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Differential Impact of Genetically Modulated Choline Transporter Expression on the Release of Endogenous Versus Newly Synthesized Acetylcholine

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Abstract

The efficient import of choline into cholinergic nerve terminals by the presynaptic, high-affinity choline transporter (CHT, SLC5A7) dictates the capacity for acetylcholine (ACh) synthesis and release. Tissue levels of ACh are significantly reduced in mice heterozygous for a loss of function mutation in *Slc5a7* (HET, $CHT^{+/-}$), but significantly elevated in overexpressing, *Slc5a7* BAC-transgenic mice (BAC). Since the readily-releasable pool of ACh is thought to constitute a small fraction of the total ACh pool, these genotype-dependent changes raised the question as to whether CHT expression or activity might preferentially influence the size of reserve pool ACh vesicles. In the current study, we approached this question by evaluating CHT genotype effects on the release of ACh from suprafused mouse forebrain slices. We treated slices from HET, BAC or wildtype (WT) controls with elevated K^+ and monitored release of both newly synthesized and storage pools of ACh. Newly synthesized ACh produced following uptake of [^{3}H]choline was quantified by scintillation spectrometry whereas release of endogenous ACh storage pools was quantified by an HPLC-MS approach, from the same samples. Whereas endogenous ACh release scaled with CHT gene dosage, preloaded [^{3}H]ACh release displayed no significant genotype dependence. Our findings suggest that CHT protein levels preferentially impact the capacity for ACh release afforded by mobilization of reserve pool vesicles.

Keywords

choline; acetylcholine; choline transporter; vesicular release

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2. INTRODUCTION

The presynaptic, high-affinity choline transporter (CHT, *SLC5A7*) is an essential determinant of signaling capacity by the neurotransmitter acetylcholine (ACh). As choline acetyltransferase (ChAT), the enzyme responsible for ACh synthesis, is not saturated at cytoplasmic levels of choline, choline uptake sets the capacity for ACh signaling, particularly with sustained or high-frequency cholinergic activity (Yamamura and Snyder, 1973; Barker et al., 1978). Consistent with this idea, CHT knockout mice (Slc5a7$^{-/-}$) die shortly after birth due to motor paralysis and respiratory failure(Ferguson et al., 2004) whereas CHT heterozygous mice (*Slc5a7*$^{+/-}$; HET) display reduced brain levels of ACh, increased fatigue during treadmill exercise (Bazalakova et al., 2007), a reduced ability to lower basal and stress-induced heart-rate relevations, and age-dependent cardiac enlargement, as well as ventricular thickening and reduced ejection fraction(English et al., 2010). In humans, reduced function genetic variation in CHT has been associated with neuromuscular dysfunction (Barwick et al., 2012), atherosclerosis(Neumann et al., 2011), cognitive impairment (Berry et al., 2014), attention-deficit hyperactivity disorder (ADHD) (English et al., 2009), and depression(Hahn et al., 2008). Interestingly, transgenic mice with additional copies of the full CHT gene harbored by a bacterial artificial chromosome (BAC) display elevated CHT protein expression, increased synaptosomal choline uptake capacity, elevated steady-state ACh levels, and increased endurance during treadmill exercise (Holmstrand et al., 2014).

CHT surface expression and choline uptake is linked to ACh release through a unique mechanism that involves the trafficking of CHT proteins on cholinergic synaptic vesicles (Ferguson et al., 2003; Ribeiro et al., 2003; Ferguson and Blakely, 2004). Biochemical and immuno-electron microscopy studies demonstrate that the majority of CHT in brain (Ferguson et al., 2003; Holmstrand et al., 2010) and at the neuromuscular junction (Nakata et al., 2004) is sequestered on small clear synaptic vesicles (SV) that also contain the vesicular acetylcholine transporter (VAChT) and other SV proteins supporting vesicular fusion, and importantly, that store ACh (Ferguson et al., 2003). With terminal depolarization, CHT-containing SVs fuse with the plasma membrane, thereby increasing the density of CHT at the presynaptic membrane, underpinning the historical observations of activity-dependent elevations in high-affinity choline uptake (Atweh et al., 1975; Simon and Kuhar, 1975) and binding of the competitive CHT antagonist hemicholinium-3 (Saltarelli et al., 1987; Yamada et al., 1991). The cytoplasmic C-terminus of CHT bears strong dileucine-type endocytic motifs that drive efficient clathrin-mediated endocytosis (Ribeiro et al., 2005; Ruggiero et al., 2012), presumably allowing the transporter to recycle back onto cholinergic synaptic vesicles, possibly via an AP3-dependent pathway (Misawa et al., 2008).

Interestingly, not all VAChT-positive, cholinergic synaptic vesicles contain CHT (Ferguson et al., 2004), suggesting that CHT may reside on, or support the formation of, a subpopulation of cholinergic vesicles, though as yet, functional distinctions between CHT-containing and CHT-deficient vesicles, have not been identified. Studies using either organic dyes or genetically-encoded fluorophores support a differentiation of synaptic vesicle pools into two broad classes on the basis of their probability for fusion with depolarization (Rizzoli and Betz, 2005). One group of vesicles consists of a small, rapidly recycling pool

that readily fuses with the plasma membrane at low levels of excitation, The second, much larger pool, requires intense and/or sustained stimulation to mobilize and fuse with the plasma membrane. As the recycling pool of CHT is likely a small fraction of the total vesicle pool and these vesicles may not be fully loaded prior to fusion (Edwards, 2007), steady-state ACh levels measured in tissue extracts presumably reflect the larger, reserve pool of vesicles, whose long cytoplasmic residence also likely insures that they achieve their maximum ACh loading capacity. In this context, we found the evidence that ACh levels tissue are reduced in CHT HET mice, but elevated in overexpressing BAC mice, a possible indication that CHT levels or activity may preferentially impact the formation or mobilization of cholinergic reserve pool vesicles.

