Modulation of sodium-coupled choline transporter CHT function in health and disease

O.A. Ojiakor a,b, R.J. Rylett a,b,c, * 

a Molecular Medicine Research Laboratories, Robarts Research Institute, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada
b Graduate Program in Neuroscience, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada
c Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada

ARTICLE INFO

Keywords:
Cholinergic
Choline transport
CHT
Neurodegeneration
Alzheimer’s disease

ABSTRACT

The sodium-coupled high-affinity choline transporter CHT plays a critical role in acetylcholine (ACh) synthesis by taking up the substrate choline from the synaptic cleft after neurotransmitter release; this conservation mechanism is the rate-limiting step for production of ACh, thereby facilitating communication by subsequent action potentials. Mice carrying a null mutation for CHT die within an hour of birth due to respiratory failure, indicating the essential role of CHT proteins for sustaining cholinergic transmission. Choline uptake activity is regulated dynamically by CHT proteins undergoing rapid trafficking between subcellular compartments and the plasma membrane where they are functionally active. CHT proteins internalize from the cell surface into the endolysosomal pathway by a clathrin-mediated mechanism, but can undergo ubiquitination and proteosomal degradation under conditions such as cellular oxidative stress. Over the years, functionally-relevant CHT polymorphisms have been linked to a range of neurological and psychiatric disorders, including ADHD and depression; the impact of these mutations and the extent to which they alter cholinergic signaling have not been addressed fully. Recent studies have identified compounds that can either promote or diminish cholinergic neurotransmission by modulating CHT function, thus having the potential to serve as pharmacological tools or therapeutic prototypes. Here, we review regulation of CHT activity, trafficking and subcellular disposition of CHT proteins, alteration of transporter function in genetic, neurological and psychiatric diseases, and investigations of compounds that modulate activity of the transporter.

1. Introduction

Solute transporters situated in neuronal plasma membranes play an important role in recovering neurotransmitters or their substrates back into neurons from the synapse following transmitter release. This serves as a conservation mechanism that can either contribute to sufficient neurotransmitter being available to mediate communication by subsequent action potentials or ensure their removal from the synapse to allow successive postsynaptic responses. Cholinergic neurons use the neurotransmitter acetylcholine (ACh) to transmit signals throughout the brain and to peripheral targets; these neurons are thus involved in a wide range of biological processes, including cognition, movement, and attentional processing (Woolf and Butcher, 2011). The enzyme choline acetyltransferase (ChAT) catalyzes the transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to choline to produce ACh in the neuronal axoplasm. Following its biosynthesis, ACh is packaged into synaptic vesicles by the vesicular ACh transporter (VACHT) and released into the synaptic cleft upon neuronal depolarization. This ACh then binds to nicotinic and muscarinic receptors to activate downstream signaling pathways, then the transmitter is cleaved by the enzyme acetylcholinesterase (AChE) into acetate and choline to limit its binding to receptors. Choline is obtained principally from the diet and is present in brain in micromolar amounts (Fernstrom, 1981; Zeisel, 1981). Choline liberated by hydrolysis of ACh adds to this extracellular pool of choline which serves as solute to be transported into cholinergic neurons by the sodium-coupled high-affinity choline transporter CHT and as substrate for ACh synthesis (Birks and MacIntosh, 1961; Collier and Katz, 1971; Tuèck, 1985). Thus, reuptake of choline by CHT proteins localized at cholinergic synapses is critical for cholinergic function and is thought to represent the rate-limiting step of ACh production (Haga, 1985).
Since the gene for CHT was cloned from Caenorhabditis elegans by Okuda and colleagues in 2000, a range of methods have been employed to study the molecular properties of the transporter (Okuda et al., 2000). It was determined that CHT belongs to the SLC5 Na\(^+\)/Cl\(^-\) dependent glucose transporter family (SLC5A7) and consists of 530 amino acids organized into thirteen transmembrane-spanning domains with an extracellular amino-terminus and a cytoplasmic carboxyl-terminus (Apparsundaram et al., 2000; Okuda and Haga, 2000; Okuda et al., 2000). The development of cell and animal models expressing modified CHT proteins has significantly advanced our understanding of function and regulation of the transporter. Importantly, this has revealed that CHT proteins are found predominantly in intracellular compartments, such as endosomes and synaptic vesicles, with only a small proportion of total CHT proteins localized to the plasma membrane where they are functionally active (Ferguson et al., 2003; Ribeiro et al., 2003; Ferguson et al., 2004). Mice carrying a null mutation for the CHT gene die within an hour of birth due to respiratory failure, highlighting the essential role played by this transporter in mediating cholinergic signaling (Ferguson et al., 2004). Remarkably, haploinsufficient CHT (+/-) mice demonstrate sustained choline uptake activity and plasma membrane levels similar to that found in wild-type CHT (+/+ ) mice, despite having a 50% decrease in CHT protein levels (Parikh et al., 2013). It is important to note that stimulation of cholinergic neurons in wild-type mice leads to mobilization of CHT proteins to the plasma membrane with elevated CHT density and increased clearance of extracellular choline, but this mobilization is attenuated in CHT (+/-) mice (Paolone et al., 2013; Parikh et al., 2013). Taken together, these findings demonstrate the critical function of CHT proteins for maintenance of cholinergic transmission.

