLA2—fatty-acyl bonds, PLC—glycerphosphophate bond, and PLD—choline phosphate ester bond). Further, lysoPL degrades lysophosphatidylcholine, which is subsequently converted to glycerolphosphocholine and further hydrolyzed to choline by a phosphodiesterase (Fig. 1a).[9] A distinct pathway occurs for the resynthesis of phosphatidylcholine. Synthesis occurs by the transfer of CDP-choline to diacylglycerol or the methylation of phosphatidylethanolamine from *S*-adenosyl-L-methionine. This reaction is catalyzed by phosphatidylethanolamine *N*-methyltransferase, which creates phosphatidylcholine (Fig. 1a).[10] Recycling of phosphatidylcholine in this manner provides a physiological choline reservoir, regardless of dietary intake. This reservoir supply of choline can be utilized in lipid membrane construction and for acetylcholine production in the presynaptic cholinergic terminal.[11,12]

Choline has been studied extensively as a neurotransmitter precursor. Briefly, this process consists of

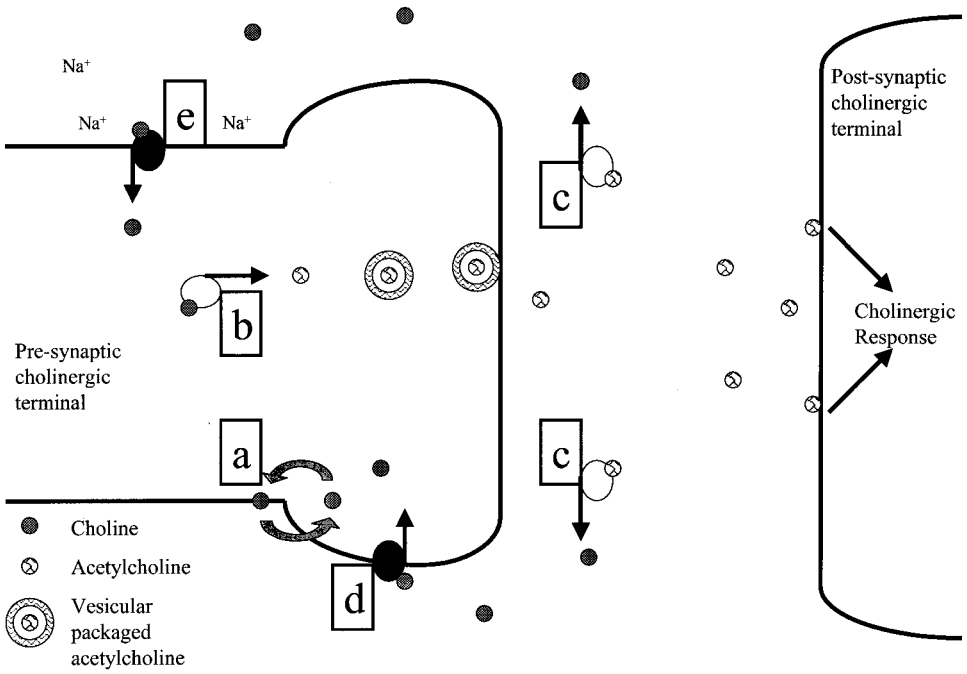

Figure 1. Depiction of choline transport and fate at cholinergic nerve terminals.

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acetylcholine synthesis from choline and acetyl–CoA via choline acetyltransferase (Fig. 1b). After release of acetylcholine into the synaptic cleft, acetylcholine is then hydrolyzed to choline and acetate by acetylcholinesterase (Fig. 1c). Choline is then free to be taken up into the presynaptic nerve terminal for recycling. This process demonstrates that the supply of choline is essential for normal cholinergic nerve functioning.

As mentioned above, choline has many physiological functions throughout the body dependent on available local choline supply. However, since choline is a charged hydrophilic cation at physiological pH (see Fig. 2), it cannot appreciably diffuse across cellular membranes in required quantities. As a result, choline transport is significant in cellular membrane construction and is the rate-limiting step for physiological cholinergic neurotransmission. The purpose of this article is to review landmark and current choline transport studies, theories in choline movement, and possible future therapeutic applications of the transporter. Specifically, this review has attempted to limit itself in scope to choline transport. There are excellent reviews on various aspects of choline and acetylcholine metabolism, structure activity studies, choline incorporation into phospholipids and the role of choline in disease. For further information on these topics, the reader is referred to the articles cited throughout the text.

PHARMACOLOGICAL EVALUATIONS

Choline Transport Mechanisms

Transport of a molecule across cellular membranes in most instances is characterized by: (1) translocation of the molecule across the membrane, (2) chemical specificity for the given ligand, (3) optical specificity, (4) competition for structurally similar compounds, and (5) Michaelis–Menten saturability. Choline transport characteristics are also demonstrated similarly: (1) translocation or uptake of choline across cellular membranes, (2) choline affinity and affinity for compounds that have a quaternary nitrogen coupled with a nitrogen–oxygen distance of approximately 3.26 Å,[13] (3) translocation of the choline R (+) enantiomer and not the S (−) enantiomer, (4) competition with similar nitrogen-methyl compounds (see Fig. 2), and (5) demonstration of Michaelis–Menten saturability.

Choline transport is demonstrated classically in two major systems classified by the degree of affinity for choline. Each system has unique defining characteristics with regard to location, ion dependence, Michaelis–Menten kinetics, and stereospecificity of analogs and inhibitors. See Table 1 for a summary of the two primary choline transport mechanisms.

This section will discuss traditional categories of choline transport and recent reports of choline transport that cannot be categorized into established classes. See Fig. 1 for an illustrative example of choline mechanisms at the cholinergic presynaptic terminal. This area demonstrates both high- and low-affinity choline transport mechanisms.

Low-Affinity Choline Transport

Traditionally, low-affinity choline transport (Fig. 1d) is defined as having a K_m greater than 30–100 μM.[11] Low-affinity choline transporters are found throughout multiple tissues[14–28] such as enterocytes, hepatocytes, placental tissue, mitochondria, and synaptosomes. Choline transport via this mechanism primarily supplies choline for the synthesis of phosphatidylcholine and other phospholipids[29] in the cellular membrane. This transporter has been considered to be linearly dependent on choline concentration, to operate by passive diffusion, and to be sodium-independent for operation.[30]

However, recent studies have demonstrated that the low-affinity transporter is carrier-mediated. For example, choline transport in rat cortical synaptosomes has been shown to be an active sodium-independent carrier-mediated process with a K_m of 144 μM and a V_{max} of 77 pmol/min/mg.[28] The authors conclude the process is carrier-mediated after observing the sodium-independent uptake of the R (+) enantiomer of α-methyl-choline and not the S (−) enantiomer. This stereospecificity suggests a protein independent of the high-affinity transporter on the synaptosome, translocating choline into the cell.