In the current study, we explore this question, examining how CHT gene dosage influences ACh release from readily-releasable versus reserve pool vesicles using brain slices prepared from CHT HET, WT, and BAC mice. CHT HET mice express approximately 50% of WT CHT levels (Ferguson et al., 2004; Bazalakova et al., 2007), whereas BAC mice express twice as much CHT protein as WT (Holmstrand et al., 2014). These studies also demonstrated that brain tissue levels of ACh are reduced or elevated in proportion to CHT protein expression. Here, we monitored the release of pre-labeled, newly synthesized ACh using radiometric approaches and assessed, in the same samples, endogenous ACh release under a double-pulse, high K^+ depolarization protocol to facilitate mobilization of reserve pool vesicles. Our results revealed a lack of genotype effect on pre-labeled ACh release whereas endogenous release tracked positively with CHT expression. We discuss our findings with respect to the physical and functional activities of CHT that can permit selective engagement of distinct vesicle pools.

3. MATERIALS AND METHODS

3.1. Mouse Models

All animal procedures were conducted under a protocol approved and reviewed annually by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). The generation and maintenance of CHT HET and BAC mice on a C57BL/6J background has been previously described (Bazalakova et al., 2007; Holmstrand et al., 2014). CHT HET and WT mice were produced by breeding HET animals. Mice (12–20 wk) of both genders were group housed (up to five per cage) on a 12:12 light/dark cycle (lights on at 0600 h) in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved mouse housing facility. Mice were provided with access to food and water *ad libitum*.

3.2. Evaluation of Preloaded and Endogenous ACh Release from Mouse Forebrain Minces

All chemical reagents, unless otherwise specified, were obtained from Sigma-Aldrich (St. Louis, MO, USA) at the highest purity available. Following rapid decapitation, brains were removed on ice and the forebrain (tissue anterior to the superior colliculus but minus olfactory bulbs) was dissected and and immediately minced with a razor blade on ice-cold steel plate. Minced tissue (50 mg) was placed into 800 μL of ice-cold, pre-oxygenated Krebs-Ringers-HEPES (KRH) buffer (130 mM NaCl, 3 mM KCl, 10 M HEPES, 1.2 mM

KH_2PO_4, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM D-glucose titrated to pH 7.4 with NaOH). To radiolabel newly synthesized ACh, the tissue was incubated with 100nM [^{3}H] choline chloride (80–85.5Ci/mmol, PerkinElmer, Waltham, MA) at 37° C for 30min. We chose 100nM [^{3}H]choline for incubations to load preferentially through the high-affinity choline uptake system supported by CHT (K_M~ 1μM) versus low affinity choline uptake mechanisms (K_M >10 μM)(Yamamura and Snyder, 1972). Following incubations, 250 μL of the tissue suspension was loaded into a Teflon chamber (300 μL capacity), juxtaposed between two glass-fiber filter discs (Whatman GF/B), in a multi-channel perfusion system (Suprafusion 2500, Brandel Inc., Gaithersburg, MD). Samples were perfused at 37°C for 1 hr at 0.5mL/min with oxygenated KRH containing 100μM physostigmine to inhibit acetylcholinesterase (AChE). Before collection of released ACh, samples were perfused non-sequestered or metabolized [^{3}H]choline prior to initiation of baseline sample collection.

To trigger ACh release, we used a double-pulse stimulation protocol (S1 and S2). Each pulse consisted of a 15 min substitution of KRH supplemented to 20 mM KCl while reducing NaCl to 113 mM. Perfusate across the experiment was collected every 5 min (2.5 mL/fraction). A portion of each fraction (100 μL) was frozen at −80°C for mass spectroscopic analysis of endogenous ACh. After each experiment, the remaining tissue in the chamber and associated filter discs were collected to estimate total [^{3}H]choline uptake. These samples were dissolved in 1 mL of 20% SDS overnight and added to 5ml of scintillation fluid (EcoScint XR, National Diagnostics, Atlanta, GA). Perfusate and tissue sample tritium content was assessed by scintillation spectrometry (TRI-CARB 2900TR, Packard BioScience Company, Meriden, CT). Total tissue [^{3}H]choline uptake was calculated by summation of perfusate and remaining tissue radioactivity. The concentration of [^{3}H]ACh release was calculated after adjustment of counts per minute (CPM) for scintillation counter counting efficiency, the specific activity of [^{3}H]choline, and volume of perfusate. The magnitude of S1 and S2 peaks was expressed as area under the curve (AUC), estimated after subtracting baseline levels using a two Gaussian method using OriginPro9.0 software (OriginLab Corporation, Northampton, MA).