Recent studies have also begun to explore CHT as a possible drug target and a number of small molecules have been discovered that modulate activity of the transporter either positively or negatively (Choudhary et al., 2017; Ennis et al., 2015). These findings could have critical implications in the treatment of diseases that affect cholinergic neurons, such as Alzheimer disease (AD) and amyotrophic lateral sclerosis (ALS) (Barron et al., 1987; Nagata et al., 1982; Oda, 1999; Selkoe, 2002; Virgo et al., 1992; Wilcock et al., 1982). Unsurprisingly, mutations at SLC5A7 have been discovered to underlie, at least in part, the impairment of cholinergic neurotransmission that occurs in some disorders, including myasthenias, cardiovascular disease, depression and attention deficit hyperactivity disorder (ADHD) (Banerjee et al., 2019; Bauché et al., 2016; English et al., 2009; Neumann et al., 2005, 2006; Okuda et al., 2002). This review focuses on studies examining the regulation of CHT-mediated high-affinity choline uptake, how CHT is affected in genetic, neurological and psychiatric diseases, as well as recent efforts to identify compounds that modulate activity of the transporter.

2. Subcellular trafficking and functional regulation of CHT

Trafficking of CHT proteins between subcellular compartments and the cell surface represents a critical means of regulating cholinergic neurotransmission as it serves as a mechanism to modulate the rate of choline uptake (Ferguson and Blakely, 2004; Ribeiro et al., 2006). We determined that about 10% of total CHT proteins form a rapid recycling pool that moves between intracellular vesicles and the plasma membrane (Ribeiro et al., 2003, 2007). CHT proteins internalize constitutively from the cell surface via a clathrin-dependent mechanism that is controlled by an atypical dileucine-like motif in its intracellular carboxyl-terminus (L531, V532); substitution of these residues with alanine (L531A and V532A) prevents this constitutive internalization of transporter proteins (Ribeiro et al., 2005).

One mechanism by which the reuptake of neurotransmitters into neurons can be regulated is by linking transporter trafficking and plasma membrane levels with neurotransmitter release. In GABAergic neurons, the GABA transporter GAT1 is trafficked in parallel with GABA-ergic neurotransmission (L531, V532); substitution of these residues with alanine (L531A and V532A) prevents this constitutive internalization of transporter proteins (Ribeiro et al., 2005).

Fig. 1. Schematic of a cholinergic synapse. The presynaptic neuron forms a cholinergic synapse with a postsynaptic neuron. In the presynaptic neuron, the enzyme choline acetyltransferase (ChAT) catalyzes synthesis of ACh from choline and acetate in the neuronal axoplasm. Vesicular acetylcholine transporters (VACHT) package ACh into synaptic vesicles which are released into the synaptic cleft upon Ca\(^{2+}\)-mediated depolarization. Released ACh then binds to nicotinic and muscarinic receptors on the postsynaptic neuron and the transmitter is cleaved by the enzyme acetylcholinesterase (AChE) into acetate and choline in the synaptic cleft. Choline liberated from ACh hydrolysis is then recovered from the synaptic cleft into cholinergic presynaptic nerve terminals via a high-affinity choline transporter CHT.
release (Deken et al., 2003). Previous studies have shown that for ChT, in addition to its constitutive trafficking to and from the plasma membrane, there exists a pool of intracellular ChT proteins that can be recruited upon neuronal depolarization. Indeed, K⁺-mediated depolarization increases the mobilization of ChT proteins to the cell surface and the size of the ChT protein recycling pool (Ribeiro et al., 2003, 2005). This observation suggests the presence of distinct vesicular organelles for ChT trafficking, with one group being responsible for mediating constitutive ChT recycling to and from the plasma membrane and another involved in the regulated recruitment of additional ChT proteins to the cell surface upon neuronal depolarization. In addition, similar to GAT1 in GABAergic neurons, there appear to be at least two subpopulations of synaptic vesicles in cholinergic neurons that can be distinguished by their ChT content; isolation of synaptic vesicles with multiple antibodies reveals that although most neurotransmitter-filled ChT-positive vesicles are VACHT-positive, only about 50% of VACHT-positive vesicles contain ChT (Ferguson et al., 2003). This may suggest that low rates of ACh release are supported by synaptic vesicles containing only VACHT, whereas prolonged excitation of cholinergic nerve endings may trigger the recruitment of the ChT-positive synaptic vesicles to increase ACh release and subsequent choline uptake (Ferguson et al., 2003; Ribeiro et al., 2006; Ennis and Blakey, 2016).

Importantly, the activity-dependent recruitment of this reserve pool of ChT-positive vesicles is required to sustain increases in cholinergic transmission during attentional performance (Apparsundaram et al., 2005), a process that is impaired in ChT (+/−) mice due to the smaller pool of intracellular ChT available for recruitment in these mice when compared to wild-type mice (Parikh et al., 2013; Paolone et al., 2013).

Moreover, negative regulators of ChT function resulting in reduced levels of cholinergic transmission have revealed the impact of changes in attentional bias on reward-related and addiction behaviours (Khosy Cherian et al., 2017). ChT may be functionally coupled to ChAT to facilitate ACh synthesis from choline transported into cholinergic neurons. ChAT exists in cytosolic and membrane-bound compartments and evidence suggests that neuronal activity can simultaneously accelerate choline uptake and increase activity of membrane-bound ChAT (Benishin and Carroll, 1983; Carroll et al., 1986; Cooke and Rylett, 1997; Docherty and Bradford, 1988; Eder-Colli et al., 1986); however, further investigations are needed to uncover properties regulating ChAT in these various subcelluar locations and how this may coordinate with ChT activity in cholinergic neurons. Taken together, these observations indicate that ChT proteins can be recruited from reserve pools to mediate compensatory increases in choline uptake when needed to maintain ACh synthesis.

