CHAPTER EIGHT

Choline on the Move: Perspectives on the Molecular Physiology and Pharmacology of the Presynaptic Choline Transporter

E.A. Ennis, R.D. Blakely

Vanderbilt University School of Medicine, Nashville, TN, United States

1Corresponding author: e-mail address: randy.blakely@vanderbilt.edu

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Abstract

Genetic, biochemical, physiological, and pharmacological approaches have advanced our understanding of cholinergic biology for over 100 years. High-affinity choline uptake (HACU) was one of the last features of cholinergic signaling to be defined at a molecular level, achieved through the cloning of the choline transporter (CHT, SLC5A7). In retrospect, the molecular era of CHT studies initiated with the identification...
of hemicholinium-3 (HC-3), a potent, competitive CHT antagonist, though it would take another 30 years before HC-3, in radiolabeled form, was used by Joseph Coyle’s laboratory to identify and monitor the dynamics of CHT proteins. Though HC-3 studies provided important insights into CHT distribution and regulation, another 15 years would pass before the structure of CHT genes and proteins were identified, a full decade after the cloning of most other neurotransmitter-associated transporters. The availability of CHT gene and protein probes propelled the development of cell and animal models as well as efforts to gain insights into how human CHT gene variation affects the risk for brain and neuromuscular disorders. Most recently, our group has pursued a broadening of CHT pharmacology, elucidating novel chemical structures that may serve to advance cholinergic diagnostics and medication development. Here we provide a short review of the transformation that has occurred in HACU research and how such advances may promote the development of novel therapeutics.

ABBREVIATIONS

[^3H] tritium-labeled compound
Acetyl-CoA acetyl coenzyme A
ACH acetylcholine
AChE acetylcholinesterase, EC 3.1.1.7
ADHD attention deficit hyperactivity disorder
BAC bacterial artificial chromosome
BAC-CHT CHT overexpression mouse line
Bmax maximum amount of drug or radioligand bound
cDNA complementary DNA
Ch choline
ChAT choline acetyltransferase, EC 2.3.1.6
cho-1 CHT gene, C. elegans
CHT choline transporter, SLC5A7 (human), Slc5a7 (rat/mouse)
CHT HET CHT heterozygous knockout mouse line
CNS central nervous system
CT1 creatine transporter, SLC6A8
DA dopamine
GABA γ-aminobutyric acid
GAT1 GABA transporter, SLC6A1
GPCR G-protein-coupled receptor
HACU high-affinity choline uptake
Hb9 motor neuron-specific promoter
HC-3 hemicholinium-3
HEK human embryonic kidney
HTS high-throughput screen
Kd dissociation constant
Ki inhibition constant
Km Michaelis–Menten constant for the substrate concentration that is required for the reaction (enzyme or transport) to occur at half Vmax
LACU low-affinity choline uptake
1. INTRODUCTION

Acetylcholine (ACh) is a neurotransmitter that interacts with muscarinic and nicotinic receptors in the central and peripheral nervous systems. Because of its ubiquitous distribution, ACh controls or modulates virtually every physiological process, from the regulation of skeletal and smooth muscle contraction, to the modulation of heart rate and the control of higher brain functions, such as attention, learning, and memory (Olshansky, Sabbah, Hauptman, & Colucci, 2008; Sellers & Chess-Williams, 2012). Not surprisingly, alterations in cholinergic signaling underlie a wide variety of disorders, from myasthenias, cardiovascular disease, and gastrointestinal disorders, to addiction, attention deficit hyperactivity disorder (ADHD), and Alzheimer’s disease (Alder, Chessell, & Bowen, 1995; Barwick et al., 2012; Berry et al., 2014; English et al., 2009). In some cases, such as with Alzheimer’s disease, which is treated with inhibitors of acetylcholinesterase (AChE), the understanding of cholinergic dysfunction has afforded opportunities for pharmacological intervention. However, achieving specificity with such agents remains a challenge and the pervasive actions of ACh often limits therapeutic potential due to dose-limiting side effects (Alt et al., 2015). Thus, though the therapeutic potential of ACh-targeted medications remains clear, much work remains
to realize the potential of improving human health through cholinergic interventions that became apparent when Dale and Loewi first identified ACh and established the principle of chemical signaling at cholinergic afferents to the heart, respectively (Dale, Laidlaw, & Symons, 1910).

In this review, we highlight a critical molecular determinant of cholinergic signaling, the presynaptic choline transporter (CHT, SLC5A7). The contribution of the CHT to cholinergic biology emerged more than 50 years ago (Clark, 1926; Dale & Dudley, 1929; Dudley, 1929) with the recognition that high-affinity choline uptake (HACU) is critical for the synthesis of ACh and cholinergic function. It was not until recently, however, that the molecular features of the transporter were defined (for earlier complementary reviews, see Bazalakova & Blakely, 2006; Ferguson & Blakely, 2004; Haga, 2014; Okuda & Haga, 2003). When one of us (RDB) was a graduate student in the laboratory of Joseph Coyle, CHT first began to be studied as a molecular entity, with ligand interaction characteristics and anatomical localization achieved through membrane binding and autoradiographic studies (Rainbow, Parsons, & Wieczorek, 1984; Sandberg & Coyle, 1985) using the radiolabeled version the CHT antagonist hemicholinium-3 (HC-3) (see later). As discussed later, the transformation of HACU “from process to molecule” not only resulted in the development of new tools for localizing and monitoring CHT proteins, it also brought into focus the sensitivity of the transporter to neuronal activity involving a unique mode of presynaptic regulation that entails “hitchhiking” on cholinergic synaptic vesicles (Ferguson et al., 2003). Following a brief review of the basic mechanisms supporting ACh synthesis, release, and inactivation, we provide in this report an overview of the research conducted to characterize HACU, and the path taken by us and other investigators, such as Joseph Coyle, to move CHT into the molecular era. The results of this work has led to defining more precisely the pharmacology of the cholinergic system and provided insights into mechanisms that underlie conditions associated with a dysfunction in cholinergic transmission.

2. ACh, HACU, AND THE BIRTH OF CHT

2.1 An Overview of the Mechanics of ACh Synapses

The synthesis of ACh in cholinergic presynaptic terminals is catalyzed by choline acetyltransferase (ChAT, EC 2.3.1.6, Fig. 1A) (Nachmansohn & Machado, 1943; Okuda & Haga, 2003; Prado et al., 2002). Given this
important role, it is not surprising that vertebrate ChAT activity mirrors the distribution of ACh macroscopically (Feldberg & Mann, 1946), with antibody studies revealing that the enzyme is localized to neuronal processes that support ACh release (Kobayashi et al., 2002; Kus et al., 2003). Although a small fraction of ChAT is membrane associated, the bulk of protein and enzymatic activity fractionates with the cytosol (Benishin & Carroll, 1983; Rylett, 1989; Tucek, 1967). ChAT catalyzes the synthesis of ACh by transferring the acetyl group from acetyl coenzyme A (acetyl-CoA) to cytosolic choline. Genetic elimination of ChAT is lethal in mice (Brandon et al., 2003) and loss-of-function mutations produce potentially fatal myasthenic disorders associated with episodic apnea in humans (Ohno et al., 2001).