3.3. Endogenous ACh Quantitation via High Performance Liquid Chromatography-Mass Spectroscopy (HPLC-MS)

All HPLC-MS experiments were performed in Mass Spectrometry Core Laboratory at Vanderbilt University. Liquid chromatography was performed using a Waters Acquity UPLC system (Waters, Milford, MA), made up of a binary solvent manager, refrigerated sample manager, and a heated column manager. Tandem mass spectrometry was performed using a TSQ Quantum Ultra triple-stage quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an Ion Max source housing, a heated electrospray (HESI) probe, and a 50 μm ID stainless steel capillary. The instrument was tuned and calibrated every four to six weeks over a mass range of *m/z* 182 to *m/z* 997 with a mixture of tyrosine peptides using the manufacturer's autotune procedure. Perfusates were freeze-dried in a vacuum rotor (DNA120 SpeedVac, Themo Savant, Holbrook, NY) and reconstituted with 100 μL of *N,N,*-dimethylacetamide:water (9:1) containing 100nM deuterium-labeled d_9 acetylcholine chloride (*N,N,N*-trimethyl-d_9, C/D/N Isotopes Inc. Quebec, Canada) as an internal standard. The reconstituted sample was sonicated for 30 min and centrifuged to remove undissolved

protein/lipid precipitation at 10,600 × *g* for 5 min. We loaded 80 μL of supernatant onto a 96-well plate in an autosampler stage for further HPLC-MS analysis. Endogenous ACh was chromatographically resolved in 3.5 min on a hydrophilic interaction liquid chromatography column (Atlantis HILIC silica, 2.1×150mm, 3μm, Waters, Milford, MA) using the following isocratic elution conditions: 15% mobile phase A (25 mM ammonium formate pH 4.0 in H_2O/CH_3OH (9:1)) and 85% mobile phase B (0.1 % (*v/v*) HCOOH in MeOH). The flow rate was maintained at 300μl/min; the column and sample tray temperatures were maintained at 40°C and 5°C respectively. The sample injection volume was 10 μL (partial loop injection mode, 10μl stainless steel loop). The mass spectrometer was operated in positive ion mode. Quantitation was based on selected reaction monitoring (SRM) detection (ACh-d_0: *m/z* 146 → 87, CE 10 V; Ach-d_9: *m/z* 155 → 87, CE 10 V) using the following optimized parameters: N_2 sheath gas 40 psi; N_2 auxiliary gas 20 psi; spray voltage 3.0 kV; capillary temperature 300 °C; capillary offset 35 V; tube lens voltage 63 V; Ar collision gas 0.5 mtorr; scan time 150 ms; Q3 scan width 0.5 *m/z*; Q1/Q3 peak widths at half-maximum 0.7 *m/z*.

Data acquisition and quantitative spectral analysis were performed using Thermo-Finnigan Xcalibur version 2.0 SUR1 and Thermo-Finnigan LCQuan version 2.5.6, respectively. Calibration curves were constructed by plotting peak area ratios (ACh-d_0/ACh-d_9) against molar concentration of ACh-d_0 for a series of calibration standards (1nM to 100nM). A weighting factor of $1/C^2$ was applied in the linear least-squares regression analysis to maintain homogeneity of variance across the concentration range (≤ 8.2% error at every level). Nominal chromatographic peak widths at half height ($W_{1/2}$) were ~ 12 sec for the HILIC column; scan times were adjusted so that each chromatographic peak represented 15–20 scan cycles. The total analysis time from injection to injection was 5 min. In order to assess matrix effects and extraction efficiency from high K^+ stimulation buffer, multiple “clean” samples made from serial dilutions of authentic standards in organic solvent were prepared alongside standards containing stimulation buffer. All samples were lyophilized, reconstituted, and analyzed in the same manner. The slopes of the response ratio vs. concentration curves were 0.0107 and 0.0102 for stimulation buffer and buffer-free standards respectively (data not shown). There were no significant differences in y-intercepts (−0.00036 for stimulation buffer vs. −0.00012 for clean standards) and no significant deviations from linearity at ACh concentrations above the lower limit of quantification (LLOQ).

3.4 Statistical and Graphical Analyses

Statistical and graphical analyses were performed with OriginPro 9.0 (OriginLab Corporation, Northampton, MA) or Prism 6.0 (GraphPad, Inc, La Jolla, CA). Results are plotted as mean values +/− SEM. Specific details for tests conducted and the number of replicates within groups (N) are provided in the Figure Legends. A *P* value < 0.05 was taken as evidence of a statistically significant effect.

4. RESULTS

4.1. Quantitative Determination of Endogenous Acetylcholine Release

In order to monitor the release of both endogenous and pre-labeled ACh from the same brain samples, we first developed HPLC-MS methods to quantify unlabeled ACh released from brain minces depolarized by elevated K^+ *in vitro*. We implemented hydrophilic interaction liquid chromatography (HILIC) followed by electrospray ionization triple quadrupole tandem mass spectrometry. Collision-induced dissociation (CID) of ACh in positive ion mode results in the cleavage of a single C–O bond, with SRM transitions *m/z* 146 → 87 and *m/z* 155 → 87 specific for the CID of ACh and ACh-d_9 standard, respectively (Fig 1A). A normal phase (HILIC) HPLC column was used to enhance the retention and chromatographic resolution of the highly polar analyte and internal standard, obviating the need for ion pairing reagents. The peak area ratios of calibration standards (ACh/ACh-d_9) were linearly proportional to the concentration of ACh over the nominal concentration range of 0.10–1000 nM with a coefficient of determination of $r \geq 0.999$ and a slope of ~0.01 (Fig 1A). Importantly, recovery of the internal standard from brain sample perfusates was quantitative, with no significant differences in the average internal standard responses of the perfusate samples vs. the calibration standards prepared in stimulation buffer. As shown in Fig 1B, both endogenous ACh and internal ACh-d_9 standard were well resolved in brain perfusates, without interfering peaks.

