CARDIOVASCULAR AND NEUROPSYCHIATRIC CONSEQUENCES OF A GENETIC LOSS OF THE HIGH-AFFINITY CHOLINE TRANSPORTER (CHT)

By

Brett Alan English

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Approved:

Professor Randy D. Blakely

Professor Dan Roden

Professor Richard Shelton

Professor David Robertson

Associate Professor Jim Sutcliffe

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LIST OF ABBREVIATIONS

-/-	Homozygous knockout
+/-	Heterozygous
ACh	Acetylcholine
AChE	Acetylcholinesterase
ANS	Autonomic nervous system
AV	Atrioventricular node
BP	Blood pressure
bpm	Beats per minute
BRS	Baroreceptor sensitivity
ChAT	Choline acetyltransferase
CHT	Choline transporter
CNS	Central nervous system
CO	Cardiac output
DA	Dopamine
DBP	Diastolic blood pressure
%FS	Fractional shortening (%)
HACU	High-affinity choline uptake
HC-3	Hemicholinium-3
HF	Heart failure
HR	Heart rate
HRV	Heart rate variability

HTN	Hypertension
i.p.	Intraperitoneal
ISO	Isoproterenol (β_1, β_2-agonist)
i.v.	Intravenous
KRH	Kreb's-Ringer's-HEPES buffer
LVIDD	Left ventricular internal dimension (diastole)
LVIDS	Left ventricular internal dimension (systole)
mAChR	Muscarinic acetylcholine receptor
MAP	Mean arterial pressure
MI	Myocardial infarction
MT	Masson's Trichrome
mBP	Mean blood pressure
nAChR	Nicotinic acetylcholine receptor
NMJ	Neuromuscular junction
PAS-H	Periodic Acid Schiff with Hematoxlyn counterstain
PE	Phenylephrine (α_1-agonist)
PNS	Parasympathetic nervous system
SA	Sinoatrial node
SCG	Superior cervical ganglia
SNP	Single nucleotide polymorphism
SNP	Sodium nitroprusside
SNS	Sympathetic nervous system
SBP	Systolic blood pressure

Tg	Transgenic
UTR	Untranslated region
VAChT	Vesicular acetylcholine transporter
VF	Ventricular fibrillation
VNS	Vagal nerve stimulation

CHAPTER I

THE HIGH-AFFINITY CHOLINE TRANSPORTER – A CRITICAL PROTEIN FOR THE MAINTENANCE OF CHOLINERGIC TONE

CHT Function and Regulation

The Cholinergic Synapse

Acetylcholine (ACh), one of the first identified neurotransmitters, was originally termed *vagusstoff* due to its actions in supporting vagal slowing of heart rate (HR) (Thayer, 2007), and has been the focus of intense research for decades (Nestler, 2001). ACh has been shown to regulate many physiologic functions both within the central nervous system (CNS) and in the periphery. Within the CNS, cholinergic signaling mediated by ACh, regulates several diverse and complex functions including cognition, arousal, mood and motor circuits (Kasa, 1986; Nestler, 2001). These CNS cholinergic projections, common to human and rodent brain, arise from several nuclei that are clustered in two areas: the basal forebrain comprising the medial septal nucleus and the nucleus basalis of Meynert, projecting to the hippocampus and cerebral cortex, and upper brain stem cholinergic nuclei which project to the thalamus and medullary nuclei such as the ventral tegmental area (**Figure 1**). Within the basal ganglia exist large numbers of cholinergic interneurons which serve as critical components of the striatal circuitry underlying components of the pyramidal motor control system (Pisani et al., 2007). In the peripheral nervous system, ACh mediates functions coordinated by the