To understand how trafficking of neurotransmitter transporters is controlled in neurons, several studies have focused on elucidating mechanisms involved in acute regulation of transporter density at the cell surface. This includes investigation of the link between transporter activity and modulation of their phosphorylation using activators or inhibitors of protein kinases and phosphatases, as protein phosphorylation is largely a dynamic process driven by transient signaling events. It has been found that acute treatment of neural cells with the protein kinase C (PKC) activator β-phorbol 12-myristate 13-acetate (PMA) leads to decreased cell surface levels of some neurotransmitter transporters, including the serotonin transporter SERT (Qian et al., 1997) and the dopamine transporter DAT (Corey et al., 1994; Foster and Vaughan, 2017; Huff et al., 2002; Kitayama et al., 1994; Zhang et al., 1997). The importance of PKC-mediated regulation of substrates which are substrates for phosphatase has further been revealed when Khoshbouei and colleagues showed that phosphorylation of DAT is necessary for amphetamine-induced dopamine efflux (Khoshbouei et al., 2004). Our laboratory showed that inhibition of serine-threonine protein phosphatase 1/2A (PP1/PP2A) with calyculin-A or okadaic acid in rat brain synaptosomes, which could lead to increased protein phosphorylation, resulted in decreased choline uptake activity (Cooke and Rylett, 1997).

Further, experiments by Gates and colleagues revealed that either activation of PKC with PMA or inhibition of PP1/PP2A decreased ChT cell surface levels and choline transport activity in mouse brain synaptosomes (Gates et al., 2004). Interestingly, these latter results differ from our findings that short-term treatment of neural cells with PMA resulted in a rapid and transient increase in the activity, plasma membrane levels and recycling of ChT proteins (Black et al., 2010). A possible explanation for these differences is that the duration of PMA-treatment may alter the effects of phosphorylation-induced changes in ChT trafficking, with acute treatment leading to increased ChT mobilization to the plasma membrane and more prolonged incubation with the drug leading to a decrease in choline uptake activity and a reduction in the amount of ChT protein at the cell surface. Importantly, under the conditions tested, inhibition of PP1/PP2A, but not PMA treatment, resulted in incorporation of [32P]phosphate into ChT protein (Gates et al., 2004). Further studies are needed to determine if the effects of PMA treatment or the modulation of other kinases are related either directly to phosphorylation of the transporter or due to modification of regulatory or interacting proteins, and the mechanisms by which this regulates ChT trafficking and choline uptake activity.

Emerging evidence suggests that cell surface levels of some transporters can be regulated by their substrates. A pioneering study by Duan and colleagues revealed that treatment of mouse astrocyte cultures with glutamate produces a rapid and dose-dependent increase in glutamate uptake activity associated with increased cell surface levels of the excitatory amino acid transporter EAAT1 (GLAST) (Duan et al., 1999). Similar observations have been made with serotonin and GABA substrates leading to increased cell surface levels and activity of SERT and GAT1, respectively (Bernstein and Quick, 1999; Ramamoorthy and Blakey, 1999). However, the opposite effect has also been reported, with exposure of both cells and rat brain synaptosomes to dopamine resulting in decreased cell surface levels of DAT (Chi and Reith, 2003).

Thus, the question emerges of how extracellular choline affects cell surface levels of ChT protein. A study by Okuda and colleagues revealed that exposure of cells to high concentrations of choline increases ChT internalization to intracellular vesicles in rat brain synaptosomes, suggesting that fluctuations in extracellular choline concentrations may affect ChT trafficking (Okuda et al., 2011). However, unlike constitutive internalization of ChT proteins that occurs by a clathrin-dependent process, substrate-induced ChT endocytosis relies on a clathrin-independent, dynamin-dependent endocytic pathway. This indicates that the small population of ChT proteins normally observed at cholinergic nerve terminal membranes may be internalized by both constitutive and substrate-mediated trafficking to synaptic and endosomal vesicles as choline concentrations at synapses are sufficiently high to saturate ChT proteins (Okuda et al., 2011).

3. ChT protein stability and degradation

An important mechanism by which choline uptake activity is regulated in cholinergic neurons is through ChT protein turnover and degradation. Rab proteins are GTPases that regulate the trafficking of cell surface proteins to and from the plasma membrane and between different intracellular organelles. Thus, these proteins represent effective molecular tools for monitoring the route by which membrane proteins are trafficked in the endolysosomal pathway (Sönichsen et al., 2006; Zerial and McBride, 2001). Following clathrin-mediated internalization, ChT proteins colocalize with Rab-5 positive early endosomes (Ribeiro et al., 2003, 2005) and can recycle back to the cell surface either rapidly by Rab-4 positive endosomes or slowly by Rab-11 positive recycling endosomes (Ribeiro et al., 2007). ChT can also be transported through Rab-7 and Rab-9 positive late endosomes to lysosomes which is the primary organelle where the transporter is degraded; this is demonstrated by treatment of neural cells with lysosome inhibitors leading to increases in the steady-state levels of ChT proteins (Cuddy et al., 2012, 2017) (see Fig. 2).