4.2. Evaluation of CHT genotype on release of newly synthesized [^{3}H]ACh versus endogenous ACh

Having developed an approach to quantify the release of endogenous ACh, we implemented a precursor ([^{3}H]choline) pre-labeling protocol to monitor, in the same samples used for HPLC-MS, the release of newly synthesized [^{3}H]ACh from forebrain minces of HET, WT and BAC mice, as described in Methods. Prior studies have found that ACh synthesis from exogenously supplied choline is CHT-dependent (Perry, 1953; Macintosh et al., 1956; Birks and Macintosh, 1961) with >90% of tritium collected following K^+ stimulation of [3H]choline preloaded samples attributable to [^{3}H]ACh (Zhang et al., 2002). In evaluation of [^{3}H]choline loading (Fig 2A), we detected no overall CHT genotype effect. We did detect a significant genotype effect for baseline [^{3}H]ACh release (average of three fractions collected prior to K^+ stimulation, Fig 2B), though in post-hoc tests only the BAC vs HET comparison reached significance. Baseline endogenous ACh release, derived from HPLC-MS analyses, also revealed a significant overall CHT genotype effect though with larger fold changes (Fig 2C), that resulted in significant differences for comparisons of both HET vs WT and HET vs BAC values.

To assess CHT genotype effects on evoked ACh release, we implemented a double pulse, high K^+ stimulation protocol (Fig 3A–B) and split samples from each run to provide for both radiometric and HPLC-MS analyses. The first high K^+ pulse (S1) in this paradigm provides a measure of initial ACh release capacity, whereas the ratio of second pulse to first pulse ACh release (S2/S1) provides a measure of reserve pool capacity. To quantify genotype effects on ACh release during S1 and S2, we calculated the AUC for each pulse relative to the baseline for that sample, and then normalized the AUC of HET and BAC samples to the

AUCs observed with WT samples (WT noted as dashed lines in Fig 3C–D). In this analysis, we found no significant genotype effects in the capacity of forebrain samples to support K^+-evoked [^{3}H]ACh release, either with the first (S1) or second high K^+ treatment (S2). In contrast, significant genotype effects were observed for endogenous ACh release in both S1 and S2 samples.

Although a CHT genotype effect was absent for K^+-evoked [^{3}H]ACh release, we found that the relationship between [^{3}H]choline uptake and [^{3}H]ACh release in individual experiments, for both S1 and S2 pulses, were well correlated (Fig 4A). The significant correlations regardless of genotype suggests that the reduction observed in the magnitude of S2 relative to S1 is unlikely to be attributable to an effect of CHT expression on mechanisms translating uptake and synthesis to release. The significantly reduced slope of the fit defining the relationship between [^{3}H]choline uptake and [^{3}H]ACh release in S2 samples compared to S1 samples suggests that S1 release is derived from readily-releasable pool vesicles that have been loaded with newly synthesized [^{3}H]ACh, whereas S2 release is derived from a pool of vesicles less efficiently labeled with [^{3}H]ACh, such as reserve vesicles loaded with endogenous ACh. Consistent with this idea, the S2/S1 ratio was higher in samples monitored by HPLC-MS versus samples monitored scintillation spectrometry (Fig 4E). However, the S2/S1 ratio did not display a CHT genotype effect, whether assessing release of [^{3}H]ACh or of endogenous ACh. These findings support the idea that CHT expression influences reserve pool capacity (higher endogenous ACh S2/S1), but does not modulate the fundamental mechanisms through which repeated depolarization leads to sustained ACh release.

5. DISCUSSION

The physiological impact of presynaptic, CHT-dependent choline uptake is ultimately achieved via the quantity of ACh released into the synapse and how well this release is maintained in relation to the temporal patterns of neuronal activation. Our prior findings indicate that steady-state levels of ACh are diminished in CHT HET mice (Bazalakova et al, 2007) and elevated in CHT BAC mice (Holmstrand et al, 2014), consistent with an important role of the transporter in providing precursor for ACh synthesis. One expectation from such findings is that cholinergic signaling, or behaviors contingent on such signaling, might be unsustainable, in HET relative to WT animals, with continued cholinergic activation, whereas BAC animals might demonstrate a greater ability to sustain responses. This is a question of translational importance given the presence of bi-directional functional variation in the human *SLC5A7* gene (Okuda et al., 2002; Neumann et al., 2006; Barwick et al., 2012). Moreover, evidence is accumulating to indicate an important relationship between CHT expression or activity for *in vivo* physiological and behavioral measures that are dependent on cholinergic signaling. Thus, CHT HET mice fail to sustain treadmill running at durations (or speeds) observed with WT animals (Bazalakova et al., 2007), whereas both motorneuron-specific CHT overexpressing mice (Lund et al., 2010) and CHT BAC mice (Holmstrand et al., 2014) display enhanced endurance for treadmill running. Similarly, HET mice cannot reset their heart rates after an imposition of treadmill exercise-induced tachycardia as fast as their WT littermates (English et al., 2010). Finally, attention-demanding tasks mobilize the trafficking of CHT in prefrontal cortex of the rat (Apparsundaram et al., 2005) and CHT HET mice display a loss in their ability to support

elevated ACh release and in attentional performance (Parikh et al., 2013), a suggestive parallel to the increased distractibility of humans expressing the loss of function CHT coding variant Ile89Val (Berry et al., 2014).