The ability of the low-affinity choline transporter to be inhibited by various choline structural analogs[19,20,22,23,25] is also characteristic of transport mechanisms. The defining substrate that demonstrates reversible uptake inhibition is hemicholinium-3.[31] Grassl demonstrated low-affinity choline uptake could be inhibited by hemicholinium-3 with an apparent K_i of ~100 μM.[22] This data is in agreement with other low-affinity uptake

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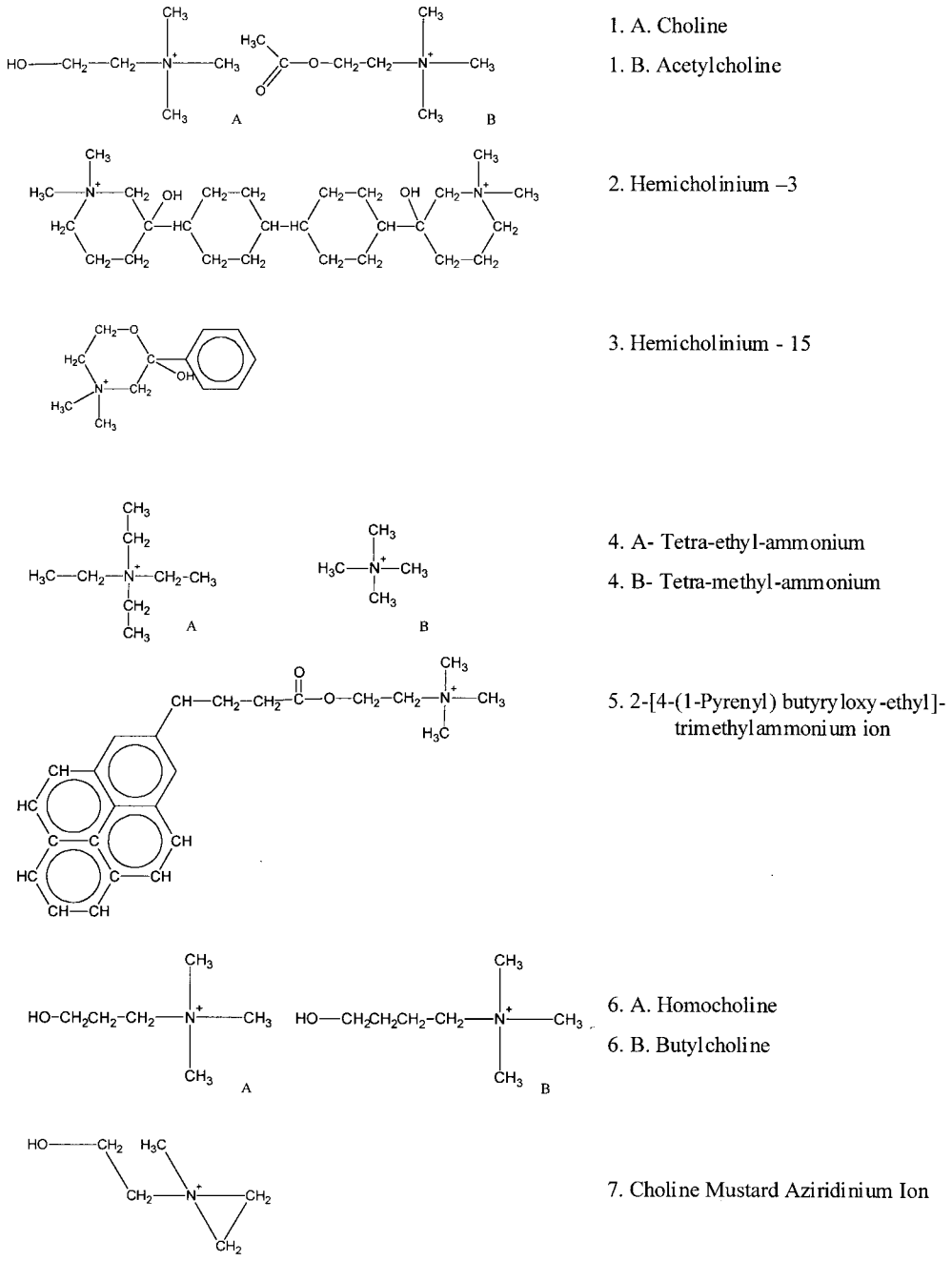

Figure 2. Structure of choline and various choline analogs utilized in pharmacological inhibition studies.

mechanisms inhibited by hemicholinium-3 in the micromolar range.[19,20,23,25]

Further evidence that low-affinity choline transport is carrier-mediated was demonstrated in rat liver basolateral plasma membranes. Choline transport in this tissue exhibits sodium-independence and temperature-dependence, with a K_m of 340 μM and a V_{max} of 1800 pmol/min/mg. Further, transport in this system is cis-inhibited by hemicholinium-3 and acetylcholine. The characteristics of choline transport demonstrated in rat liver basolateral plasma membranes demonstrate characteristics of protein-mediated transport.

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Table 1

Choline Transport Mechanism Comparison

Low-Affinity
• Choline $K_m \sim >30–100\,\mu M$
• Location: ubiquitous
• Function: provide choline for phospholipid synthesis
• Hemicholinium $K_i \sim 100\,\mu M$ (μM range)
• Na^+-independent
High-Affinity
• Choline $K_m \sim <10\,\mu M$
• Location: presynaptic cholinergic nerve terminal
• Function: provide choline for acetylcholine synthesis
• Hemicholinium $K_i \sim 0.001–0.1\,\mu M$
• Na^+, Cl^--dependent

Choline transport in the low-affinity system is typically independent of sodium.[19,21–25,28] For instance, choline transport at maternal and fetal interfaces of guinea-pig placenta is carrier-mediated with a K_m of 120 μM. Replacement of sodium with tris or lithium, abolishing the sodium gradient, has no effect on choline transport.[19]

Not all studies demonstrate low-affinity choline uptake and sodium-independence, as is evident in isolated enterocytes of guinea pig. Choline transport in this system has a K_m of 119 μM and uptake is reduced when sodium is replaced in the medium by potassium. Transport is also reduced by inhibition of the Na^+/K^+ ATPase by oubain.[25] Similar studies[14,15] indicate not all low-affinity choline uptake is sodium-independent.

In summary, low-affinity choline transport is typically characterized by sodium-independent carrier-mediated uptake. The average affinity for choline is >20–30 μM and is a saturable process. Furthermore, low-affinity choline uptake is defined by stereospecificity and can be inhibited by similar nitrogen-methyl compounds.