Subcellular trafficking and degradation patterns of plasma membrane proteins can be altered by diverse conditions, such as cellular stress.
conditions or mutations. For example, exposure of cells to oxidative stress switches the degradation pathway of the glucose transporter GLUT1 from the lysosome to proteasome (Fernandes et al., 2011), while a single point mutation in the low-density lipoprotein receptor switches the degradation of its mature protein from the proteasome to the lysosome (Martín de Llano et al., 2006). Our laboratory demonstrated that CHT activity is reduced in neural cells exposed to cellular stress by the peroxynitrite (ONOO-) donor SIN-1, and that blocking proteasome but not lysosome function with inhibitors attenuates the inhibition of choline uptake activity observed in these cells (Cuddy et al., 2012). Proteasomal degradation is normally signalled by the addition of a chain of ubiquitin molecules to lysine sidechains of a target protein; this is catalyzed sequentially by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) in an ATP-dependent manner (Wang and Robbins, 2014). E3 ubiquitin ligases are involved in recognizing the substrate and thus in determining the specificity of the protein targeted for proteasome degradation. We showed that CHT proteins in SIN-1-treated cells were ubiquitinated, with this potentially mediating signaling for proteosomal degradation (Cuddy et al., 2012). In other studies, it was demonstrated that the ubiquitin ligase Nedd4-2 can mediate ubiquitination of plasma membrane CHT proteins and negatively regulate levels of the transporter at the cell surface and reduce choline uptake activity (Yamada et al., 2012). It is not known, however, if ubiquitination of CHT proteins by Nedd4-2 is able to direct CHT proteins to the proteasome for degradation or if this post-translational modification regulates trafficking of transporters between the plasma membrane and subcellular compartments.

4. Human genetic findings for SLC5A7/CHT

There has been considerable interest in the CHT gene (SLC5A7) and the role that mutations may play in relation to neurological and psychiatric disorders. Molecular cloning of SLC5A7 from C. elegans (Okuda et al., 2000) was followed in 2002 by the discovery of a functionally relevant, nonsynonymous SLC5A7 single nucleotide polymorphism (SNP) in an Ashkenazi Jewish population (Okuda et al., 2002). The functional properties of this CHT variant (Ile89Val) were assessed following its expression in mammalian cell lines. This mutant CHT has a 40–50% decrease in choline transport rate compared to wild-type, despite there being normal levels of the protein at the plasma membrane; the loss-of-function is related to a decrease in Vmax for uptake rate with no change in the affinity for solute, suggesting an impaired translocation mechanism. Another polymorphism, located in the 3′ untranslated region of SLC5A7 (3′SNP) was revealed in a subsequent study to be associated with peripheral cholinergic neuron dysregulation, measured by variability in heart rate and depressive symptoms (Neumann et al., 2005, 2006).

In 2009, a large case-controlled study by English and colleagues identified that the Ile89Val and 3′SNP variants, both polymorphisms that affect CHT functional activity, were associated with ADHD in a Vanderbilt/Chicago cohort of patients diagnosed with the disorder (English et al., 2009). In this cohort, there was a two-to three-fold elevation of the Ile89Val allele, as well as a significant increase in the 3′ SNP major allele, observed primarily in male Caucasians relative to healthy control subjects and published allele frequencies. Other studies tested the hypothesis that CHT capacity is linked to attentional performance by assessing distractibility in Ile89Val subjects using both self-report measures of attention and a laboratory task that measured...
sustained attention with and without distraction (Berry et al., 2014, 2015; Sarter et al., 2016). Ile89Val participants showed an increased vulnerability to distraction on both self-report measures and laboratory task performance compared to controls, indicating that the cholinergic system plays an important role in resisting distraction and that decreased cholinergic signaling might lead to attentional deficits in ADHD subjects (Berry et al., 2014; Sarter et al., 2016). In support of this, other studies have shown that in response to attentional demands, there is an increase in the capacity and density of CHT proteins in synaptic membranes in the right prefrontal cortex of attention-task-performing rodents expressing wild-type CHT (Apparsundaram et al., 2005). Importantly, Ile89Val carriers exhibit reduced activation in this cortical region in response to attentional demands, compared to control subjects (Berry et al., 2015).

Both cortical and subcortical cholinergic systems are also known to be involved in facilitating sensory motor-gating and in mediating arousal and attentional processing (Kobayashi and Isa, 2002; Sarter and Bruno, 1999). Interestingly, the Ile89Val variant showed significant association with depression severity in a cohort of 110 patients with major depressive disorder and subjects carrying the 3′SNP polymorphism associated with enhanced CHT function showed reduced depressive symptoms, indicating that cholinergic signaling deficits may also be implicated in the disorder (Hahn et al., 2008; Neumann et al., 2006). One possibility is that hypofunctional CHT leads to decreased cholinergic tone in the central nervous system, which in turn alters receptor sensitivity and impacts risk for depression. Indeed, hypofunctional CHT (+/−) mice display reduced sensitivity to the muscarinic receptor antagonist scopolamine in a locomotor behavior task (Bazalakova et al., 2007). Future studies are needed to replicate these findings and examine the impact of CHT-associated genetic alterations in depression. These studies could also identify subpopulations of patients that can benefit from more targeted pharmacotherapy. Moreover, the comorbidity that exists between depression and ADHD may suggest that altered cholinergic tone is shared by and underlies both disorders (English et al., 2009; Hahn et al., 2008).