Following synthesis, cytosolic ACh is packaged into synaptic vesicles by the vesicular ACh transporter (VACHT, SLC18A3), a H+/ACh antiporter embedded in the vesicle membrane. Intracellular acidification of
cholinergic, as well as other, synaptic vesicles is accomplished through ATP hydrolysis by a H$^+$ pumping, vacuolar type ATPase (Breer, Morris, & Whittaker, 1977). Upon vesicular fusion, ACh is released into the extracellular space where it is rapidly hydrolyzed to yield acetate and choline by AChE (EC 3.1.1.7). The choline then rapidly diffuses from the synapse or is accumulated by presynaptic terminals where it is recycled for ACh synthesis.

2.2 From ACh to HACU

As studies indicate that ChAT is not saturated by cytosolic levels of choline (Haga & Noda, 1973), the availability of choline is rate limiting in the production of ACh. However, because acetyl-CoA contributes to cell metabolism in a number of ways, its intracellular levels are normally quite high. The quaternary nitrogen of choline, which is obtained largely through dietary sources (Cohen & Wurtman, 1976; Jope & Jenden, 1979), precludes the passive transfer of choline across the plasma membrane of cells, necessitating the presence of an efficient transport system for this ACh precursor (Bligh, 1952) (Fig. 1A). The functional importance of choline uptake became apparent in studies documenting a decline in ACh release after persistent stimulation of cholinergic terminals in sympathetic ganglia bathed in choline-free medium (Mulder, Yamamura, Kuhar, & Snyder, 1974; Perry, 1953). In Perry’s study, ganglia were exposed to an AChE inhibitor, which further diminished the capacity of the synapse to recycle choline after ACh release and hydrolysis. Interestingly, a comparison of ACh levels in stimulated vs unstimulated ganglia revealed equal amounts of tissue ACh, in spite of the time-dependent decline in ACh release from the stimulated tissue. These data led to the theory that exogenous choline is critical for ACh synthesis and that ACh resided in two compartments, a readily releasable pool and a reserve pool, that supports the need for rapid vs sustained ACh release (Mulder et al., 1974; Perry, 1953). Birks, Macintosh, and Sastry reported a similar decline in ACh after repeated stimulation of cat cervical ganglion and found that the amount released could be enhanced by the perfusion with blood containing choline (Macintosh, Birks, & Sastry, 1958). Such findings led to the hypothesis that a choline transport system that is necessary for ACh synthesis. This idea was supported by later studies demonstrating that choline uptake is saturable in the presence of normal dietary levels of choline (Brunello, Cheney, & Costa, 1982). Additionally, as the concentration of choline in the extracellular fluid was estimated to be
\( \sim 10^{-5} \text{M} \) (Garguilo & Michael, 1996), a specific HACU appeared to be responsible for the transport of choline as amino acid and sugar transporters work in the presence of mM concentrations of substrate (Mulder et al., 1974; Perry, 1953).

Once the critical nature of HACU in ACh synthesis and release was established, investigators undertook radiotracer studies to determine the nature of choline influx into tissues. Brain slices and other tissues incubated with \( ^{14} \text{C} \)choline were found to accumulate choline to concentrations higher than that in the incubation medium, suggesting an energy-dependent transport system (Hodgkin & Martin, 1965; Martin, 1968). Incubations with high, 5 \( \mu \text{M} \), and low, 0.1 \( \mu \text{M} \), concentrations of choline yield results that were best described by two separate Michaelis–Menten constants, implying two separate transport systems. While these initial uptake studies with \( ^{14} \text{C} \) choline proved the existence of a low-affinity choline uptake (LACU) system, the low specific activity of the radiolabeled substance precluded the identification of a high-affinity transporter. The subsequent introduction of higher specific activity \( ^{3} \text{H} \) choline allowed studies to be conducted at concentrations of substrate low enough to definitively identify a HACU site in tissue preparations known to contain cholinergic terminals (Marchbanks, 1968, 1969). Because of the high capacity of the LACU system, care must be taken when working at or above choline concentrations of choline that saturate HACU if the aim of the experiment is to selectively study the higher affinity site.

As indicated earlier, an additional source of choline results from ACh hydrolysis (Okuda & Haga, 2003). Presynaptic deposition of CHT proteins occurs with ACh vesicle fusion (see later), a process thought to have developed to ensure localization of the transporter near the release sites. Such a localization facilitates recapture of AChE-derived choline before the precursor can diffuse away from the synapse.

Studies with synaptosomes yielded valuable information on the characteristics of HACU (Diamond & Kennedy, 1969; Simon & Kuhar, 1976; Yamamura & Snyder, 1973). In 1966, Whittaker described the “characterization of ACh-containing particles as pinched-off nerve endings (synaptosomes)” (Whittaker, 1965). With this preparation, he and Marchbanks noted a rapid uptake of choline that was recovered after lysing the synaptosomes in a hypotonic solution. These findings supported the presence of a transmembrane choline uptake process. Choline transport across the synaptosomal membrane was shown to be specifically dependent on the concentration of \( \text{Na}^+ \), indicating uptake is driven coupling to a
transmembrane Na\(^+\) gradient that is maintained by Na\(^+\)/K\(^+\) ATPase. When monitored as a function of time and extrasynaptosomal choline concentration, the rate of choline “flux” was shown to have two components, a nonlinear one that is sensitive to choline concentration, and a linear one that is nonsaturable, with an estimated \(K_M\) of 232 \(\mu\)M (Marchbanks, 1968). Accumulation of \([^{14}\text{C}]\text{ACh}\) occurs when incubating synaptosomes with radiolabeled \([^{14}\text{C}]\text{choline}\) (Potter, Glover, & Saelens, 1968).

The synthesis of \([^{14}\text{C}]\text{ACh}\) in synaptosomes, like choline transport, is dependent on the presence of Na\(^+\) and Cl\(^-\) and unaffected by oxotremorine or ouabain (Marchbanks, 1969). As an aside, the Cl\(^-\) sensitivity of HACU initially led our group to use homology-based cloning strategies to search, in vain, for the gene encoding CHT in the family of transporters responsible for norepinephrine (NE), dopamine (DA), and serotonin uptake, all of which, unlike glutamate transporters and many other solute transporters, exhibit Cl\(^-\) dependence (Amara, 1992). Importantly, ACh synthesized from radiolabeled choline is found not only in the cytoplasm but also in the synaptic vesicle fraction, suggesting it can enter into a releasable neurotransmitter pool. The synaptosomal uptake of choline was examined using conditions previously employed to selectively identify HACU and for tracking cholinergic regions in tissues. Low- and high-affinity choline uptake processes were identified in synaptosomes, with the latter shown to be associated with ACh synthesis (Yamamura & Snyder, 1972). The HACU process in synaptosomes is saturable, and dependent on Na\(^+\) (Diamond & Kennedy, 1969; Simon & Kuhar, 1976; Yamamura & Snyder, 1973). It was also reported that synaptosomal HACU is optimal at 37°C, at a pH of 8.6, and in the presence of both Na\(^+\) and Cl\(^-\) (Diamond & Kennedy, 1969). Based on these and other findings, it was postulated that HACU is mediated by a “macro molecule with a high binding capacity for choline” and that this site is capable of supporting “sodium-dependent high-affinity choline uptake” (Marchbanks, 1968).