High-Affinity Choline Transport

Unlike the low-affinity system that provides choline for membrane phospholipids, the high-affinity system (Fig. 1e) is localized to pre-synaptic cholinergic nerve terminals and possibly coupled with acetylcholine synthesis.[32] Autoradiography with [^{3}H]-hemicholinium binding demonstrates high concentrations of high-affinity choline uptake sites in the interpeduncular nucleus, caudate putamen, and the olfactory tubercle. The distribution of binding sites correlates well with brain areas rich in cholinergic nerve terminals[33,34] and remains similar throughout development.[35] Further, traditional high-affinity choline transport is defined as carrier-mediated, sodium-dependent,[26] possibly chloride-dependent,[36] and having a $K_m \sim <10\,\mu M$.[11]

The high-affinity choline uptake mechanism provides choline for acetylcholine synthesis within the presynaptic terminal[26] and may be regulated by cytoplasmic acetylcholine concentrations.[37] However, there is controversy as to whether the transporter in cholinergic nerve terminals is independent or functionally coupled with presynaptic choline acetyltransferase, acetylcholinesterase, or butylcholinesterase**.** Considering the distribution of choline binding sites, demonstrated by [^{3}H]-hemicholinium binding, there is suggested to be a positive relationship to acetylcholinesterase location in rat brain.[33] Selective inhibition of acetylcholinesterase decreases acetylcholine synthesis[38] and the V_{max} of choline transport[34] by approximately 50%. In contrast, when a selective butylcholinesterase inhibitor is utilized, a small, though significant, rise in choline V_{max} is observed. This may suggest the two enzyme systems are coupled to the transport protein working in a divergent manner. An alternative explanation may be that acetylcholine synthesis and choline uptake are suppressed after increased concentrations of synaptic cleft choline are present, secondary to decreased acetylcholinesterase activity.

There is also evidence that choline transport may be linked to intracellular choline production via choline acetyltransferase.[39] Raiteri et al.[40] showed that choline acetyltransferase and the high-affinity choline uptake transporter are located in close proximity on rat brain cholinergic nerve terminal membranes. Further, the rate of synthesis of [^{3}H]-acetylcholine from [^{3}H]-choline appears to approximate the maximal activity of the high-affinity choline uptake system.[41] However, this coupling has been kinetically challenged[42,43] and the sodium-dependent high-affinity choline uptake system in the cardiac ganglion of the horseshoe crab provides choline to the presynaptic terminal, but is not linked to acetylcholine production. This was demonstrated by the lack of [^{3}H]-acetylcholine production after the addition of [^{3}H]-choline to the extracellular environment.[44]

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While further work needs to be completed to demonstrate a tangible coupling between terminal choline uptake and acetylcholinesterase function, choline uptake remains the rate-limiting regulatory step in acetylcholine synthesis.[45] Nevertheless, considering the relative constant plasma and brain levels of choline,[7] and that adequate acetylcholine synthesis occurs with choline concentrations approximately one-tenth of the high-affinity transporter K_m,[46] it is unlikely significant reductions in acetylcholine synthesis will occur in the absence of pathophysiology.

The dependence of high-affinity choline transport on sodium is well documented.[15,26,41,44,47–56] The landmark study by Yamamura and Snyder[26] demonstrates that replacement of sodium by either tris or lithium decreased [^{3}H]-choline (near the K_m of the transporter) uptake in rat synaptosomes by ~95%. Simon and Kuhar[57] verify these results in synaptosomes and further demonstrate chloride- and energy-dependence. Additionally, replacement of external sodium with lithium or potassium resulted in decreased choline accumulation by human erythrocytes[56] of ~60%. These studies demonstrate, after a kinetic correction for low-affinity transport, that high-affinity choline transport in synaptosomes is dependent on a sodium-choline co-transporter or, at minimum, an energy-, sodium-, chloride-dependent uptake system.

Similar to the low-affinity choline transport mechanism, the high-affinity mechanism can also be inhibited by hemicholinium-3. However, inhibition sensitivity is much greater in the high-affinity system. Barker and Mittag[32] demonstrated hemicholinium-3 inhibition of high-affinity choline transport in rat forebrain synaptosomes with an apparent K_i of ~0.08 μM. This data is in strong agreement with other established K_i values of 0.001–0.1 μM.[27,35,58] Since hemicholinium-3 is not transported,[59] it is a superb tool in autoradiography for determination of cholinergic binding sites.[35] Therefore, the determination of hemicholinium-3 K_i values is an effective tool in evaluation of high- vs. low-affinity choline uptake mechanisms.

In summary, high-affinity choline transport is typically characterized by sodium-dependent carrier-mediated uptake in presynaptic cholinergic nerve terminals. The average affinity for choline is $\ll$20 μM and is a saturable process. Furthermore, high-affinity choline uptake is defined by high sensitivity to inhibition by hemicholinium-3.

Unique Choline Transport Mechanisms

Not all choline transporters can be classified into the traditional systems. Table 2 lists choline transport mechanisms characterized in a variety of tissues. One good example of a unique choline transport mechanism is the basic amine transporter found at the blood–brain barrier.

For choline to maintain its physiological role in the CNS, it must cross the blood–brain barrier. The barrier regulates the exchange of hydrophilic compounds between plasma and the CNS and is comprised of a layer of endothelial cells connected by tight junctions at the brain capillaries and the choroid plexus epithelium. The endothelium junctions (*zonulae occludens*) are 100 times tighter than junctions of other capillary endothelia.[86] Thus, the barrier has many of the same properties of a continuous cell membrane that allows lipid-soluble molecular diffusion across the membrane, whereas hydrophilic solutes demonstrate minimal blood–brain barrier permeation.[87]

Given that: (1) the brain has a high demand for choline, (2) the brain is not able to synthesize choline *de novo* in sufficient amounts for cholinergic functioning,[88] (3) physiological plasma levels of choline enter the brain in linear fashion,[89,90] and (4) choline is a charged cation at physiological pH, brain cholinergic function appears intimately dependent on choline transported from the plasma.[91,92]

Choline transport at the blood–brain barrier has been demonstrated in vivo[80,93–95] and in vitro[74,96,97] to be carrier-mediated and saturable. Considerable differences have been observed in the literature regarding the actual affinity for the carrier. Early studies have choline K_m with a range of 180–450 μM,[93,98–101] but more recent studies suggest the K_m to be much lower and approximate the range of some high-affinity mechanism.[76,80] Using a rat brain microvessel endothelial cell line (RBE4), the K_m was calculated to be 20 μM,[76] demonstrating close agreement to rat brain in situ perfusion study K_m calculation of ~4 μM.[80] These earlier studies may have overestimated the K_m of the transporter secondary to the utilization of low specific activity choline {[^{14}C-methyl]choline (40–60 mCi/mmol)} in brain uptake index experiments. Utilization of low specific activity choline would add approximately 50–75 μM of unlabeled choline to the injectate and consequently decrease the apparent affinity by self-inhibition.[80]

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Table 2

Choline Transport Mechanisms Characterized in a Variety of Tissues. The Table Is Arranged by Decreasing K_m for Choline. The V_{max} Data Are Adapted from Original Studies to Units of pmol/min/mg When Possible. When Two K_m Values Are Shown in the Table for One Tissue, the First Is the Calculated K_m in the Presence of Na^+ and the Second Is Calculated Without Na^+. However, Exceptions to K_m Data Are as Follows: (1) Ref. [66], Values Refer to #1 Random Males, #2 Random Females, #3 Females at Proesterus, and #4 Females at Esterus; (2) Ref. [19], Values Refer to #1 Maternal Side, #2 Fetal Side and Ref. [61], Values Refer to #1 opuB Mutation, #2 opuC Mutation; (3) Ref. [26], Values Refer to #1 Corpus Striatum, #2 Cerebral Cortex