The clearest findings related to mutations in SLC5A7 have emerged in a genetically heterogenous group of rare disorders of the neuromuscular junction (NMJ) associated with variable fatigability and weakness of skeletal muscle, generally classified under the term ‘congenital myasthenic syndromes’ (CMS). Using whole-exome sequencing to define disease-causing mutations within three families, a recent study by Wang and colleagues revealed the presence of autosomal recessively-acting CHT mutations in patients with a severe CMS phenotype (Wang et al., 2017). In vitro work conducted by transfecting HEK 293 cells with cDNA constructs containing these missense mutations showed that the steady-state levels of CHT proteins present were not significantly different between wild-type CHT and the mutant SLC5A7 variants, indicating that these mutations do not alter either translation or protein stability. However, cell-surface biotinylation and immunoblot approaches revealed a decrease in the plasma membrane levels of all three CHT variants when compared with wild-type CHT, demonstrating an attenuation in trafficking of the transporter to the cell surface. Further investigation into the effects of these mutations on CHT-dependent choline uptake activity revealed a significant decrease in CHT activity for two of the variants (p.Ser94Arg and p.Val112Glu) compared with wild-type CHT in transiently transfected HEK 293T cells (Wang et al., 2017). Taken together, these findings indicate that the CMS-associated deficits in these recessively acting SLC5A7 variants are likely due to the combined deleterious outcomes on both CHT cell surface trafficking and transporter activity. Indeed, another study by Bausch and colleagues shows that loss-of-function mutations of SLC5A7 in six unrelated families is the underlying cause of a recessive form of CMS, indicating that CHT dysfunction may underlie a clinical spectrum of the disorder (Bausch et al., 2016).

5. CHT in Alzheimer disease (AD)

Current research supports a crucial role for cholinergic transmission in some neurological and psychiatric diseases. One particularly salient example is AD, which is characterized by the loss of basal forebrain cholinergic neurons coinciding with impaired higher cognitive function. Although there is progressive and widespread loss of function of a number of neuronal networks in the brains of AD patients, cholinergic neurons of the basal forebrain possibly exhibit the earliest, most severe and most consistent functional decline that occurs in this disorder (Auld et al., 2002; Chen and Mobeley, 2019; DeKosky and Marek, 2003; Mufson et al., 2003). Several studies have examined how high-affinity choline uptake is altered in AD. One study performed using purified synaptosomes from necropsy brains of late-stage AD patients showed a 50% decrease in choline transport in frontal cortex nerve terminals compared with controls (Rylett et al., 1983). Another study reported widespread loss of cholinergic projections and increased high-affinity choline uptake in the remaining cholinergic nerve terminals, suggesting that there may be upregulation of CHT activity in the remaining cholinergic terminals in some cortical areas (Slotkin et al., 1990). Differences in the findings between these studies could reflect the stage of disease of study participants and the brain areas assessed. Mechanistic studies have been designed to address the effects of β-amyloid (Aβ) – toxic peptides that comprise a major component of AD pathology – on CHT activity. Some report an increase in choline uptake following Aβ treatment (Bales et al., 2006; Kristóffórová et al., 2006), whereas others reveal an impairment in choline uptake (Apelt et al., 2002; Cuddy et al., 2015, 2017; Klingner et al., 2003; Opazo et al., 2006; Payette et al., 2007) or no effect (Hartmann et al., 2004; Melo et al., 2002). These findings likely reflect differences in the in vitro models studied and Aβ preparations used. Future studies that make use of high-specificity CHT protein probes are needed to examine whether the dynamic levels of cell surface CHT proteins or choline uptake activity is impaired in AD, as well as to elucidate mechanisms by which impairment occurs.

CHT trafficking can be modulated by its interaction with other proteins such as amyloid precursor protein (APP), an integral membrane protein whose proteolysis leads to the generation of Aβ peptides. APP is an established binding partner for CHT and has been shown to be implicated in regulating the presynaptic localization of CHT proteins and their internalization by endocytosis from the cell surface (Wang et al., 2007). The Swedish mutation of APP (K595N/M596L) (APPsw) increases by 10-fold the cleavage of cellular APP by the enzyme β-secretase (BACE) within the trans-Golgi network and secretory pathway; this mutant form of APP is thus considered to be causal for a subset of early onset familial AD (Haas et al., 1995; Thinakaran et al., 1996). Our studies revealed that CHT interacts significantly less with APPsw than APPwt although both forms of APP inhibit CHT function to a similar extent by decreasing CHT cell surface levels and choline uptake activity in SH-SY5Y cells, but by different mechanisms (Cuddy et al., 2015). Altered CHT function that occurs when the transporter is coexpressed with APPsw is related primarily to the interaction between CHT and APP, whereas APPsw-induced CHT inhibition is due to the increased Aβ production caused by the accelerated cleavage of this mutant APP. Interestingly, this Aβ-mediated decrease in CHT cell surface levels and choline uptake activity was attenuated when Aβ was neutralized using an anti-Aβ antibody directed at the N-terminal amino acids 1–16 of Aβ, but not by an antibody directed at the mid-region amino acids 22–35 of Aβ (Cuddy et al., 2015). This suggests that Aβ immunotherapy may have additional therapeutic consequences of preventing or slowing cholinergic dysfunction in AD.

The role of oxidative stress (OS), which occurs when the generation of reactive oxygen and nitrogen species (ROS/RNS) exceeds cellular antioxidant defenses, is an active area of investigation in relation to age-related neurodegenerative diseases such as AD (Tónnies and Trushina, 2017; Wang and Michaelis, 2010). Because cholinergic neurons of the basal forebrain undergo early and consistent functional decline in the
progression of AD, it is possible that OS plays a role in the selective vulnerability of these neurons, as multiple lines of evidence have implicated OS in contributing to the early pathological lesions that occur in the disease (Casadesus et al., 2004; Castellani et al., 2002; Smith et al., 1997, 1994). We showed that the peroxynitrite-donor SIN-1 impairs CHT activity and decreases cell surface levels of the transporter (Cuddy et al., 2012; Pithong et al., 2008), suggesting that CHT dysregulation may be involved in OS-associated dysfunction of cholinergic transmission. Interestingly, while exposure of cells to SIN-1 does not inhibit choline uptake by a direct effect on the protein, we did find that preventing clathrin- or dynamin-mediated endocytosis of CHT attenuated the effects of SIN-1 on CHT activity, suggesting that peroxynitrite impairs high-affinity choline uptake by increasing CHT internalization (Cuddy et al., 2012). These studies also showed that movement of CHT proteins in endolysosomal compartments was not altered by SIN-1 treatment (Cuddy et al., 2013). The enhanced internalization of CHT proteins in SIN-1-treated cells may represent a more generalized mechanism by which neurons prevent damage of cell surface transporters or remove damaged proteins from the plasma membrane when exposed to OS. Indeed, we reported that SIN-1 treatment also led to internalization of the transferrin receptor (Pithong et al., 2008), another cell surface receptor that also undergoes clathrin-mediated internalization (Hanover et al., 1984).