Although a CHT genotype influence on steady-state tissue levels of ACh might be expected, given the rate-limiting nature of choline uptake for ongoing ACh synthesis, tissue levels are generally thought to reflect the abundance of stable stores of neurotransmitter (e.g. reserve pool). Indeed, previous studies have indicated that the readily releasable pool of ACh that is preferentially loaded with newly synthesized neurotransmitter is thought to be a small fraction of total stores (Searl et al., 1990; Parsons, 1993). Additionally, long-residence pools of vesicles have sufficient time to achieve equilibrium between internal pH and accumulated ACh, and under such circumstances, the rate of vesicular uptake of ACh should be inconsequential on steady state ACh levels, suggesting that a compensatory change in the density of VAChT per vesicle does not explain elevated tissue levels of ACh. Indeed, brain ACh levels (vs readily releasable pools) of ACh in VAChT +/− mice are not diminished (actually they are elevated) (Prado et al, 2006). Moreover, genetic loss of the vesicular glutamate transporter type 3 (VGLUT3), which is co-expressed on CNS cholinergic vesicles, and can enhance vesicle loading over short time periods through glutamate buffering of intra-vesicle pH, does not significantly reduce tissue levels of ACh, though a reduction of evoked, preloaded [^{3}H]ACh release is observed (Gras et al, 2008).

These perspectives suggested to us that CHT protein may play a more active role in the formation or stabilization of reserve pool cholinergic synaptic vesicles. The CHT C-terminus possesses a strong, dileucine-type endocytic sequence (Ribeiro et al., 2005; Ruggiero et al., 2012) that can facilitate clathrin-mediated endocytosis and possibly enhance budding from endosomal compartments, raising the possibility that CHT-accumulated adaptors such as AP-3 (Misawa et al., 2008) may facilitate the budding of a subpopulation of cholinergic synaptic vesicles. The nature of these vesicles, besides their ability to store ACh, has not been defined, though one clear possibility is that they deliver CHT to the plasma membrane under circumstances of high or sustained cholinergic activity (Ferguson et al., 2003), leading to enhance choline uptake and maintained ACh synthesis. One study (Ribeiro et al., 2007), indicated that CHT can rapidly recycle to the plasma membrane, suggestive of residence on readily releasable vesicles or transit through a recycling endosomal pathway. This study, however, involved the study of transfected CHT in cultured cells that do not form synapses, and the pool of recycling CHT was a small proportion of the total cell content. Our findings that CHT protein expression impacts to a greater degree the release capacity of endogenous ACh versus pre-labeled ACh, suggests that CHT, directly or indirectly, impacts reserve pool capacity, more so than the capacity or content of the readily releasable pool. We acknowledge that our studies are limited by inference of vesicle pool participation through the evaluation of vesicle contents, versus monitoring the number of vesicles in distinct pools directly. We have reported that in the rodent forebrain, approximately 50% of cholinergic vesicles have VAChT and CHT, whereas 50% possess only VAChT. The use of techniques that can establish the amount of ACh in different pools of cholinergic vesicles as a function of genotype and that can distinguish between the fusion of the two vesicle populations independent of content are needed to gain more refined insights.

The concept of functionally distinct neurotransmitter vesicle pools arose initially from biochemical studies with Torpedo electric organ preparations that defined both size differences among vesicles that store ACh (Zimmermann and Denston, 1977; Luz et al., 1985; Whittaker, 1990; Parsons, 1993), as well as different sensitivities of ACh pools to VAChT blockade with vesamicol that can also seen with mammalian preparations (Searl et al., 1990; Parsons, 1993). In subsequent years, studies with fluorescent organic dyes and genetically-encoded reporters further established the concept of multiple vesicle pools of distinct fusion probability supporting neural signaling (Rizzoli and Betz, 2005). Readily-releasable, or rapidly recycling synaptic vesicles, are thought to sustain release at low levels of neuronal firing, whereas reserve pool vesicles require stronger or more sustained stimulation to mobilize a more significant fraction of neurotransmitter stores. Although in some systems, a more interior spatial localization of vesicles within the terminal may contribute to the definition of reserve vesicles, cholinergic vesicles at the frog neuromuscular junction appear to be interspersed (Rizzoli and Betz, 2004), and evidence has accumulated for distinct molecular influences that define the differential mobilization probability of vesicle pools (Gaffield et al., 2006; Akbergenova and Bykhovskaia, 2007; Kim and Ryan, 2010). Our findings in this study suggest that CHT may contribute to the molecular specification or loading of reserve pool vesicles, possibly by routing membrane through an endosomal sorting path that ultimately becomes tightly tethered to the presynaptic cytoskeleton. In preliminary studies (D.S. Matthies and R.D. Blakely, unpublished findings), we have examined the distribution of synaptic vesicles at cholinergic synaptic vesicles at the neuromuscular junction of WT and *cho-1* (worm ortholog of CHT) deficient nematodes by electron microscopy, obtaining evidence that while no change in the total population of vesicles is evident, the spatial localization vesicles is affected.