Reference	Transporter Location	K_m (μM)	V_{max}	HC-3 IC_{50} (μM)	Ion Dependence	Inhibitors
41.	Rat atria	0.2	6000 pmol/min/mg		Na^+	
47.	Mice cortical synaptosomes	0.9	71 pmol/mg/min		Na^+	
60.	Human Y79	0.93	19.6 pmol/min/mg		Na^+	Ethanolamine decreased
	retinoblastoma cells	9.74	79.25 pmol/min/mg			by 10-fold
61.	*Bacillus subtillis*	1.0	2100 pmol/min/mg			
		38	7500 pmol/min/mg			
26.	Rat brain synaptosomes	1.43	45×10^{-3} pmol/mg/min	1.1	Na^+	Potassium cyanide,
		3.06	30×10^{-3} pmol/mg/min			dinitrophenol, iodoacetamide,
						N-ethylmaleimide,
						Ach, atropine,
						chlorpromazine, bretylium,
						guanethidine, neostigmine
62.	Rat brain synaptosomes	1.44			Na^+	
48.	Rat lung Type II	1.51		1.7	Na^+	
	epithelial cells	18.6				
28.	Rat cortical synaptosomes	1.6	12 pmol/min/mg		Na^+	
		139	139 pmol/min/mg			
54.	*Torpedo marmorata*	1.7		25	Na^+, Cl^-,	Temperature-sensitive
	vesiculated					
	presynaptic plasma					
	membrane fragments					
63.	*Hymenolepis diminuta*	2.0	0.15 pmol/mg/min		K^+	HCO_3-dependent[73]
		20	2.0 pmol/mg/min			
64.	Rat brain synaptosomes	2.04	22 pmol/min/mg			
44.	Horseshoe crab	2.2	0.16 pmol/mg/min	+	Na^+	
	cardiac ganglion	92.3	3.08 pmol/mg/min			
65.	Guinea-pig brain	3.6	89 pmol/mg/min	1.0	Na^+	Propranolol, phentolamine,
						1-Norepinephrine
						stimulates uptake
49.	A549 lung	4		0.1	Na^+	
	membrane vesicles					

(*continued*)

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Continued.

Reference	Transporter Location	K_m (μM)	V_{max}	HC-3 IC_{50} (μM)	Ion Dependence	Inhibitors
66.	Rat forebrain microvessels	6.1	10.6 pmol/mg/min	14		
		12.6	28.4 pmol/mg/min			
		14.0	54.9 pmol/mg/min			
		2.6	19.2 pmol/mg/min			
67.	Human erythrocytes	7.4	14.2 μmol/hr/cell			
68.	Simian erythrocytes	8.5	0.2 nmol/10^{10} cells/min			Decamethonium
51.	Cultured fibroblasts	10		+	Na^+	
50.	14-day-old embryonic chick ventricles	10	77 pmol/mg/min	+	Na^+	Low temperature
69.	Rat diaphragm	11.8	0.61 pmol/mg/min	Cooperative effector	Ca^+	
70.	PC-12 cells	12	270 pmol/mg cell protein/min	(−)	Na^+	
52.	Bovine brain capillaries	17.8 non-saturable to 200 μM	11.3 pmol/mg/min		Na^+	Oubain
71.	Rat lung alveolar Type II epithelial cells	18	213 ± 44 pmol/min/μL cell water	100	Independent	*N*,*N*′-Dimethylethanolamine (apparent K_i, 7 μM), *n*-decylcholine (apparent K_i, 0.5 μM).[72]
53.	Mouse cortical neurons	19.8	334 pmol/mg/min	+	Na^+	Monoiodoacetate
74.	Mouse brain capillaries	20	423 pmol/mg/min		Independent	
75.	Human glioma cells	20	56 pmol/min/10^6 cells	+		$HgCl_2$, phosphodiesterase inhibitors, acetylcholine
76.	Rat brain microvessel endothelial cell line (RBE4)	20		50	Independent	1-Methyl-4-phenylpyridinium, 1-methyl-4-1,2,3,6-tetrahydropyridine, clonidine, procainamide, tetramethylammonium
26.	Rat brain striatum synaptosome mitochondria	29	195×10^{-3} pmol/mg/min			
56.	Human erythrocytes	30	0.22 μM/L cell/min	+	Na^+	Cs^+, Rb^+, K^+, Li^+[77]
15.	Superior cervical sympathetic ganglia	30.7	286 pmol/min/mg	+	Na^+	
16.	Rat mammary-gland epithelial cells	35	20.6 pmol/min/mg	+	Ca^{2+}	Na, K, Li, sucrose
18.	*Schistosoma mansoni*	36	270×10^{-3} pmol/mg/min			

78.	Caco-2 cells	39	0.14 nmol/min/mg	+	Ca^{2+}[79]	pH-dependent, etilfrin, atropine, atenolol, clonidine
80.	In situ brain perfusion, rat capillary endothelium	40	2.4–3.1 pmol/min/mg	57	Independent	Mn^{2+}, Cd^{+},[81] and Na^{+}[80]
82.	*Staphylococcus aureus*	46	54 pmol/min/mg			
17.	Rat jejunum, in situ ligated loop	47	4.1 nmol/ml icf/min	+		2,4-Dinitrophenol
14.	Renal inner medulla	80	120 pmol/μcell water/min	+	Independent	Ethanolamine, *N*,*N*-dimethylethanolamine K^{+} addition (50 mM) lowered transport 19%
83.	Chick intestinal segments	110	25 nM/mL tissue water/min	+	Independent	Na^{+}, *N*,*N*′-dimethylethanolamine, atropine, tubocurarine, decamthonium, thiamine. Others in the study provide <50% inhibition
25.	Guinea-pig isolated enterocytes	119	208 pmol/mg/min		Na^{+}	Actimycin, ouabain
19.	Perfused guinea-pig placenta	120 130	80 pmol/min/mg 70 pmol/min/mg	+	Independent	Ethanolamine, *N*,*N*-dimethylethanolamine
24.	Isolated perfused rat liver	170	0.84 mM/min/g			
20.	Rat proximal tubule—stop flow microperfusion	180	0.43 pmol/cm/sec		Independent	Amines with high pK_a's
23.	Rat liver inner mitochondrial membrane	220	0.4 nmol/mg/min	17		Hemicholinium-15, quinine, quinidine
22.	Human placental brush-border membranes vesicles	300	30 nmol/ng/min	100	Independent	Imipramine, verapamil, propranolol, quinine, flurazepam, amiloride, and ritodrin
21.	Rat liver basolateral membrane vesicles	340	1800 pmol/mg/min	+	Independent	Acetylcholine
84.	Cerebrospinal fluid ventriculocisternal perfusion		70.5 ng/min (T_{max})			K_t 2.2 μg/min ouabain, hexamthonium, Li^{+} increases transport out—frog choroids plexus.[85]

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While the K_m of the transporter nears that of the system in high-affinity synaptosomes, other characteristics bear greater resemblance to low-affinity systems. These characteristics include sodium-independence, no evidence of enzymatic coupling, and hemicholinium inhibition. For example, removal of sodium from the extracellular medium demonstrated no significant change in choline uptake in mouse brain capillary endothelial cells.[74] These results have also been verified using in situ rat brain perfusions with sodium-free perfusate.[80,81,98] Further, the basic amine transporter does not appear to be coupled to the enzyme acetylcholinesterase. Drewes and Singh[102] demonstrate no inhibition of choline brain influx after administration of the acetylcholinesterase inhibitors soman and sarin.