### 2.3 HC-3: Key Reagent in the Definition of HACU

Key to the evaluation of HACU in synaptosomes was the discovery of HC-3 (Birks, Macintosh, & Sastry, 1956; Diamond & Kennedy, 1969; Schueler, 1955). Long and Schueler synthesized and characterized the hemicholiniums, a collection of aromatic compounds containing a bis-quartenary ammonium (Fig. 1A). The bis-quartenary ammonium compounds became of interest when they, like curare, displayed anticholinergic effects in ex vivo
nerve-tissue preparations from rat (Barlow & Ing, 1948), and a lethal toxicity that could be reversed by artificial respiration. The commonality in structure gave rise to a study of the structure–activity relationship around the hemicholinium pharmacophore. These studies noted that the third in this series of agents (HC-3) was the most toxic, making it the most widely examined member of this group. It was found that HC-3 is toxic across a number of animal species, with its administration causing respiratory depression, tonic and/or clonic convulsions, and death. Schueler demonstrated that the convulsions were due to anoxia, as they are prevented by artificial respiration, suggesting that these toxic effects might be mediated primarily by inhibition of peripheral cholinergic transmission. Because these toxic responses to HC-3 are similar to those observed with other agents that interfere with cholinergic signaling, and the HC-3 structure consists of two, ring-embedded, choline-like moieties, it has become an invaluable tool for defining the pharmacology of the transport site and the biology of the cholinergic system.

In one of the earliest HC-3 studies, Birks, Macintosh, and Sastry observed that ACh synthesis is inhibited when minced brain tissue was exposed to this agent and that this effect is reversed by the presence of choline in the incubation medium. They concluded from this work that HC-3 appears to target HACU rather than ChAT (Birks et al., 1956), noting “An alternative explanation would be that HC3, and other hemicholiniums, may compete with choline transport by a specific carrier system into interneuronal sites of acylation.”

2.4 HC-3-Binding Sites: HACU Enters the Molecular Era

Coyle’s group (Sandberg & Coyle, 1985) capitalized on the availability of [3H]HC-3 to identify HACU-binding sites using the method pioneered by Pert and Snyder for studying opioid receptors (Pert & Snyder, 1973). Ironically, the identification of opioid receptor-binding sites was achieved in a fortuitous departure of Pert and Snyder from studies of choline uptake in the myenteric plexus, with their HACU work being published thereafter (Pert & Snyder, 1974). Coyle’s decision to study HACU reflected his interests in neurotransmitter transport processes in general, and his appreciation for the involvement of cholinergic systems in cognitive disorders. In his last years of medical school at Johns Hopkins, Coyle’s studies with Solomon Snyder capitalized on transport studies in the synaptosome preparation to demonstrate a neurotransmitter role for DA as distinct from its role as a precursor for NE (Coyle & Snyder, 1969). In joining Nobel laureate Julius
Axelrod at the NIMH for postdoctoral studies, the focus of Coyle’s work transitioned to studies of the ontogeny of mechanisms associated with DA and NE synthesis and reuptake (Coyle, 1972; Coyle & Axelrod, 1972; Coyle & Snyder, 1969; Snyder & Coyle, 1969). After establishing his own laboratory at Johns Hopkins, Coyle developed an interest in neurodegenerative disorders, including Huntington’s disease and Alzheimer’s disease. As a model of Huntington’s disease, Coyle studied the ability of intrastriatal kainic acid, a glutamate receptor agonist, to lesions intrinsic cholinergic and GABAergic neurons, sparing fibers of passage, yielding a neuropathology that resembles that seen in Huntington’s disease (Coyle & Schwarcz, 1976). In contrast, Alzheimer’s disease is characterized by a major loss of cholinergic neurons of the basal forebrain, depriving the neocortex of cholinergic innervation, leading Coyle, Price, and Delong, in their landmark study (Whitehouse et al., 1982), to label this condition “a disorder of cortical cholinergic innervation.”

In 1985, Sandberg and Coyle, published a characterization of $[^3]H$HC-3-binding sites in rat brain membranes (Sandberg & Coyle, 1985). Prior studies by Simpson and Smart of radiolabeled choline binding to hippocampal synaptosomes had revealed Na$^+$-dependent-binding sites that were sensitive to low concentrations of HC-3 (Simpson & Smart, 1982). Rainbow and Yamamura’s groups first capitalized on the availability of $[^3]H$HC-3 to identify anatomically defined binding sites in brain preparations (Rainbow et al., 1984; Vickroy, Fibiger, Roeske, & Yamamura, 1984; Vickroy, Roeske, Gehlert, Wamsley, & Yamamura, 1985; Vickroy, Roeske, & Yamamura, 1984). In the Coyle studies (see also Manaker, Wieczorek, & Rainbow, 1986; Vickroy, Roeske, et al., 1984), HC-3 binding to rat forebrain synaptic membranes was found to be saturable, reversible, pH dependent, and of high affinity, with a $K_d$ of 35 nM and a $B_{max}$ of 56 fmol/mg. Binding conditions were optimal in the presence of 200 mM NaCl, in keeping with the Na$^+$ and Cl$^-$ dependence of HACU. The rank order of potency of HACU inhibitors was preserved in competition studies of $[^3]H$HC-3-binding sites. Choline competed for HC-3 binding with a $K_i$ of 40 μM and $[^3]H$HC-3-binding distribution followed the expected distribution of cholinergic terminals, suggesting that the $[^3]H$HC-3-labeled site represented the endogenous HACU transporter. Moreover, transection of the fornix, severing the septohippocampal projection, produced a loss of hippocampal $[^3]H$HC-3-binding sites along with a loss of ChAT activity. Thanks to these and other studies by a number of investigators, the molecular entity responsible for HACU began to emerge, though it would be
another 15 years before the CHT gene and protein were identified (Apparsundaram, Ferguson, & Blakely, 2001; Apparsundaram, Ferguson, George, & Blakely, 2000; Okuda et al., 2000).

Using this radioligand, Coyle’s group would make a number of important contributions relating to the regulation of HACU. Lowenstein & Coyle, 1986 demonstrated that $[^3]$HHC-3-binding sites in brain are sensitive to certain drugs, paralleling effects obtained when HACU was examined ex vivo. This finding validated further the value of $[^3]$HHC-3 as a probe for studying HACU/CHT and for identifying changes in CHT density that foreshadow changes in surface expression of CHT proteins following depolarization (Ferguson et al., 2003) (Fig. 1B). Saltarelli, Lowenstein, and Coyle (1987) reported that an increase in the density of $[^3]$HHC-3-binding sites occurred in the presence of elevated K$^+$, suggesting that the binding site can transition between open and occluded states. Alternatively, this findings might indicate that CHT vesicles that are tethered to the membrane can fuse under these conditions, exposing $[^3]$HHC-3-binding sites. Further studies revealed the presence of Ca$^{2+}$-dependent mechanisms and phospholipases and their ability to modify HACU in brain slices and to alter the membrane density of $[^3]$HHC-3-binding sites (Saltarelli, Lopez, Lowenstein, & Coyle, 1988; Saltarelli, Yamada, & Coyle, 1990; Yamada, Saltarelli, & Coyle, 1988a; Yamada, Saltarelli, & Coyle, 1989; Yamada, Saltarelli, & Coyle, 1991b). These findings are consistent with the notion of a highly dynamic state for CHT availability. The physiological and pathophysiologic relevance of these finding was indicated by the discovery of rapid elevations in $[^3]$HHC-3-binding sites in association with drug induced (Yamada, Saltarelli, & Coyle, 1991a).