In this study, we implemented a protocol permitting the measurement of both previously synthesized, endogenous ACh and newly synthesized ACh released from brain preparations by elevated extracellular K+, and compared these two modes of ACh release assessment as a function of CHT genotype. As expected, significantly greater quantities of released ACh were detected using radiometric versus HPLC-MS approaches (50–70 pM vs 20–80 nM, respectively), likely owing to the short time afforded for prelabeling (30 min) and the low exogenous choline concentration used, favoring uptake by CHT versus low affinity, non-neuronal choline uptake systems. Our findings of a stronger relationship between [^{3}H]choline uptake and first pulse (S1) evoked release of [^{3}H]ACh as compared to second pulse release (S2), as well as a larger fractional release (S2/S1) with endogenous ACh, supports the idea that S1 contains a pool of vesicles packaged with newly synthesized ACh that has with higher release probability, whereas S2 reports release from vesicles mobilized by repeated depolarization. We found that changes in CHT density, as induced by changes in the number of *Slc5a7* functional copies in HET, WT and BAC mice, most strongly influenced the capacity for endogenous ACh release. Additional support for this idea comes from the reduced dependence of [^{3}H]ACh release by variations in [^{3}H]choline loading, as denoted by the significantly reduced slope of the correlation plot in Fig 4A. These findings suggest that the quantity of readily releasable vesicles, or their loading with ACh, is CHT-independent, whereas the size of the vesicular reserve, loaded with endogenous ACh prior to precursor loading are strongly influenced by CHT expression.

Both S1 and S2 fractions demonstrated a significant dependence on CHT expression when endogenous ACh was measured, but not when pre-labeled [^{3}H[]ACh was quantified. The S1 pulse depletes a larger fraction of releasable [^{3}H]ACh vs endogenous ACh, consistent with the S1 pulse as mobilizing the bulk of readily releasable vesicles that contains newly synthesized ACh, though a proportion of reserve pool vesicles may also be mobilized and contribute to the ACh released. The S2 pulse releases a lower quantity of newly synthesized ACh, consistent with release largely by mobilized reserve pool vesicles filled with previously synthesized ACh. These vesicles presumably have less access to, or capacity for storage of, newly synthesized ACh, possibly because these vesicles are already "fully loaded". In relation to this idea, it would be helpful to know to what degree the reserve pool contributes in our paradigm to the endogenous release of ACh in the S1 stimulation. Answering this question will require the ability to monitor reserve pool vesicles independent of their contents. At present, there is not a molecular tag that we can use to demarcate cholinergic reserve pool vesicles versus the cholinergic readily releasable vesicles, though from our data a reasonable possibility is that CHT itself may be one such protein, a possibility that needs to be explored in further studies.

Since our findings of a CHT dependence on ACh release arise in animals with lifelong changes in CHT expression, we cannot rule out the possibility that changes in release derive from compensations arising that lead to changes in the mechanics of ACh release and signaling. Indeed, we have reported changes in both muscarinic (Bazalakova et al., 2007) and nicotinic receptor (Paolone et al., 2013) densities and/or activation in HET vs WT mice. However, against this idea, we detected no changes in synthesis (ChAT) or metabolism (AChE) capacities in either the HET (Bazalakova et al., 2007) or BAC model (Holmstrand et al., 2014). Additionally, we found no CHT genotype dependence on the S2/S1 ratio, suggesting that critical determinants of reserve pool mobilization (e.g. Ca^{2+} entry/buffering, cytoskeletal anchoring) appear normal. Nonetheless, as noted above, future studies that monitor cholinergic synaptic vesicle pools directly, versus the ACh that these vesicles release, should provide important data to address the interesting idea that CHT impacts the capacity for ACh release in two ways, one by providing precursor choline to drive elevated ACh synthesis and storage and another by increasing reserve pool size. It will also be important to evaluate whether regional dependence exists for the CHT expression relationships we report (e.g. striatal vs hippocampal vs frontal cortex), as our studies made no attempt to separate contributions of various forebrain cholinergic populations whose intrinsic firing and regulatory control mechanisms may influence the degree to which the transporter modifies ACh release. Lastly, other transporters whose activities are driven by the Na^+ gradient, and therefore likely to function at the cell surface, have been reported to reside on synaptic vesicles (Renick et al., 1999; Geerlings et al., 2001). We speculate that these proteins may similarly contribute to the capacity for release of neurotransmiiters from readily releasable vs reserve pool compartments.

6. CONCLUSIONS

By monitoring the release of both endogenous and newly-synthesized ACh in three mouse strains varying in CHT protein expression, we have obtained evidence that CHT expression impacts ACh release to a greater degree from reserve versus readily releasable pools of

cholinergic vesicles, studied in mouse forebrain minces *ex vivo*. Our findings support a more complex contribution of CHT to the capacity of cholinergic neurons to sustain cholinergic signaling than the well-known role as provider of precursor for ACh synthesis.

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Highlights

- Transgenic mice that under or over-express the presynaptic choline transporter demonstrate selective alterations in the evoked release of neurotransmitter acetylcholine that depends on acetylcholine synthesis history
- The evoked release of newly synthesized acetylcholine is insensitive to changes in the expression level of choline transporters, whereas the release of reserve stores of acetylcholine scale with the level of transporter protein.
- Variations in the level of choline transporter proteins may impact the capacity for sustained acetylcholine release independent of changes in choline uptake activity.