Similar to the K_m, the IC_{50} of hemicholinium-3 (Fig. 2.2) for the blood–brain barrier choline transporter is closer to low-affinity transport mechanisms when compared to synaptosomal transport. In vitro, the calculated K_i for hemicholinium-3 is approximately 50 μM,[76] in agreement with in vivo calculations of ~55 μM.[80] This estimate exceeds the high-affinity choline transporter K_i by one to two orders of magnitude.

The previously described data depict choline transport across the blood–brain barrier as unique when compared to traditional models. Transport of choline into brain is carrier-mediated and saturable, similar to both mechanisms. The affinity for choline is similar to the high-affinity mechanism, whereas the characteristics of sodium-independence, non-coupling with acetylcholinesterase, and the hemicholinium IC_{50} are similar to low-affinity choline transport. The amalgamate of characteristics at the blood–brain barrier may suggest a unique transport protein or unique transport characteristics based on physiological location and the requirement for exogenous choline in the central nervous system.

Monovalent Cation Competition

The relationship of sodium and choline transport is complex. Not only do the traditional models diverge in dependence, there are also exceptions for each mechanism. Examples of these exceptions include intermediate-affinity sodium-independent transport at the blood–brain barrier[80] and low-affinity sodium-dependent choline uptake in guinea-pig enterocytes.[25] Many studies use the replacement of sodium with lithium, tris, or potassium in the extracellular environment to assess sodium dependency. However, this method does not determine if sodium is competing with choline for binding to the transporter or acting as a co-transport requirement. Competition of sodium and choline has been demonstrated at the blood–brain barrier by isoosmotically replacing sodium with sucrose. After replacement, permeability (uptake across the barrier) of choline increases by approximately 50%. Further, this increase is not a result of barrier deterioration or non-specific transporter interactions.[80,81] Reduction of choline uptake was seen in the presence of monovalent cations (cesium, lithium, and potassium)[80] and divalent metal cations [manganese(II) and cadmium][81] at the blood–brain barrier. Martin[77] also demonstrated monovalent cation competition in erythrocytes. The cation affinity for the carrier decreased in the following order: cesium > rubidium > potassium > lithium and sodium. The above studies demonstrate that monovalent and divalent cations have the ability to compete with choline at the binding site and that sodium likely acts to compete with the active site rather than acting as a co-factor in choline uptake.

Active Site Characterization: Structure–Activity Relationships

Two primary characteristics of molecule transport are specificity for the given ligand and competition for similar compounds, both of which are defining of a transporter active site. The specificity of choline transport has been broadly investigated using choline, hemicholinium-3, and various other nitrogen-methyl compounds. Previous reports by Roberts and Tamaru,[103] Lerner,[13] and Batzold et al.[104] are earlier comprehensive reviews to which readers are referred in this regard. Table 2 is a comprehensive list of choline transport studies for multiple tissues and organisms with details of kinetic values, hemicholinium-sensitivity, ion-dependence, and comments related to inhibitors. Figure 2 shows structures discussed in this section.

Significant information regarding the choline-binding site has been gathered through evaluation of choline structural analog inhibition studies. Figure 3 illustrates a simple two-dimensional rendition of choline and the corresponding transporter binding pocket (active site). While there may be significant differences in molecular space between the

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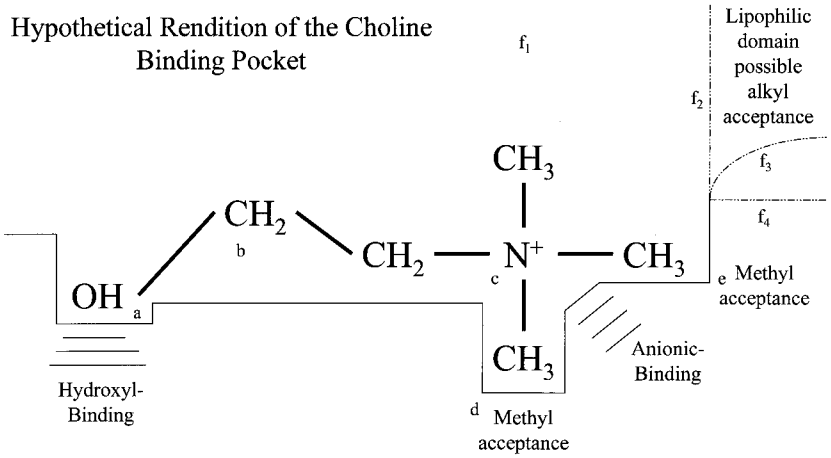

Figure 3. Theoretical two-dimensional rendition of choline-binding site.

high-affinity, low-affinity, and blood–brain barrier transport binding sites, some universal principals of transporter binding requirements apply.

The interaction between the quaternary nitrogen and the binding site (Fig. 3c) may be the area of primary attraction for choline (Fig. 2.1a) and other ligands. Using tetraethylammonium and tetramethylammonium (Fig. 2.4a,b) as inhibitors of choline uptake, Simon et al.[105] demonstrated significant uptake blockade in synaptosomes. When the structures are evaluated, quaternary ammonium recognition is a major transport feature. Similarly, a pyrene derivative, 2-[4-(1-pyrenyl) butyryloxy-ethyl]-trimethylammonium ion (Fig. 2.5), is a competitive inhibitor with a 20-fold greater affinity than choline itself[106] in synaptosomes as well. The increased affinity of this compound may be a result of the compound's hydrophobicity interacting with the membrane core[13] or an adjacent hydrophobic binding region (Fig. 3f).

Of particular interest, there may be a second anionic binding site close to the primary anionic site. This has been suggested by the following data. When two quaternary nitrogens are separated by carbon chains they have 50- to 100-fold greater affinity for the carrier (in synaptosomes) than choline.[107,108] Chain length between the two quaternary nitrogens is apparently related to the distance between the anionic binding sites, as an increase in chain length corresponds to an increased affinity in the erythrocyte.[109] Furthermore, hemicholinium-3 is a bis-compound with two quaternary nitrogens and a specific and potent competitive antagonist, notably in the high-affinity system.[110] When compared to the similar monomer compound, hemicholinium-15 (Fig. 2.3), the bis-compound inhibits choline transport 100 to 300 times more effectively in the high-affinity transporter.[105]

Two hydrophilic anionic sites, separated by an essentially planar but conformationally flexible, hinged cationic hydrophobic domain, have been identified with close-packed sphere molecular models. The attachment of choline to either anionic binding site resulted in dual-site cooperation by enveloping the ligand with a Venus fly-trap mechanism and a subsequent interior release of choline for synaptosomal high-affinity transport.[103] Figure 4 illustrates a potential mechanism of choline transport, which is further discussed below.