### 3. CHT MOLECULAR BIOLOGY AND REGULATION

#### 3.1 Early Efforts to Identify CHT Proteins

To our knowledge, Marchbanks was the first to report the successful reconstitution of synaptic HACU from solubilized proteins (King & Marchbanks, 1982; Marchbanks, 1982). In their studies this group reported evidence for both HACU and LACU, suggesting that either a single transporter can exist in two states or multiple carriers/subunits were reconstituted in parallel. Yamada and colleagues in the Coyle lab (Yamada, Saltarelli, & Coyle, 1988b) solubilized brain membranes and retained $[^3]$HHC-3-binding sites, suggesting a possible approach for characterizing further the CHT protein. Rylett, 1988 used $[^3]$Hcholine mustard to label proteins of the Torpedo
electroplax electric organ, a preparation rich in cholinergic terminals, yielding species of 42, 58, and 90 kDa. Labeling of the two smaller species was absent when conducted in the presence of HC-3. Breer and colleagues (Breer, Knipper, & Kahle, 1989; Knipper, Boekhoff, & Breer, 1989) using monoclonal antibodies that block HACU in insect preparations, purified an 80 kDa species that could support HC-3-sensitive HACU on liposome reconstitution. Deglycosylation studies (Knipper, Kahle, & Breer, 1991) indicated that the core protein has a mass of ~65 kDa. Although these studies did not yield sequence information or progress toward complementary DNA (cDNA) or gene isolation, the findings fit reasonably well with the size of CHT identified once antibodies were developed from the cloned transporter (see later).

3.2 Cloning and Characterization of CHT cDNAs and Genes

Efforts to clone mRNAs that encode CHT proteins accelerated in the early 1990s, with the elucidation of first members of the SLC6 transporter gene family (Broer & Gether, 2012; Guastella et al., 1990; Pacholczyk, Blakely, & Amara, 1991). Because it was known that both the GAT1 GABA transporter (SLC6A1) and the antidepressant-sensitive NE transporter (NET, SLC6A2) require extracellular Na\(^+\) and Cl\(^-\) to drive neurotransmitter uptake, the Na\(^+\)/Cl\(^-\) dependence of HACU suggested the CHT would be a member of the SLC6 gene family. Indeed, there was one report of the cloning of a CHT as a member of the SLC6 family (Mayser, Schloss, & Betz, 1992). Ultimately the cDNA for this protein revealed it to be a creatine, not a choline, transporter (CT1, SLC6A8) (Guimbal & Kilimann, 1993; Schloss, Mayser, & Betz, 1994).

Okuda and colleagues were the first to report cDNAs encoding CHT proteins (Okuda et al., 2000), identifying CHO-1 in C. elegans and a rat species termed CHT1. Currently the abbreviation CHT is preferred as there does not appear to be other gene products that can mediate HACU. Surprisingly, the sequence of these cDNAs identified the transporter as a member of the SLC5 gene family (SLC5A7), which encodes, among others, Na\(^+\)-dependent glucose transporters (SGLTs) (Fig. 2A). Shortly thereafter, we reported sequences of human and mouse CHT (Apparsundaram et al., 2001, 2000). Our studies predicted human and mouse CHT proteins to contain 580 amino acids and to be an N-glycosylated species comprised of 13 transmembrane domains (TMDs), with an extracellular amino terminus and an intracellular carboxyl terminus (Fig. 2B). Canonical phosphorylation
Fig. 2  CHT gene and predicted protein primary structure from cDNA cloning. (A) SLC5 gene family containing various glucose transporters and the CHT. SGLT, sodium-dependent glucose transporter; NIS, sodium–iodide transporter; SMCT, sodium-dependent monocarboxylate transporter; SMIT, sodium-dependent myo-inositol transporter; SMVT; sodium-dependent multivitamin transporter. (B) Predicted secondary structure of human CHT with for protein kinase C (PKC), protein kinase A (PKA), and N-linked glycosylation sites indicated. Dark circles indicate amino acid residues conserved in human, mouse, rat, and nematode choline transporters. Panel (A) reprinted from Wright, E. M. (2013). Glucose transport families SLC5 and SLC50. Molecular Aspects of Medicine, 34, 183–196. Copyright (2013), with permission from Elsevier. Panel (B) reprinted from Apparsundaram, S., Ferguson, S. M., George, A. L., Blakely, R. D. (2000). Molecular Cloning of a Human, Hemicholinium-3-Sensitive Choline Transporter. Biochemical and Biophysical Research Communications, 276, 862–867. Copyright (2000), with permission from Elsevier.
sites for protein kinase C and protein kinase A were identified, along with 12 other serines and 10 threonines that hold potential for noncanonical phosphorylation (Apparsundaram et al., 2001). The topology of CHT was supported by cysteine-scanning analysis, with a three-dimensional model of CHT generated based on vSGLT guiding ongoing structure–function studies (Okuda et al., 2012). Although a CHT monomer is believed to be the functional unit supporting HACU, a homodimerization motif, GXXXG (Russ & Engelman, 2000), has been identified in TMD 12. Cross-linking and immunoprecipitation studies (Okuda et al., 2012), and the dominant-negative character of human CHT mutations, have provided experimental evidence that CHT may in fact oligomerize in vivo (Barwick et al., 2012).

### 3.3 Molecular Mechanisms of CHT Regulation

Once the sequence of CHT was established, highly specific antibodies could be produced that can selectively detect this protein (Ferguson et al., 2003; Guidry, Willison, Blakely, Landis, & Habecker, 2005; Harrington, Hutson, & Southwell, 2007; Hoover, Ganote, Ferguson, Blakely, & Parsons, 2004; Kobayashi et al., 2002; Kus et al., 2003; Lips, Pícel, Haberberger, & Kummer, 2002; Nakata, Okuda, & Misawa, 2004; Proskocil et al., 2004). These studies revealed CHT localization to be highly enriched in the presynaptic terminals of cholinergic neurons, including projections of the mammalian basal forebrain, striatum, medial habenula, and motor neurons. Such studies added higher resolution to the distribution of CHT first defined using $[^3H]$HC-3 autoradiography.

The availability of CHT antibodies also led to the discovery of a novel mechanism that supports activity-dependent trafficking of CHT to the presynaptic membrane (Fig. 1B) (Ferguson & Blakely, 2004; Ferguson et al., 2003). Ferguson et al. reported that subcellular fractionation studies of brain CHT protein reveal an enrichment of the transporter in membranes that cofractionate with synaptic vesicle markers, including VAChT. Immune electron microscopy studies confirmed a predominant localization of CHT to synaptic vesicles in cholinergic terminals (Ferguson et al., 2003; Holmstrand, Asafu-Adjei, Sampson, Blakely, & Sesack, 2010; Nakata et al., 2004), with a much lower expression on the presynaptic plasma membrane. Using a surface biotinylation approach, Ferguson et al. (2003) demonstrated that depolarization of synaptosomes with $K^+$ leads to a translocation of CHT protein to the plasma membrane and that this is
dependent on Ca\(^{2+}\) influx and synaptic vesicle fusion machinery. These studies led to the creation of a model suggesting that CHT traffics to the cell surface in an activity-dependent manner due to its high steady-state residence on cholinergic synaptic vesicles where the transporter, lacking a Na\(^{+}\) gradient to support choline uptake, is inactive. Immunodepletion studies revealed that CHT protein is present in only a fraction (~50% in brain preparations) of cholinergic vesicles labeled with VACHT protein, suggesting a unique subpopulation of ACh storage vesicles that are responsible for CHT surface trafficking. Parikh and colleagues in the Sarter group provided evidence that through this mechanism CHT trafficking is a key feature of activity-dependent elevations in HACU that support the continued ACh release needed for sustained attention (Apparsundaram, Martinez, Parikh, Kozak, & Sarter, 2005; Parikh, St Peters, Blakely, & Sarter, 2013; Sarter & Parikh, 2005).