6. Discovery of compounds that modulate CHT activity

Although it has been decades since CHT was identified as playing a major role in cholinergic signaling by serving as the rate-limiting step of ACh production, studies that manipulate CHT function pharmacologically or genetically to modulate cholinergic activity have appeared more recently. In 1969, a pioneering study by Diamond and Kennedy revealed that high-affinity choline transport in synaptosomes from guinea pig brain samples was inhibited by the competitive antagonist hemicholinium-3 (HC-3) (Diamond and Kennedy, 1969). It is now known that while inhibiting choline transport activity, HC-3 may also perturb CHT trafficking resulting in retention of transporters at the cell surface. Another potent, non-selective inhibitor of CHT, termed ML352, that inhibits choline uptake by a direct effect on the protein, we did find that pre-treatment with ML352 promoted an increase in high-affinity choline uptake (Ruggiero et al., 2012). Two studies reported similar findings that repeated administration of another compound, MKC-231, promoted an increase in high-affinity choline uptake in hippocampal synaptosomes which correlated with cognitive improvement in memory and learning-impaired rats (Bessho et al., 2008; Takashina, Bessho et al., 2008; Takashina et al., 2008).

In an effort to identify and characterize novel molecular tools that modulate high-affinity choline uptake, Choudhary and colleagues recently performed a large screening analysis using compounds based on the CHT positive allosteric modulator MKC-231 (Bessho et al., 2008; Takashina et al., 2008), the negative allosteric modulator ML352 (Ennis et al., 2015) and a compound library comprising 2753 molecules (Choudhary et al., 2017). This resulted in the discovery of nine previously unknown active and structurally distinct positive allosteric modulators of CHT, as well as three novel negative allosteric modulators. Collectively, these discoveries provide new tools to test the hypothesis that modulation of CHT activity may be a central therapeutic mechanism for treating disorders associated with either cholinergic hyper- or hypo-function.

Importantly, characterizing compounds that modulate CHT transport function may also reveal new therapeutic targets for enhancing cholinergic transmission in the early stages of disorders like AD when cholinergic signaling is impaired, but cholinergic neurons remain viable (Mufson et al., 2003). Indeed, promoting cholinergic signaling remains a critical aspect of AD therapy as cholinesterase inhibitors that block ACh degradation, such as donepezil, galantamine and rivastigmine, do not appear to modify the disease progression or prevent neuron loss. As CHT controls the rate-limiting step of ACh production and changes in its expression and trafficking have been shown to modulate cholinergic transmission (Haga, 1971; Murrin and Kuhar, 1976), a potential strategy for improving cholinergic function in AD could be through the use of drugs that enhance CHT activity or promote its trafficking to the plasma membrane. Thus, such drugs may be beneficial in priming efforts towards the development of future targets for AD therapy.

7. Conclusion

Due to their unique and widespread organization in the nervous system, cholinergic neurons are important in the maintenance of key biological functions, including cognition, movement and attentional processing. A wealth of literature supports the role of the high-affinity choline transporter CHT in maintaining cholinergic neurotransmission through the uptake of choline into cholinergic neurons, with this process being the rate-limiting step of ACh synthesis. CHT protein function and trafficking have been shown to modulate cholinergic function in vivo, such as enhancement of learning and memory in aged transgenic Tg2576 mouse brain expressing the Swedish mutation of human β-amyloid precursor protein (Apelt, J., Kumar, A., Schliebs, R., 2002. Impairment of cholinergic neurotransmission in adult and aged transgenic Tg2576 mouse brain expressing the Swedish mutation of human β-amyloid precursor protein. Brain Res. 953, 17–30. https://doi.org/10.1016/S0006-8993(02)01262-4).

Acknowledgements

This research was supported by research grants from the Natural Sciences and Engineering Research Council (Canada) and the Canadian Institutes of Health Research to RJR. OAO is the recipient of a Studentship from the Alzheimer Society of London and Middlesex (Canada).

References

Apelt, J., Kumar, A., Schliebs, R., 2002. Impairment of cholinergic neurotransmission in adult and aged transgenic Tg2576 mouse brain expressing the Swedish mutation of human β-amyloid precursor protein. Brain Res. 953, 17–30. https://doi.org/10.1016/S0006-8993(02)01262-4.
Castellani, R., Hirai, K., Aliev, G., Drew, K.L., Nunomura, A., Takeda, A., Cash, A.D., Bazalakova, M.H., Wright, J., Schneble, E.J., McDonald, M.P., Heilman, C.J., Levey, A.I., Benishin, C.G., Carroll, P.T., 1983. Multiple forms of choline-O-acetyltransferase in cholinergic nerve endings of the Torpedo. J. Neurosci. 19, 10193–10200. https://doi.org/10.1124/jnc.19.23.10193.