This pharmacological and molecular data suggest the choline transporter may be coupled with a second transporter in close proximity or, as discussed previously, there may be functional coupling to one of the following enzymes: (1) choline acetyltransferase, (2) acetylcholinesterase, or (3) butylcholinesterase.

The other significant location of choline binding to the transporter is the hydroxyl-binding site (Fig. 3a). This site has significant restrictions. If the hydroxyl group is replaced with a methyl group[104] or deleted,[72] a considerable decrease in affinity is noted for choline in synaptosomal and alveolar epithelial cells, respectively. Additionally, it has been suggested that there is a space limitation for

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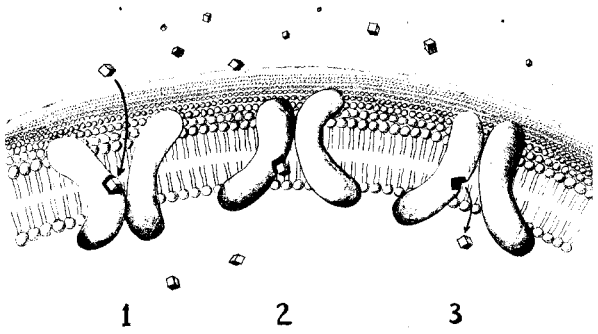

Figure 4. Hypothetical representation of choline transport across a membrane. The transport protein may act as a mechanism with reciprocating gates. Adapted from Krupka and Deves.[125]

hydroxyl binding, which may function to exclude acetylcholine from the high-affinity choline-binding site.[13] This area may be considered to be the primary responsible site of erythrocyte reversible non-competitive inhibition by ethanol, *n*-butanol, *n*-hexanol, *n*-octanol, and *n*-decanol.[111] Moreover, once the ligand has bound to the site, the hydroxyl bond is fixed and unable to rotate.[112]

The anionic attraction with the quaternary nitrogen allows rotation of the methyl groups (Fig. 3c). However, there appear to be three areas of binding for alkyl groups surrounding the nitrogen (Fig. 3d–f). Deves and Krupka[112] demonstrated free rotation around the ligands' trialkyl ammonium group in erythrocytes. Of the three binding areas for methyl groups, there appear to be restrictions on the size of the carbon chain that can be accommodated. Significant size restrictions are found on two methyl acceptor locations (Fig. 3d, e) and are restricted to ethyl groups in synaptosomes.[105] However, the third methyl acceptor group (Fig. 3f) can accommodate a propyl group in erythrocytes[112] or an octyl group at the blood–brain barrier[95] (Allen, unpublished data). For example, we have found that substitution of an octyl group for a single methyl group on the choline nitrogen results in a >25-fold increase in affinity of the carrier for this choline analog at the blood–brain barrier. Similar studies in our laboratory have demonstrated that this alkyl addition to the choline molecule can overcome the strict hydroxyl ion-binding requirement discussed above. Taken together, these findings suggest the third group is interacting with potential lipophilic domains (Fig. $3f_{2-4}$) or the extracellular aqueous environment (Fig. $3f_1$), facilitating binding and/or transport. The only restriction consistent in all methyl acceptor sites is the rejection of branched methyl groups. This data suggests that the third site is the least restrictive acceptor site, allowing projection away from the transporter.

The distance between the quaternary nitrogen and the hydroxyl group is also space-limited (Fig. 3b). Using Dreiding molecular models, the optimum calculated distance for binding between the two is approximately 3.26 to 3.30 Å.[13,104] This has been substantiated by inhibition studies through an evaluation of comparative IC_{50}'s, where: (1) two methyl moieties between the nitrogen and hydroxyl group of choline exhibit an IC_{50} of 0.63 μM, (2) three methyl moiety separation, i.e., homocholine (Fig. 2.5a), had an IC_{50} of 2.2 μM, and (3) four methyl moiety separation, i.e., butyl choline (Fig. 2.5b), demonstrated an IC_{50} of 310 μM. These data suggest the distance between the two charged binding sites is restricted by chain size and limited to two to three intermediate carbons. Furthermore, choline analogs with nitrogen to oxygen distances of 3.4 to 3.7 Å demonstrated IC_{50} values ranging from 1.4 to 22 μM in synaptosomes.[104] This data is in agreement with an inhibition study evaluating five harmala alkaloids in blocking high-affinity choline transport.[113] Transport inhibition with the alkaloids revealed the optimum distance between the hydrogen-bonding and quaternary nitrogen moieties as approximately 3.6 to 3.7 Å, with the compounds displaying a K_i in the range of 3.4–36.2 μM.[113]

The interaction between choline and the carrier is significant. The strength has been demonstrated by the determination of ionic bond half-saturation constants. The charged cationic substrates had a higher affinity in erythrocytes by a factor of 2000 when compared to their uncharged carbon analogs, i.e., (a) choline—6.3 μM vs. 3,3-dimethyl-1-butanol—16 mM and (b) 2-dimethylaminoethanol—19 μM vs. isoamyl alcohol—45 mM. The authors suggest that to account for the unusually strong ionic bonds, the close association between the carrier site and the substrate may be related to exclusion of water of hydration.[114] Comparable results have been observed at the blood–brain barrier as well.[95]

The development of an irreversible inhibitor of the choline transporter has simplified and clarified kinetic evaluation of choline transport under various conditions. One of the first identified irreversible inhibitors is the choline mustard aziridinium ion

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(Fig. 2.6). Rylett and Colhoun[115,116] demonstrated this agent as a specific, irreversible inhibitor of choline transport in rat brain synaptosomes. Similar to hemicholinium-3, the high-affinity transport mechanism has greater sensitivity to this compound ($IC_{50} \sim 0.94\,\mu M$) when compared to the low-affinity mechanism ($IC_{50} \sim 29\,\mu M$). A second irreversible inhibitor developed and studied is hemicholinium mustard.[117] This ligand, unlike the choline mustard, inhibits high-affinity choline transport but not low-affinity transport.[118] The characterization of irreversible inhibitors has not only aided in calculation of choline transport kinetic determinants, but also served as a probe for transporter location, a model of cholinergic hypofunction,[119] and in the molecular characterization of the choline transporter.[120]

Conformation of the Carrier

To determine the actual conformation of the choline transporter during substrate transport, the elegant work of Deves and Krupka in erythrocytes should be examined. Figure 4 shows a theoretical drawing of choline transport based on works from these authors. Initially, after characterizing choline transport across erythrocytes,[112] they compared choline affinity on inner and outer membrane surfaces. A fivefold difference in affinity cannot account for asymmetrical transporter distribution, as the concentrations of inward-facing and outward-facing carriers are similar.[121] Furthermore, the carrier sites were found to have distinct specificities. The outer site is complementary to the structure of choline, whereas the inner site lacks the hydrophobic region (Fig. 3f). Additionally, the inner site preferentially binds enlarged trimethylammonium and hydroxyethyl groups[122] and the compound dibutylaminoethanol.[123]

Kinetically, individual reaction rates for the two inward-facing forms, the free carrier and the bound complex, have been evaluated. The pseudo-first-order rate constant for the complex with a substrate analog, di-*n*-butylaminoethanol, has been demonstrated to be approximately twofold greater than the free carrier. Furthermore, the authors suggest that reorientation and disassociation of the carrier–substrate complex occurs more quickly than reorientation of the free carrier.[121] Taken together, the authors suggest this data defines three different conformational states during the transportation of choline (Fig. 4). First, there exists an outward-facing carrier; second, a choline-bound inward-facing complex; and last, an outward-facing carrier.[124]

The carrier has been further defined by *N*-ethylmaleimide inhibition studies. Using this lipophilic compound, Krupka and Deves[125] demonstrated that the reactive thiol group of the transport protein is bound outside the substrate site, interferes with translocation of choline, and binds to the internal side only when the carrier is exposed internally. This data supports earlier studies suggesting the reactive thiol group is located in a lipophilic environment and is more reactive with an inward-facing carrier.[126] The authors suggest the aggregate of this data describes choline transport across erythrocytes via a reciprocating gated channel that allows influx and efflux of the substrate.