The presence of CHT on cholinergic synaptic vesicles suggests they may target to presynaptic regions via the same mechanism that traffics synaptic vesicle proteins from the cell soma to neuronal terminals. Support for this is provided by studies (Matthies, Fleming, Wilkes, & Blakely, 2006) indicating a somatic retention of C. elegans CHO-1 in a kinesin mutant (unc-104) that results in retention of other synaptic vesicle proteins. Evidence was also accumulated that the synthesis and export of CHT to cholinergic terminals is under retrograde influences by target-derived signals (Krishnaswamy & Cooper, 2009). High steady-state localization of CHT to synaptic vesicles also raises questions about how the transporter localizes to this compartment. Studies in transfected cells with CHT mutants revealed a dileucine motif in the CHT C-terminus that promotes efficient endocytosis of surface transporters (Ribeiro et al., 2003, 2005, 2006). Ribeiro and colleagues provided evidence in cell systems that CHT endocytosis is clathrin mediated (Ribeiro et al., 2003). Using a genetic mouse model system, Misawa and colleagues found that the clathrin adaptor protein AP-3 may be responsible for CHT export from intracellular membranes and endocytosis (Misawa et al., 2008). Rylett’s group (Cuddy et al., 2012) reported that the peroxynitrite donor SIN-1 can influence CHT endocytosis, ubiquitylation, and degradation, suggesting that oxidative stress may influence cholinergic signaling by targeting CHT away from synaptic vesicles and toward a degradative, proteosomal pathway.

The ability of kinases and phosphatases to regulate ACh synthesis was established by biochemical studies prior to the cloning of CHT and the identification of putative phosphorylation sites. For example, the Ser/Thr
phosphatase inhibitors calyculin A and okadaic acid concentration de-ependently reduce ACh synthesis in rat hippocampal slices (Issa, Gauthier, & Collier, 1996). Their mechanism of action is unrelated to inhibition of ChAT but could, in part, be explained by a decrease in HACU. The availability of CHT antibodies permitted metabolic phosphate labeling of CHT proteins followed by immunoprecipitation, which led to the accumulation of evidence supporting phosphorylation of hippocampal and striatal CHT after calyculin A and okadaic acid treatment in parallel with reductions in HACU and surface CHT levels (Gates, Ferguson, Blakely, & Apparsundaram, 2004). The kinases responsible for CHT phosphorylation have yet to be identified. Moreover, it has yet to be proven whether phosphorylation is a critical determinant of activity-dependent trafficking. An emerging area of CHT research concerns the identification of proteins associated with the transporter (Bales et al., 2006; Fishwick & Rylett, 2015; Misawa et al., 2008; Okuda, Konishi, Misawa, & Haga, 2011; Ribeiro et al., 2003; Xie & Guo, 2004; Yamada, Imajoh-Ohmi, & Haga, 2012). Given evidence for CHT phosphorylation noted earlier and the data on the localization of CHT to cholesterol-rich plasma membrane microdomains (Cuddy, Winick-Ng, & Rylett, 2014), it will be interesting to learn whether, or which of, these protein associations are modulated by CHT phosphorylation, and whether specific membrane compartments support these interactions.

4. CHT CONTRIBUTIONS TO CHOLINERGIC FUNCTION AND DYSFUNCTION IN VIVO

4.1 CHT Genetic Animal Models

The lethality of administered HC-3 is presumably due to an inability to sustain ACh synthesis and release. To validate an essential requirement for CHT in sustaining cholinergic signaling, we mutated the Slc5a7 gene locus in mice to produce animals with no functional capacity to synthesize the transporter (CHT KO) or with only one functional allele (CHT HET) (Ferguson et al., 2004). At birth, CHT KO pups appear normal, but within 30 min they display abnormal breathing, become cyanotic (Fig. 3A), and paralyzed. They typically die within an hour of birth. In these animals we detected a complete loss of HC-3-sensitive [3H]ACh synthesis from exogenous [3H]choline. Though there is a total absence of CHT protein (Fig. 3B) in the KO, cholinergic signaling at the neuromuscular junction was normal
at the beginning of recordings, but there was a progressive loss of spontaneous and evoked end-plate potentials, which is consistent with a failure to synthesize and release ACh. Compensatory changes in AChE or ChAT activity were not detected in these animals. The CHT KO mice display alterations in the organization of axonal inputs to muscle fibers, with a
broader spread and increased branching of motor axons, consistent with a role for ACh in the development of cholinergic inputs to muscle. A similar pattern was observed in ChAT KO mice (Lin et al., 2005).

Although these studies demonstrated an essential requirement for CHT in cholinergic signaling capacity, the lack of viability of CHT KO mice precludes tests of a requirement for CHT in older animals. Such experiments await the development of animals subject to conditional gene elimination. However, in *C. elegans*, because many genes essential to life in vertebrates do not have such devastating effects, we examined the contribution of the transporter to cholinergic biology and behavior in worms deficient in expression of the CHT ortholog CHO-1 (Matthies et al., 2006). Because the standard culture of worms on a lawn of OP-50 bacteria provides these animals with a food source high in choline, *cho-1* mutant worms lack an essential requirement for HACU, though they did display a significant, 40% reduction in whole animal ACh levels. In the worm, as in humans, ACh supports neuromuscular contraction, making it possible for movement assays to be used to examine modulation of cholinergic signaling in vivo. When grown on OP-50, *cho-1* mutant animals display normal patterns of movement. When grown on HB101 bacteria that contain low amounts of free choline, and when subjected to conditions that require high rates of movement, the *cho-1* animals suffered premature fatigue, consistent with a time-dependent loss of neuromuscular cholinergic signaling capacity. Together, these studies demonstrate the critical role of CHT and its phylogenetic orthologs in sustaining ACh synthesis and release in vivo.

The lethality associated with the CHT KO in mouse demonstrated the absolute requirement for CHT in sustaining life. There may be many sites of cholinergic signaling responsible for this lethality, such as the neuromuscular junction, spinal cord, and brainstem cholinergic controls of motor circuits, none of which can be readily studied with this model. We therefore pursued a rescue strategy, expressing CHT selectively in motor neurons of CHT KO mice under the control the Hb9 promoter (Lund et al., 2010). This succeeded in prolonging the life of the CHT KO animals by as much as 24 h. It is possible that the limited strength of the Hb9 promoter and the low amount of CHT produced in the rescue line was insufficient to sustain animals for a longer period. It is also possible that the descending central nervous system (CNS)/spinal cholinergic circuits become critical after the first day of life for driving the necessary motor
rhythms needed for respiration vs the contributions that motor neurons make to the execution of muscle contraction.