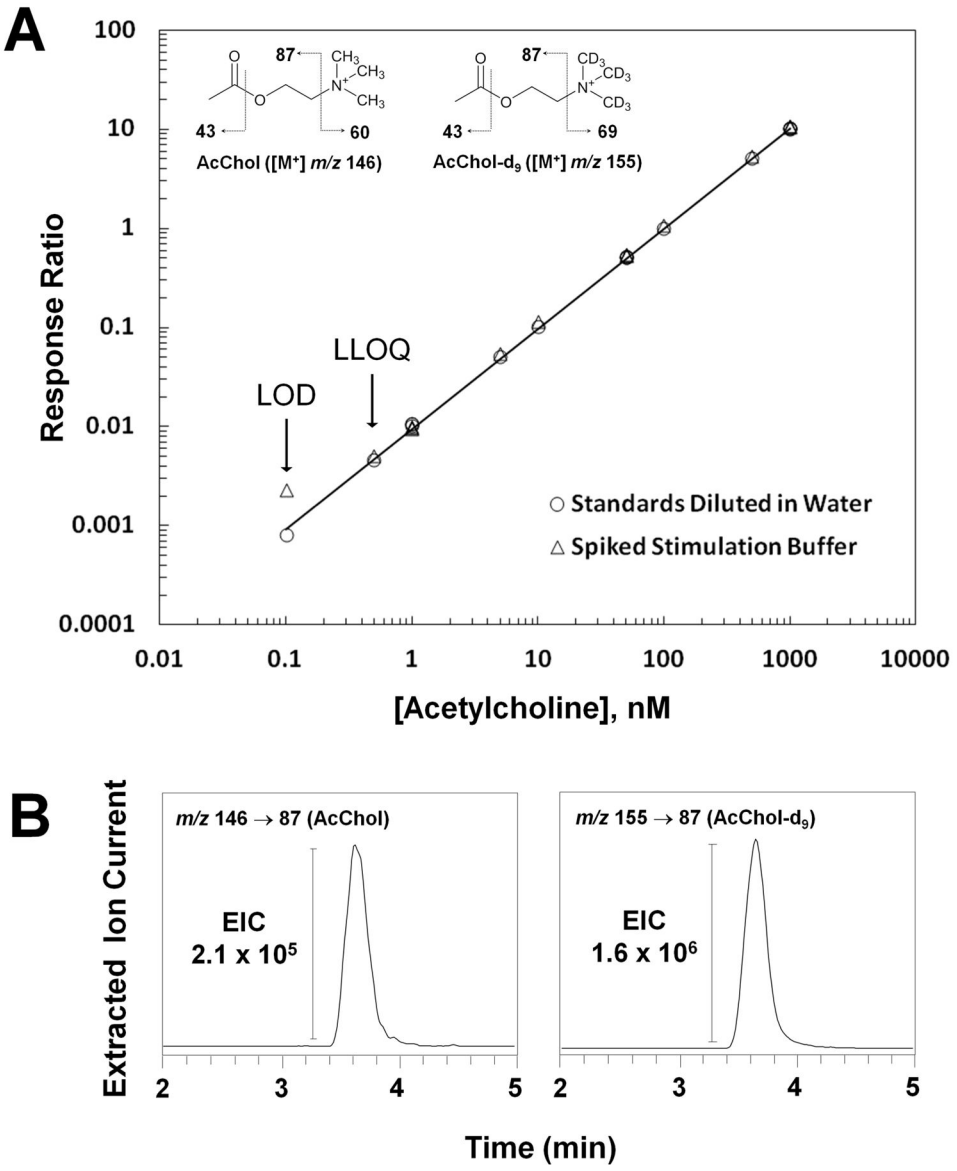

Figure 1. HPLC-MS analysis of acetylcholine

(**A**) Selected reaction monitoring (SRM) based ion chromatogram calibration curves. Peak area ratios (ACh/ACh-d_9) were plotted against analyte concentrations for a series of standards prepared in water (○) and in high K^+ stimulation buffer (△) over the nominal concentration range of 100 pM to 1 μM. A weighting factor of 1/concentration was applied to maintain homogeneity of variance across the concentration range. Precursor ions were activated at a collision energy of 15 V and at an indicated argon pressure of 1.5×10^{-3} torr. Detection was based on the SRM transitions *m/z* 146 → 87 (ACh) and m/z 155 → 87 (ACh-d_9). The dashed lines in the chemical structures represent the proposed sites of collision-induced fragmentation (inset). (**B**) SRM ion chromatograms of brain perfusate spiked with 100 nM acetylcholine-d_9 (1 pmol injected). Perfusate samples were lyophilized to dryness and reconstituted in dimethylacetamide/water (9:1).

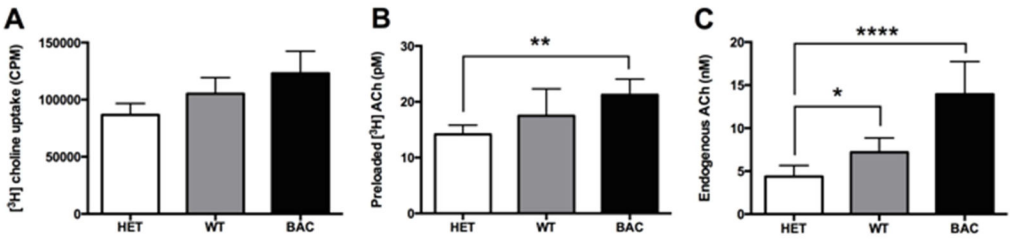

Figure 2.