MOLECULAR CHARACTERIZATION

Molecular Identification

Pharmacological evaluation of choline transport has provided information on location, transport kinetics, and characteristics of choline transport. However, actual molecular characterization of the choline transporter was elusive until recently. Little advancement in characterization had been accomplished, secondary to the transporter's membrane-bound nature and the lack of an irreversible high-affinity ligand.[127] In the early 1990s, Breer and colleagues utilized [^{3}H]-hemicholinum-3 binding and identified an 80-kDa polypeptide as the choline transporter in the locust nervous system.[128] Further, they were able to develop monoclonal antibodies to the polypeptide and reconstitute transport activity into proteoliposomes. Unfortunately, the antibodies failed to cross-react with the mammalian models, rodent or *Torpedo* tissue.[129]

Further progress was completed with partial characterization of the choline transporter in rat brain striatum using [^{3}H]-choline mustard aziridinium ion. This ligand irreversibly bound to two non-glycosylated polypetides with apparent masses of 58 and 35 kDa.[127] This data complemented sodium-dependent, hemicholinium-sensitive identification of two polypeptides from the synaptic membranes of the *Torpedo marmorata* with molecular masses of 42 and 58 kDa (with the 42 kDa being labeled predominantly and possibly belonging to a subunit containing the choline-binding site).[130]

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Over time, further information regarding the molecular structure of the choline transporter has been elucidated using sequence information from rat striatum, murine models, and human tissues.

Using the *Caenorhabditis elegans* genome project, the high-affinity choline transporter cDNA was cloned and employed to isolate the corresponding cDNA from rat brain. The authors found the corresponding rat mRNA was restricted to cholinergic neurons and, when expressed in *Xenopus laevis*, had essential characteristics of the high-affinity cholinergic transporter in nerve endings.[131]

By using the choline transporter sequence information from previous studies in rats and humans,[131–133] a mouse choline transporter was identified. The authors used reverse transcriptase of spinal cord mRNA and confirmed the sequence with assembled mouse genomic DNA. The genomic characteristics of the murine choline transporter included: (1) encoding for 580 amino acids, (2) 13 transmembrane domains, (3) the N-terminus located extracellularly, and (4) the C-terminus located intracellularly. When compared to known choline transporter sequences, the following homology was revealed: with regard to the human sequences, exons are preserved as well as a 93% amino acid homology; and with regard to rat, a 98% homology was noted.[134]

In human tissue, molecular cloning and functional characterization of the high-affinity choline transporter was accomplished. When expressed in *Xenopus oocytes*, the human clone demonstrated sodium- and chloride-dependent, high-affinity choline uptake. Further, the uptake was sensitive to the specific inhibitor hemicholinium-3, with a K_i of 1.3 nM.[132]

Computer Molecular Modeling

While molecular identification of the choline transporter provides information regarding the mechanism of ligand binding, mapping of the choline transporter active site provides additional information regarding obligatory binding requirements. To further characterize the in vivo choline transporter, computer-generated models have facilitated determination of structural binding activity.

We have used choline analog inhibition data gathered from in situ rat brain perfusion studies to generate a three-dimensional computer choline transport model at the blood–brain barrier (Allen, unpublished data).[135–137] Comparative molecular field analysis conducted using the Tripos, Inc. software SYBYL allows determination of structural force fields and prediction of substrate binding affinity by the transporter active site. These computer-based models incorporate the same structural and chemical requirements elucidated by pharmacological inhibition studies (i.e., anionic, hydroxyl, and hydrophilic binding). This data correlates with pharmacological inhibition data and provides a validated predictive model for new compounds that have significant interaction with this transport system.

Molecular characterization and computer modeling studies have demonstrated specific molecular characteristics of the choline transporter in various models. The cloning data and development of computer-generated predictive models act as valuable tools for: (1) assessing choline transport in pathophysiological conditions, (2) predicting structural requirements for ligands, and (3) increasing the possibility of using this cationic transporter as a drug delivery vector. Expansion and further refinement of these models will enhance the ability to identify new lead compounds which may interact with the transporter, possibly as a drug delivery vector as described below.

PHARMACOLOGICAL APPLICATIONS

Choline Transporter as a Drug Delivery Vector

Treatment of disease often relies on therapeutic agents reaching and entering targeted tissue. Utilization of the choline transporter as a drug delivery vector may provide a mechanistic answer for distributing drugs to areas high in choline transporters. However, in order for drugs to be delivered efficaciously via this mechanism, the following requirements must be met: (1) the carrier must be free to bind the drug, (2) the agent must meet structural binding requirements, and (3) translocation of the drug across the membrane must occur.

The choline transporter has been shown to have utility as a delivery vector by Goldenberg and Begleiter,[138] who showed distribution of therapeutic alkylating agents in lymphoblasts. Since that time, the choline transporter at the blood–brain barrier has been suggested as a possible brain trans-

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port vector since it has high transport capacity and adequate transfer rate from blood to brain.[139,140]

Considering physiological plasma concentrations of choline are approximately 25% of the blood–brain barrier K_m, the carrier is not physiologically saturated and is capable of transporting drugs from the plasma to brain.[80,89,90] The choline transporter has demonstrated the ability to competitively bind to potentially therapeutic agents, i.e., derivatives of isoarecolone and lobeline,[140] lithium,[17,80] procainamide, quinine, and serotonin.[98] Actual transport of these therapeutic agents across the blood–brain barrier has not been demonstrated. We have demonstrated binding[141] and transport of a novel nicotine derivative into the brain using the in situ rat brain perfusion method, suggesting this transport system may work as such a vector (Allen, unpublished data).

Utilization of choline as a cell surface ligand on nanoparticles for targeted delivery of drugs across the blood–brain barrier has been suggested.[142] Further, choline analogs on the surface of the nanoparticles may account for the three- to four-fold increases of nanoparticle brain uptake in studies by Fenart et al.[143] The authors demonstrate that when charged nanoparticles are coated with a lipid bilayer containing dipalmitoyl phosphatidyl choline, significant improvement in brain distribution is observed. Furthermore, when albumin, normally excluded from the brain, is similarly coated, a significant 27-fold increase in brain uptake is demonstrated. The authors point out that in the presence of erythrocytes, a significant decrease in brain transport is seen, possibly due to a nanoparticle (NP)–erythrocyte interaction.[143] This data is consistent with the nanoparticle interacting with physiological choline transporters. Considering the K_m of the choline transporter of erythrocytes is approximately 40% less than that found at the blood–brain barrier,[56,80] it may be expected that choline ligand interactions would preferentially occur at the erythrocyte-binding site, decreasing apparent brain uptake.