The CHT KO mouse line also affords a unique opportunity to evaluate the impact of reduced CHT availability on ACh synthesis, signaling, and behavior via the study of CHT HET animals (Bazalakova et al., 2007; Ferguson et al., 2004). In our initial CHT KO study we found that forebrain \[^3H\]HC-3 binding and synaptosomal uptake were unaffected by loss of one copy of the Slc5a7 gene, even though CHT protein levels were reduced by half. The results suggest posttranslational mechanisms that compensate for reduced total protein expression. This conclusion was supported by studies revealing a shift in the subcellular distribution of CHT proteins, the elevation of cell surface pools at the expense of intracellular levels, and a maintenance of normal HACU (Parikh et al., 2013). The normal level of HC-3 binding observed in CHT HET mice also demonstrates that \[^3H\]HC-3 binding cannot be used, without qualification, to infer CHT protein density. Rather, HC-3 binding appears to reflect the density of surface resident transporters, where the HC-3-binding site is exposed in an “open out” conformation. Reflecting back on the earlier studies by Coyle and his group where in vitro manipulations rapidly altered \[^3H\]HC-3 density, it seems this was due to the induced fusion of cholinergic synaptic vesicles tethered to plasma membrane fragments. Alternatively, these changes could reflect a shift in conformation of plasma membrane-resident CHT from an inactive, inward-facing conformation that cannot bind the ligand, to an active, outward facing conformation, to which HC-3 can attach. Further studies are needed to explore these possibilities, with the results revealing with respect to the mechanics of CHT regulation.

As for phenotypes of CHT HET mice, these animals initially appear normal, growing to normal size with a normal lifespan and fertility (Bazalakova et al., 2007). The CHT HET KO mice also exhibit normal rates of horizontal locomotion, though they display an increase in vertical activity (rearing). Normal behavior is observed in the rotarod, Morris water maze, elevated plus maze, and light–dark tests, suggesting that loss of one Slc5a7 allele has no effect on balance and motor learning, spatial learning and memory, or anxiety. These data indicate that the compensations that maintain normal rates of HACU in the CHT HET mouse preclude the emergence of gross behavioral alterations. Despite these compensations, reductions are evident in striatal M1 receptors as well as cortical and striatal M2 muscarinic receptors (Bazalakova et al., 2007). In contrast, there are elevations in cortical
α4β2 nicotinic receptors. Together, these findings indicate that demands for normal cholinergic signaling in vivo are not fully met in the CHT HET context. Notably, CHT HET mice display premature fatigue on the treadmill test (Bazalakova et al., 2007) (Fig. 3C), reminiscent of the motor deficits seen in cho-1 mutant nematodes where the animals fail to sustain normal rates of swimming behavior (Matthies et al., 2006). The CHT HET mice also display reductions in scopolamine–induced hyperactivity (Bazalakova et al., 2007), as well as cocaine– and nicotine–induced DA release in vivo (Dong, Dani, & Blakely, 2013). Further studies are need to define whether these changes derive from ongoing deficits in ACh release (Paolone et al., 2013) or involve changes in synapse structure imposed by CHT heterozygozity during development. The CHT HET mice demonstrate a basal tachycardia, although they exhibit a normal heart rate elevation upon exercise (English et al., 2010). When CHT HET mice are removed from the treadmill, they fail to reset their heart rates as quickly as wild-type animals, reinforcing the demand–dependent contribution of CHT to cholinergic signaling, in this case for bradycardia. The hearts of CHT HET mice are enlarged and display ventricular thickening and age-dependent fibrosis, suggesting that the basal tachycardia throughout life remodels structural features of the heart. These changes are reminiscent of some observed in cardiovascular disease in humans. Finally, CHT HET mice also demonstrate deficits in attention–demanding cognitive tasks (Parikh et al., 2013; Zurkovsky et al., 2013).

Genetic manipulations have also provided insights into the consequences of abnormally elevated CHT expression. In the course of our studies aimed at restoring motor neuron CHT expression in CHT KO animals, we also generated animals with motor neuron–specific overexpression of CHT (Lund et al., 2010). Analysis of these animals revealed an increased capacity for treadmill running as well as increased compound muscle action potentials. In a separate effort, we also established a model of global, constitutive CHT overexpression via genomic integration of a bacterial artificial chromosome (BAC) containing the full-length Slc5a7 gene (Holmstrand et al., 2014). The BAC–CHT mice express two– to threefold more CHT protein binding throughout the body and a comparable elevation in neuronal HACU. Immunocytochemical analyses revealed a lack of ectopic transporter expression. We have also found BAC–CHT mice to support elevated depolarization–induced ACh release (Iwamoto, Calcutt, & Blakely, manuscript in preparation). Like the motor neuron–specific CHT
overexpressors (CHT expressed by the Hb9 promoter on an otherwise wild-type background), BAC-CHT mice display reduced fatigue in the treadmill test (Fig. 3D). Additionally, these mice display increased horizontal activity in the open field, decreased spontaneous alterations in the Y-maze, and reduced time in the open arms of the elevated plus maze, consistent with an anxiety phenotype. The CHT HET and BAC-CHT mouse models are important new tools for defining the contributions to cholinergic behavior that are made by alterations in CHT expression and/or activity (Table 1).

4.2 CHT Gene Contributions to Human Disorders

The cloning of human CHT cDNAs and mapping of the SLC5A7 chromosomal locus (2q12.3) (Apparsundaram et al., 2001, 2000; Okuda et al., 2000) made possible a search for potential functional polymorphisms and mutations that could affect disease risk. The first such polymorphism to be identified (rs1013940) is an A to G transition at nucleotide 265 of the cDNA that produces an Ile to Val substitution at amino acid 89 in TM3 (Okuda, Okamura, Kaitsuka, Haga, & Gurwitz, 2002). It is reported to be present with an allele frequency of 6% in a small Ashkenazi Jewish cohort. Significant ethnic differences exist in allele frequency (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs1013940), ranging from 1–15%. This is a significantly higher frequency of coding variation than is observed for other neurotransmitter transporters, raising questions as to whether significant risk for one or more disorders associated with disrupted cholinergic signaling is influenced by CHT dysfunction. Indeed, our group has found the Val89 variant to be overrepresented in subjects with major depressive disorder (Hahn et al., 2008) and ADHD (particularly the combined type). With respect to depression, preclinical studies indicate a role for elevated cholinergic signaling in mood/anxiety-like behaviors in mice (Mineur et al., 2013) and suggest differential Slc5a7 expression may contribute to behavioral changes in rats bred for low- vs high-anxiety traits (Diaz-Moran et al., 2013). In relation to attentional dysfunction, Berry et al. (2014) reported a significant association of the Ile89Val polymorphism with distractibility, both in self-reports and in psychometric evaluations. Using a functional magnetic resonance imaging approach, these same investigators found evidence that the Val89 variant is associated with a redistribution of cortical activation in an attention-demanding task (Berry, Blakely, Sarter, & Lustig, 2015). Moreover, a 3′ untranslated region variant in the human
### Table 1  Impact of Genetic Alterations of CHT in Mice