Denu, S.L., Wang, D., Quick, M.W., 2003. Plasma membrane GABA transporters reside on distinct vesicles and undergo rapid regulated recycling. J. Neurosci. 23, 1563–1568. https://doi.org/10.1523/jneurosci.23-05-1563.2003.

DeKosky, S.T., Marek, K., 2003. Looking backward to move forward: early detection of neurodegenerative disorders. Science 302, 830–834. https://doi.org/10.1126/science.1087009.

Diamond, I., Kennedy, E.P., 1969. Carrier-mediated transport of choline into synaptic nerve endings. J. Biol. Chem. 244, 3258–3263. Retrieved from https://www.jbc.org.

Docherty, M., Bradford, H.F., 1988. Choline acetyltransferase in mammalian synaptosomes: evidence for an integral membrane-bound form. Neuroscience. Int. 13, 119–127. https://doi.org/10.1017/s014769168880011-8.

Duan, S., Anderson, C.M., Stein, B.A., Swanson, R.A., 1999. Glutamate induces rapid upregulation of astrocyte glutamate transporter and cell-surface expression of GLAST. J. Neurosci. 19, 1019–10200. https://doi.org/10.1523/jneurosci.19-23-10193.1999.

Eder-Colli, L., Amato, S., Froment, Y., 1986. Amphiphilic and hydrophilic forms of Choline-O-acetyltransferase protein in the electric organ. Neuroscience 19, 275–287. https://doi.org/10.1016/0306-4522(86)90188-1.

English, B.A., Hahn, M.K., Gizer, I.R., Mazei-Robison, M., Steele, A., Kurnik, D.M., Brown, M., Heilman, C.J., Blakely, R.D., 2004. Choline transporter gene variation is associated with attention-deficit hyperactivity disorder. J. Neurodev. Disord. 1, 252–263. https://doi.org/10.1016/s1669-0993(08).30012-4.

Ennis, E.A., Blakely, R.D., 2016. Choline on the move. Perspectives on the molecular physiology and pharmacology of the presynaptic choline transporter. Adv. Pharmacol. 175–213. https://doi.org/10.1016/s0065-2194(16)30009-2.

Ferguson, S.M., Blakely, R.D., 2004. The choline transporter restores: new roles for choline transport in the brain. J. Neural. Transm. 111, 84–103. https://doi.org/10.1007/s00702-003-1061-1.

Ferguson, S.M., Savchenko, V., Apparsundaram, S., Zwick, M., Wright, J., Heilman, C., Yi, H., Levey, A., Blakely, R.D., 2003. Vesicular localization and activity-dependent trafficking of presynaptic choline transporters. ACS Chem. Neurosci. 6, 417–427. https://doi.org/10.1021/cn0100819.

Ferguson, S.M., Bazalakova, M., Savchenko, V., Tapia, J.C., Wright, J., Blakely, R.D., 2004. Lethal impairment of cholinergic neurotransmission in hemicholinium-3-sensitive choline transporter knockout mice. Proc. Natl. Acad. Sci. U. S. A. 101, 7826–7831. https://doi.org/10.1073/pnas.0403120101.

Fernandes, R., Hossoya, K., Abe, H., 1994. Reactive oxygen species downregulate glucose transport system in retinal endothelial cells. Am. J. Physiol. Cell Physiol. 266, C212–C218. https://doi.org/10.1152/ajpcell.1994.266.1.c212.

Foster, J.D., Vaughan, R.A., 2017. Phosphorylation mechanisms in dopamine transporter regulation. J. Chem. Neuroanatom. 83–84, 10–18. https://doi.org/10.1016/j.jchemneu.2019.06.004.

Gates, J., Ferguson, S.M., Blakely, R.D., Apparsundaram, S., 2004. Regulation of choline transporter surface expression and phosphorylation by protein kinase C and protein phosphatase 1/2A. J. Pharmacol. Exp. Therapeut. 310, 536–545. https://doi.org/10.1124/jpet.104.066795.

Haas, C., Lemerce, A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannert, L., Selkoe, D.J., 1995. The Swedish mutation causes early-onset Alzheimer’s disease by β-secretase cleavage within the secretory pathway. Nat. Med. 1, 1291–1296. https://doi.org/10.1038/nm0195-1291.

Haga, T., 1971. Synthesis and release of [14C] acetylcholine in synaptosomes. J. Neurochem. 18, 781–798. https://doi.org/10.1111/j.1471-4159.1971.tb01208.x.

Hahn, M.K., Blackford, A.J., Haman, K., Mazzei-Robison, M., English, B.A., Mazei-Robison, M.P., Blakely, R.D., Fredrick, C.H., Steele, A., Hazeldow, L., Fentress, H.M., Sanders-Bush, E., Shelton, R., 2008. Multivariate permutation analysis associates multiple
polymorphisms with subtypes of major depression. Gene Brain Behav. 7, 487–495. https://doi.org/10.1111/j.1601-0022.2007.00080.x.

Hanover, J.A., Willingham, P.C., Postan, I., 1984. Kinetics of transit of transferrin and epithelial growth factor through clathrin-coated membranes. Cell 35, 283–293. https://doi.org/10.1016/0092-8674(84)90006-0.

Hartmann, J., Erb, C., Ebert, U., Baumann, K.H., Popo, A., König, G., Klein, J., 2004. Central cholinergic system in human heterozygous M140V mutant protein kinase in knock-in presenilin-1 transgenic mice. Neuroscience 125, 1009–1017. https://doi.org/10.1016/j.neuroscience.2004.02.038.

Huff, R.A., Vaughan, R.A., Kahar, M.J., Uhl, G.R., 2002. Phobol esters increase dopaminergic transporter phosphorylation and decrease transport Vmax. J. Neurochem. 86, 225–232. https://doi.org/10.1002/jn.4990860231.