Choline Transport in Physiological and Pathophysiological Conditions

Alzheimer's Disease

In Alzheimer's disease, the regulation of acetylcholine synthesis and cholinergic functioning is greatly diminished and disrupted. The deterioration of cholinergic neurons in the nucleus basalis[144,145] and choline acetyltransferase diminution[146,147] in cholinergic neuronal projection areas is a hallmark characteristic of this disease. This cholinergic disruption supports a central hypothesis of Alzheimer's treatment: increasing acetylcholine [by either enhancing choline acetyltransferase or inhibiting acetylcholinesterase (Fig. 1b,c)] will enhance cholinergic functioning and subsequently improve patient outcomes.

Indeed, acetylcholinesterase inhibitors have been shown to improve cognition and reduce agitation, apathy, anxiety, hallucinations, delusions, and aberrant motor behavior. Pharmacotherapy in Alzheimer's disease patients is out of the scope of this article. For more detail relating to pharmacotherapy options in Alzheimer's patients, the reviews of Rojas-Fernandez et al.[148] and Cummings[149] may be helpful.

The high-affinity choline transporter has been used as a specific marker for cholinergic functioning. This marker is more specific for synaptosome high-affinity carriers than acetylcholine, which undergoes rapid hydrolysis, or choline, which is ubiquitous. High-affinity choline uptake activity was reduced in 13 postmortem Alzheimer's diseased brains, as evidenced by [^{3}H]-hemicholinium binding,[150] and in uptake experiments with diseased human synaptosomes.[151] Further, amyloid-beta peptide interacts directly with the transporter, can inhibit transport activity, and make the transporter more sensitive to proteolytic degradation.[152] This reduction in synaptosomal high-affinity choline transport in Alzheimer's disease may be illustrative of late-stage cholinergic cell death.

However, in a second study, an upregulation of the carrier was found via autopsy within a more sensitive timeframe, two hours after death.[153] A concentration-dependent increase in choline flux was also demonstrated in PC12 cells exposed to beta-amyloid.[154] This data suggests the transporter may remain functional in beta-amyloid neuronal cells and may upregulate to compensate for reduced synaptic cholinergic activity. Further, increased choline permeability in amyloid-beta diseased neurons may result in exhaustion of intracellular cholinergic choline reservoirs and subsequent disruption of acetylcholine production.[154]

For the choline transporter to maintain upregulated status, additional quantities of substrate must be supplied or downregulation will occur. It has been suggested that phosphatidylcholine degradation and subsequent efflux of choline may be a

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primary source. This has been demonstrated in erythrocytes from patients with Alzheimer's disease[155] and bovine retina capillary pericytes.[156] Further, the breakdown of neuronal membranes has been demonstrated[157] and may account for the observed increase in cerebrospinal fluid choline concentrations in Alzheimer's patients.[158]

Brain choline levels may also increase from a greater influx at the blood–brain barrier. Brust[159] demonstrated an increase of choline permeability at the barrier after a 14-day treatment of scopolamine. The author suggests that the choline transporter is regulated by cholinergic innervation of brain endothelial cells, thus it may be possible to hypothesize that because high-affinity choline uptake is upregulated, blood–brain barrier permeability increases as well.

While current medical strategies have succeeded in diminishing symptoms of Alzheimer's, they have only been palliative and inadequate in reversing disease progression.[153,160] The high-affinity choline transporter in Alzheimer's disease fibroblasts can be stimulated in transport activity by caffeine, dexamethasone, and nicotine,[161] suggestive of modifiable choline transport, and thus may be a potential therapeutic target for both acetylcholine synthesis support and disease treatment.

Hypoxia

Hypoxia from in vivo ischemic events results in transient increases in the amount of free total cortical choline,[162,163] presumably from the impairment of the energy-dependent enzyme choline acetyltransferase (Fig. 1b). Further increases are seen from the production of choline from phospholipids (Fig. 1c). This production does not depend on energy, and subsequently is not impaired in hypoxia.[164] The review by Scremin and Jenden[165] goes into more detail about hypoxia and the effects on choline availability and distribution.

Of particular interest to this report, ischemia results in the release of arachacadonic acid that can block high-affinity choline transport in synaptosomes.[166] Acidosis of comparable levels seen in brain ischemic events (pH $\sim$ 6.0) also significantly reduces choline uptake.[167] As may be expected, the amount of acetylcholine produced during these events also significantly decreases.[162]

Upon reperfusion of ischemic conditions, the amount of acetylcholine production rebounds to levels higher than control. This has been suggested to relate to acetyl-coA restoration being faster than choline concentration normalization.[162] However, it may also be related to disinhibition of the high-affinity carrier and the subsequent intracellular reintroduction of the substrate for acetylcholine production.

Aging

Controversy exists in the literature on the status of choline transport in aging. High-affinity choline uptake is demonstrated to be lower in aged rat brain synaptosomes,[168,169] older humans,[170] and either insensitive[168] or reduced with regard to hemicholinium-3 binding.[171] However, high-affinity sodium-dependent choline transport into synaptosomes is unaffected by the age of the rat (2–30 months).[172] Similar discrepancies exist at the blood–brain barrier. Using the brain uptake index method, the basic amine choline transporter was found to have decreased capacity and an increased affinity.[101,173] However, using in situ perfusions, minimal significant differences were noted in brain permeability to choline in 3-, 12-, 24-, and 28-month-old rats.[94] The explanation for the disparity among the above data may be found in the purity of synaptosomal preparations, and with regard to the perfusions, the sensitivity of the method.

Choline transport in pathophysiological conditions has major significance and may be the target of future pharmacotherapeutic agents for the treatment of disease. Future work must also expand on choline uptake in other significant pathophysiological conditions such as diabetes,[100] chronic renal failure,[67] traumatic brain injury,[174] Down's syndrome,[175] and ethanol intoxication.[176]

CONCLUSION

Choline is an essential nutrient found ubiquitously throughout all tissues. However, it is limited in its distribution by transport mechanisms. These mechanisms have been studied extensively and loosely grouped into two categories. Further studies in various tissues have provided data supporting unique mechanisms independent of either category individually. These unique mechanisms are probably not independent proteins, as they share characteristics of both types of choline transport systems.

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With scientific advancement, the ability to glean information regarding choline transporters will continue to be enhanced. Pharmacological inhibition studies, molecular characterizations, and computer modeling have facilitated the creation of rationally high-affinity ligands for the transporter, and further refinement of our understanding of these transport systems will be potentially invaluable for pharmacological approaches. These ligands not only provide further conformation of the carrier in vivo, but may be the basis for delivering and targeting drugs to cholinergic tissues in pathophysiological conditions.

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