<table>
<thead>
<tr>
<th>Genetic Manipulation</th>
<th>Species</th>
<th>Observed Changes</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>CHT KO</td>
<td>Mouse</td>
<td>Early postnatal lethality</td>
<td>Ferguson et al. (2004)</td>
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<tr>
<td></td>
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<tr>
<td>CHT KO</td>
<td><em>C. elegans</em></td>
<td>40% Less ACh than wild-type worms</td>
<td>Matthies et al. (2006)</td>
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<tr>
<td></td>
<td></td>
<td>Exhibit premature paralysis during swimming when grown on choline poor bacteria</td>
<td></td>
</tr>
<tr>
<td>CHT HET KO</td>
<td>Mouse</td>
<td>Grossly normal</td>
<td>Bazalakova et al. (2007)</td>
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<tr>
<td></td>
<td></td>
<td>Impaired performance on the treadmill</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Reduced sensitivity to scopolamine in the open-field task</td>
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<tr>
<td></td>
<td></td>
<td>Reduced density of M1 and M2 mAChRs in specific brain regions</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Decreased ACh release after basal forebrain stimulation</td>
<td>Parikh et al. (2013)</td>
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<td></td>
<td></td>
<td>Impaired performance on sustained attention task</td>
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<tr>
<td></td>
<td></td>
<td>Attenuated ACh release during SAT task concurrent with normal performance</td>
<td>Paolone et al. (2013)</td>
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<td></td>
<td></td>
<td>Increased α4β2 nAChR density in cortex</td>
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<td></td>
<td></td>
<td>Diminished dopamine levels in the NAc</td>
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<tr>
<td></td>
<td></td>
<td>Decreased DA levels after nicotine or cocaine administration</td>
<td>English et al. (2010)</td>
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<tr>
<td>Hb:9 CHT overexpressor</td>
<td>Mouse</td>
<td>Increased survival time on CHT KO background</td>
<td>Lund et al. (2010)</td>
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<tr>
<td></td>
<td></td>
<td>Increased performance on treadmill task when expressed on wild-type background</td>
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SLC5A7 transcript (rs333229) has been associated with heart rate variability (Neumann, Lawrence, Jennings, Ferrell, & Manuck, 2005), which is reminiscent of the support for heart rate under basal and stress conditions that was observed in CHT HET mice (English et al., 2010). This variant has also been associated with subclinical measures of carotid atherosclerosis (Neumann et al., 2012). Most recently, Barwick and colleagues identified a loss of function, dominantly acting coding mutation that truncates the transporter’s C-terminus, producing a hereditary motor disorder (Barwick et al., 2012). In vitro functional studies accompanying this report indicate a likelihood that this mutation appears to influence the assembly of CHT oligomers, affecting protein levels and CHT trafficking.

Finally, several studies provide evidence of changes in CHT gene or protein expression in the context of mutations in other genes linked to brain disease. For example, CHT gene expression, as well as that of the nicotinic alpha 7 receptor, has been reported to be downregulated in mice deficient in maternal Ube3a expression, generated to model Angelman’s syndrome (Low & Chen, 2010). It is unknown whether these changes reflect Ube3a-linked ubiquitination pathways. Yamada and colleagues found the ubiquitin ligase Nedd4-2 to interact with CHT and modulate transporter surface expression and activity in transfected cells (Yamada et al., 2012). Thus, alterations in CHT expression and function appear to contribute to motor, cardiovascular, mood, and cognitive disturbances. Further profiling of molecular pathways linked to variation in CHT gene expression (Ye et al., 2014) will undoubtedly characterize further the impact of CHT modulation and help elucidate ACh-associated disease risk.

<table>
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<th>Genetic Manipulation</th>
<th>Species</th>
<th>Observed Changes</th>
<th>References</th>
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<tbody>
<tr>
<td>6-OHDA treated Mouse</td>
<td>Impaired performance on object recognition</td>
<td>Zurkovsky et al. (2013)</td>
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<td></td>
<td>Impaired performance on attentional set shifting paradigm</td>
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<tr>
<td>BAC-CHT overexpression Mouse</td>
<td>Diminished fatigue and increased speeds on treadmill task</td>
<td>Holmstrand et al. (2014)</td>
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<td></td>
<td>Decreased time in open arms of elevated plus maze</td>
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5. ADVANCES IN CHT PHARMACOLOGY

5.1 The Search for Novel CHT Modulators: MKC-231

For over 50 years, HC-3 has been the primary pharmacological tool for studying the contribution of CHT to HACU and cholinergic signaling. Because of its dual quaternary amines, HC-3 penetrates the CNS poorly and, although it effectively targets CHT in accessible preparations, it interacts with other molecular targets with varying affinities (Mandl & Kiss, 2006; Yuan, Wagner, Poloumienko, & Bakovic, 2004). Because it is known that HC-3 is a competitive CHT antagonist, binding to the site that recognizes ACh, efforts have been expended to identify novel CHT-targeting pharmacophores. Among these is 2-(2-oxopyrrolidin-1-yl)-N-(2,3-dimethyl-5,6,7,8-tetrahydrofuro[2,3-b]quinolin-4-yl)acetoamide (MKC-231), a positive modulator of CHT and cholinergic signaling in vivo (Murai et al., 1994). Studies indicate that MKC-231 reverses hippocampal ACh depletion after lesion of cholinergic projections and improves working memory deficits detected in the T-maze. Additionally, MKC-231 elevates HACU after lesioning and reverses deficits in a spatial learning task (Bessho et al., 1996). Moreover, this compound enhances evoked ACh release and HC-3 binding in vivo, but has no biochemical or behavioral effects in nonlesioned preparations. These findings are consistent with either a ceiling effect of MKC-231 on HACU in normal animals, or indirect actions that require a preexisting pathological state for elaboration (Bessho, Takashina, Eguchi, Komatsu, & Saito, 2008; Takashina, Bessho, Mori, Eguchi, & Saito, 2008). Because the effects of MKC-231 are still present even after it has been cleared from the brain, the response to repeated dosing appears to be either indirect or a consequence of MKC-231-induced CHT-dependent compensations that overcome the deleterious effects of a lesion. Although experiments with unlabeled MKC-231 and surface plasmon resonance suggest that the ligand has measurable affinity for CHT (Takashina, Bessho, Mori, Kawai, et al., 2008), studies are lacking that demonstrate specificity with respect to other targets, that interactions correlate with CHT distribution, or are lost with cholinergic lesions that reduce CHT levels. Additionally, studies are lacking with radiolabeled MKC-231 that could allow further evaluation of ligand-binding kinetics and modes of CHT interactions. Given that virtually all of the reports with this compound are based on in vivo studies with lesioned animals and that modulation of HACU and HC-3 binding is highly sensitive to the state of cholinergic neuron activation, it seems likely...
that the responses to MKC-231 are due to indirect actions of this agent rather than to a direct interaction with CHT.

