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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.

1. 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, 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;

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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.

2. Materials and methods

2.1. 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/6 J 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*.

2.2. 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 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 100 nM [^{3}H] choline chloride (80–85.5 Ci/mmol, PerkinElmer, Waltham, MA) at 37° C for 30 min. We chose 100 nM [^{3}H]choline for incubations to load preferentially through the high-affinity choline uptake system supported by CHT (K_M~ 1 μM) 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 h at 0.5 mL/min with oxygenated KRH containing 100 μM 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 5 mL of scintillation fluid (EcoScint XR, National Diagnostics, Atlanta, GA). Perfusate and tissue sample tritium content was assessed by scintillation spectrometry (TRI-CARB 2900 TR, 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).

2.3. Endogenous ACh quantitation via high performance liquid chromatography-mass spectroscopy (HPLC-MS)

All HPLC-MS experiments were performed in Mass