Impact of CHT genotype on choline uptake and ACh release from mouse forebrain minces *ex vivo*. (A) Genotype influences on uptake of [^{3}H]choline. One-way Analysis of Variance (ANOVA) indicates a non-significant genotype effect (P<.09, N=8 for all genotypes) (B) Genotype influences on basal [3H]ACh release. One-way ANOVA indicates a significant genotype effect (P<.01, HET N=8, WT N=14, BAC N = 8). Dunnet's multiple comparison test indicates a significant difference between HET and BAC values (**=P<.01). (C) Genotype influences on basal endogenous ACh release. One-way ANOVA indicates a significant genotype effect (P<.0001, HET N=7, WT N=13, BAC N=7). Dunnet's multiple comparison test indicates a significant difference between HET and WT (*=P<.05) and HET and BAC (****=P<.001) values.

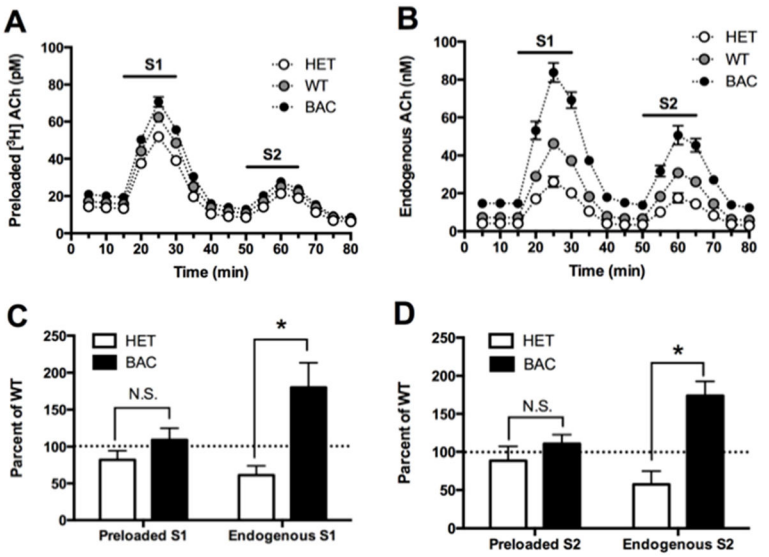

Figure 3.

Impact of CHT genotype on K^+-evoked ACh release from mouse forebrain minces *ex vivo*. (A) Time plot of [^{3}H]ACh concentration obtained in a double pulse, high K^+ stimulation paradigm, assessed by scintillation spectrometry. (B) Time plot of endogenous ACh concentration obtained in a double pulse, high K^+ stimulation paradigm, assessed by HPLC-MS. N in (A) and (B) =7 for all genotypes. (C) Pre-labeled and endogenous ACh release from S1 pulses shown in (A) and (B). No genotype effect was evident for pre-labeled ACh comparing HET to BAC samples, both normalized to WT (two-tailed Student's t-test, *P>0.1*), whereas endogenous S1 ACh release exhibited a significant genotype effect (Student's two-tail t-test, * = *P<0.05*). (D) Pre-labeled and endogenous ACh release from S2 pulses shown in (A) and (B). No genotype effect was evident for pre-labeled ACh comparing HET to BAC samples, both normalized to WT (two-tailed, Student's t-test, *P>0.1*), whereas endogenous S1 ACh release exhibited a significant genotype effect (Student's two-tail t-test, * = *P<0.05*).

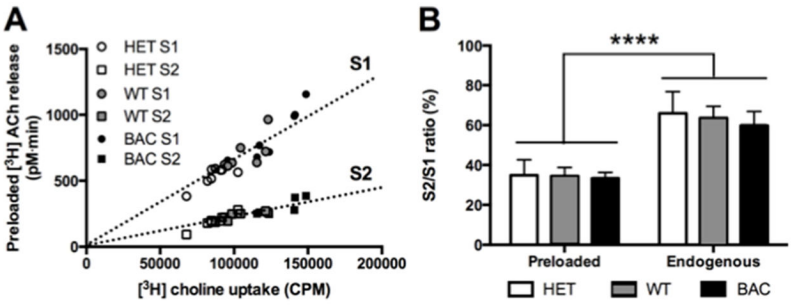

Figure 4.

Influence of CHT gene dosage on the relationship between choline uptake and ACh release. (A) [^{3}H]Choline uptake is significantly correlated with K^+-evoked [^{3}H]ACh release inclusive of different CHT genotypes. S1 correlation Pearson r^2=0.855, P<.0001, S2 correlation, Pearson r^2=.816, P<.0001. Dotted line = linear regression. Significantly reduced slope of S2 correlation (.003) as compared to S1 (.008) (Analysis of Covariance, P<.0001) supports a diminished dependence of S2 [^{3}H]ACh release on variations in choline uptake. (B) Ratio of second pulse release to first pulse (S2/S1) as a function of mode of detection and genotype. Two-way ANOVA indicates a significant difference between assessment mode (preloaded versus endogenous) (****=P<.0001) with no significant genotype or interaction effects. N=8 for preloaded values across all three genotypes, N=7 for endogenous values across all three genotypes.