5.2 The Search for Novel CHT Modulators: ML352

The findings with MKC-231, despite the continued uncertainty about its mechanism of action, stimulated further the idea that novel compounds which interact with the CHT will provide important insights into transporter physiology and regulation, and could point the way for the development of new therapeutics. The emergence of allosteric agents targeting G-protein-coupled receptors (GPCRs) (Conn, Lindsley, Meiler, & Niswender, 2014; Nickols & Conn, 2014) also suggested that compounds could be developed that target CHT with better specificity and brain penetration than HC-3. Such agents would target sites on the transporter protein other than the orthosteric choline-binding site. To examine this possibility we first sought to overcome obstacles that limit the use of traditional high-throughput screens (HTSs) for identify such agents. These limitations include the low surface expression of CHT at steady state in transfected cells and the costs and handling complications associated with assays based on radioactive choline uptake. By expressing the human CHT C-terminus as a fusion to a plasma membrane reporter protein followed by mutagenesis studies, we identified two amino acids (Leu531Val532) that meet criteria as a dileucine-type trafficking motif. When this sequence was mutated (LVAA), CHT protein exhibited elevated surface expression, overcoming one limitation for a high-throughput assay (Ruggiero et al., 2012). These findings reinforced prior findings of Ribeiro and colleagues who identified the same sequences as sites of CHT endocytic control (Ribeiro et al., 2003, 2005, 2007).

To address the need for a nonisotopic CHT activity assay that is compatible with an HTS format, we considered the possibility that the electrogenicity of the CHT transport cycle would allow for such an approach (Iwamoto, Blakely, & De Felice, 2006). Indeed, when HEK 293 cells stably transfected with the CHT LVAA mutant were incubated with a membrane potential-sensitive dye, significant elevations in whole cell fluorescence were detected upon addition of choline (Ruggiero et al., 2012). This assay was then employed to screen over 300,000 compounds for their ability to modify choline–dependent membrane depolarization, with the aim of identifying agents that could either augment or inhibit CHT function only in the presence of choline while not affecting non-transfected cells (Ennis et al., 2015) (Fig. 4).
Fig. 4 CHT directed high-throughput screening (HTS) and the resulting, novel inhibitor, ML352. (A) HTS workflow depicting the sequence of the nine major phases of the screen with the respective number of compounds that entered (small left hand boxes) and exited (small right hand boxes) each phase. (B) Molecular structure of ML352, a specific noncompetitive inhibitor of CHT. Panels (A) and (B) republished with permission from Ennis, E. A., Wright, J., Retzlaff, C. L., McManus, O. B., Lin, Z., Huang, X., … Blakely, R. D. (2015). Identification and characterization of ML352: A novel, noncompetitive inhibitor of the presynaptic choline transporter. ACS Chemical Neuroscience, 6(3), 417–427. doi: 10.1021/cn5001809. http://pubs.acs.org/doi/pdf/10.1021/cn5001809.
Hits in our initial effort to identify novel CHT inhibitors were confirmed as potent CHT inhibitors in traditional $[^3]H$choline uptake assays, using both cells and mouse brain synaptosomes. Based on their chemical structure or physicochemical properties, compounds that were not considered drugable were discarded. Five structural classes of potential allosteric inhibitors were identified, with one class subjected to chemical diversification and functional analysis (Bollinger et al., 2015; Ennis et al., 2015). From this effort came (N-((3-isopropylisoxazol-5-yl)methyl)-4-methoxy-3-((1-methylpiperidin-4-yl)oxy)benzamide), ML352 (Fig. 3), as the most potent inhibitor of CHT.

Transport and binding studies revealed that ML352 blocks HACU and HC-3 binding noncompetitively. Occupancy of CHT by ML352 and HC-3 is mutually exclusive, suggesting that ML352 stabilizes a non-transporting conformation that cannot bind HC-3. This could occur if ML352 moved CHT to an “inward-facing conformation.” It also appears that ML352 induces a conformation that diminishes cell surface redistribution. This is suggested by an elevation of surface transporters after exposure of CHT transfected cells to ML352 in biotinylation assays (Ennis et al., 2015) (Fig. 5).

Tests of many other GPCRs, ion channels, and transporters, including ACh receptors and LACU, indicate that ML352 interacts selectively with HACU/CHT (Ennis et al., 2015). Pharmacokinetic studies indicated a favorable profile for in vivo use, with limited metabolism and significant brain penetration (Ennis et al., 2015). Currently, we are engaged in studies to elucidate the neurochemical, physiological, and behavioral effects of ML352, with an ultimate goal of using the molecule to attenuate symptoms of disorders thought to involve excess or inappropriate cholinergic signaling. A reduction in HACU, as with HC-3, should limit the availability of choline for presynaptic ChAT, thereby decreasing the synthesis and release of ACh. Although reducing ACh signaling can be hazardous, disorders such as DYT1 dystonia that are associated with excess cholinergic signaling may benefit from such a drug. DYT1 dystonia is a common form of primary dystonia associated with the DYT1 mutation in the torsin1A and is characterized by abnormal involuntary contracting of musculature that causes twisting and turning of the body. Though the specific process by which the disease develops is still unknown, dysfunction in sensory integration controlled by the basal ganglia has been implicated (Breakefield et al., 2008; Eskow Jaunarajs, Bonsi, Chesselet, Standaert, & Pisani, 2015; Sciamanna et al.,...
Fig. 5 See legend on opposite page.
We are particularly interested in the use of novel CHT antagonists such as ML352 for the treatment of dystonia, as both centrally and peripherally acting anticholinergics are already employed for this purpose (Jankovic, 2013; Patel & Martino, 2013). CHT antagonism using HC-3 has proven effective in restoring alterations in synaptic plasticity that are evident in a DYT1 dystonia mouse model (Martella et al., 2009; Sciamanna et al., 2012).

6. CONCLUSION

The elucidation of the CHT gene, and predictions of transporter protein structure, has provided new opportunities for investigating many of the poorly understood dimensions of cholinergic signaling. Key areas for future studies include the generation of high-resolution structures of CHT in the apo state, and when complexed with ligands (eg, choline, HC-3, ML352), elucidation of the mechanisms by which CHT is targeted to synaptic vesicles, and identification of CNS circuits where human SLC5A7 polymorphisms influence disease risk. We hypothesize that the novel CHT pharmacology we have developed could ultimately include CHT activators and molecules that could be beneficial in the treatment of disorders with reduced cholinergic tone, such as Alzheimer’s disease.

Given his important contributions to the understanding of CHT biology and, more generally, his career-long attention to the molecular foundations of brain signaling and disease, we are delighted to offer this review as a tribute to Joseph T. Coyle.

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**Fig. 5** Pharmacological properties of CHT inhibitor ML352. (A) ML352 inhibition of CHT-specific choline uptake in HEK 293 cells transfected with LVAA CHT \( K_i = 92 \pm 2.8 \text{ nM} \) and mouse forebrain synaptosomes \( K_i = 172 \pm 12 \text{ nM} \). (B) ML352 noncompetitively inhibits CHT-mediated choline uptake in mouse forebrain synaptosomes. Addition of ML352 induces a decrease in \( V_{\text{max}} \) at saturating concentrations of choline without a change in \( K_M \). (C) Representative Western blot of biotinylation experiment demonstrating an increase in surface expression of CHT in human CHT transfected HEK 293 cells after a 15-min incubation with 5 \( \mu \text{M} \) ML352 or HC-3. Panels (A)–(C) reprinted with permission from Ennis, E. A., Wright, J., Retzlaff, C. L., McManus, O. B., Lin, Z., Huang, X., … Blakely, R. D. (2015). Identification and characterization of ML352: A novel, noncompetitive inhibitor of the presynaptic choline transporter. ACS Chemical Neuroscience, 6(3), 417–427. doi: 10.1021/cn5001809. http://pubs.acs.org/doi/pdf/10.1021/cn5001809.
CONFLICTS OF INTEREST
The authors have no conflicts of interest to declare.

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