Khoshbouei, H., Sen, N., Guptray, B., Johnson, L., Lund, D., Gerey, M.E., Gali, A., Javitch, I.A., 2004. N-terminal phosphorylation of the dopamine transporter is required for amphetamine-induced eF8. PLoS 2. https://doi.org/10.1371/journal.pbio.0020078.

Kizayama, S., Dohi, T., Uhl, G.R., 1994. Phobol esters alter functions of the expressed transporter. Eur. J. Pharmacol. 268, 115–119. https://doi.org/10.1016/0014-2999(94)80180-5.

Klingner, M., Apelt, J., Kumar, A., Sorger, D., Sabri, O., Steinbach, J., Scheunemann, M., et al., 2019. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j.i jdevneu.2003.08.001.

Kobayashi, Y., Ista, T., 2002. Sensory-motor gating and cognitive control by the brainstem cholinergic system. Neural Network. 15, 731–741. https://doi.org/10.1016/s0893-6080(02)00203-x.

Koshy Cherian, A., Kucinski, A., Pitchers, K., Yegla, B., Parikh, V., Kim, Y., Valuskova, P., et al., 2003. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j. i jdevneu.2003.08.001.

Krylova, T., Kucinski, A., Pitchers, K., Yegla, B., Parikh, V., Kim, Y., Valuskova, P., et al., 2003. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j. i jdevneu.2003.08.001.

Krylova, T., Kucinski, A., Pitchers, K., Yegla, B., Parikh, V., Kim, Y., Valuskova, P., et al., 2003. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j. i jdevneu.2003.08.001.

Krylova, T., Kucinski, A., Pitchers, K., Yegla, B., Parikh, V., Kim, Y., Valuskova, P., et al., 2003. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j. i jdevneu.2003.08.001.

Krylova, T., Kucinski, A., Pitchers, K., Yegla, B., Parikh, V., Kim, Y., Valuskova, P., et al., 2003. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j. i jdevneu.2003.08.001.

Krylova, T., Kucinski, A., Pitchers, K., Yegla, B., Parikh, V., Kim, Y., Valuskova, P., et al., 2003. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j. i jdevneu.2003.08.001.

Krylova, T., Kucinski, A., Pitchers, K., Yegla, B., Parikh, V., Kim, Y., Valuskova, P., et al., 2003. Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse with β-amyloid pathology. Int. J. Dev. Neurosci. 21, 357–369. https://doi.org/10.1016/j. i jdevneu.2003.08.001.
Tuček, S., 1985. Regulation of acetylcholine synthesis in the brain. J. Neurochem. 44, 11–24. https://doi.org/10.1111/j.1471-4159.1985.tb07106.x.

Virgo, I., de Belleroce, J., Rossi, M., Steiner, T.J., 1992. Characterisation of the distribution of choline acetyltransferase messenger RNA in human spinal cord and its depletion in motor neuron disease. J. Neurol. Sci. 112, 126–132. https://doi.org/10.1016/0022-510X(92)90141-7.

Wang, B., Yang, L., Wang, Z., Zheng, H., 2007. Amyloid precursor protein mediates presynaptic localization and activity of the high-affinity choline transporter. Proc. Natl. Acad. Sci. Unit. States Am. 104, 14140–14145. https://doi.org/10.1073/pnas.0704070104.

Wang, H., Saler, C.G., Refai, O., Hardy, H., Barwick, K.E.S., Akpolat, U., Kvarnung, M., Chioza, B.A., Harlalka, G., Taylan, F., Sejersen, T., Wright, J., Zimmerman, H.H., Karakaya, M., Stüve, B., Weis, J., Schar, A., Russel, M.A., Abdul-Rahman, O.A., Chilton, J., Blakely, R.D., Baple, E.L., Cirak, S., Crosby, A.H., 2017. Choline transporter mutations in severe congenital myasthenic syndrome disrupt transporter localization. Brain 140, 2838–2850. https://doi.org/10.1093/brain/aws249.

Wang, X., Robbins, J., 2014. Proteasomal and lysosomal protein degradation and heart disease. J. Mol. Cell. Cardiol. 71, 16–24. https://doi.org/10.1016/j.yjmcc.2013.11.006.

Wilcock, G.K., Eizirik, M.M., Bowen, D.M., Smith, C.C.T., 1982. Alzheimer’s disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. J. Neurol. Sci. 57, 407–417. https://doi.org/10.1016/0022-510X(82)90045-4.

Woolf, N.J., Butcher, L.L., 2011. Cholinergic systems mediate action from movement to higher consciousness. Behav. Brain Res. 221, 488–498. https://doi.org/10.1016/j.bbr.2009.12.046.

Yamada, H., Imajoh-Ohmi, S., Haga, T., 2012. The high-affinity choline transporter CHT 1 is regulated by the ubiquitin ligase Nedd4-2. Biomed. Res. 33, 1–8. https://doi.org/10.2220/biomedres.33.1.

Zeisel, S.H., 1981. Dietary choline: biochemistry, physiology, and pharmacology. Annu. Rev. Nutr. 1, 95–121. https://doi.org/10.1146/annurev.nu.01.070181.000525.

Zerial, M., McBride, H., 2001. Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. 2, 107–117. https://doi.org/10.1038/35052065.

Zhang, L., Coffey, L.L., Reith, M.E.A., 1997. Regulation of the functional activity of the human dopamine transporter by protein kinase C. Biochem. Pharmacol. 53, 677–688. https://doi.org/10.1016/S0006-2952(96)00898-2.