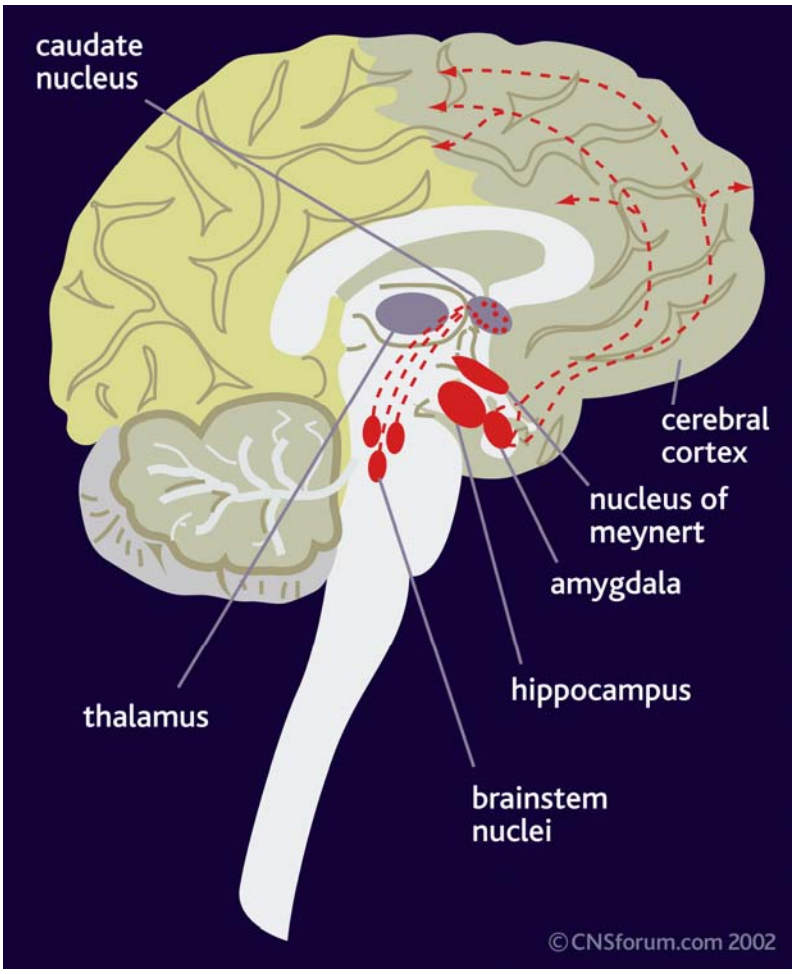

Figure 1. Cholinergic pathways in the brain. The basal forebrain comprises the medial septal nucleus, the nucleus basalis of Meynert, the vertical nucleus of the diagonal band and the horizontal limb of the diagonal band (not shown). These nuclei project to the hippocampus and cerebral cortex. Upper brain stem cholinergic neurons include the pedunculopontine, laterodorsal tegmentum, medial habenula and parabigeminal nuclei and project predominantly to the hippoampus and midbrain ventral tegmentum area (VTA). Cholinergic interneurons are found in the striatum, nucleus accumbens, olfactory tubercle, and the islands of Calleja. BFC, basal forebrain complex; IPN, interpeduncular nucleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus; VTA, ventral tegmental area. Adapted from Piccioto et al. (2002) and www.CNSforum.com, 2002. □

autonomic nervous system (**Figure 2A**), respiration, gut motility and exocrine secretions (Burnstock, 1979; Lefkowitz, 1996). Within the ANS, ACh is the predominant neurotransmitter at the preganglionic projections originating from within the CNS at the level of the medulla, for both the sympathetic (SNS) and parasympathetic nervous systems (PNS), mediating post-ganglionic effects via nicotinic acetylcholine receptors (nAChRs) (**Figure 2B**) (Lefkowitz, 1996). At the post-ganglionic synapse, PNS neurons utilize ACh with their post-synaptic effects mediated by mAChRs, while SNS neurons utilize NE, with their post-synaptic effects mediated by adrenergic receptors (Lefkowitz, 1996).

The availability of ACh for cholinergic transmission involves a highly coordinated process of ACh synthesis, vesicular packaging, vesicular release, hydrolysis and reuptake into the presynaptic nerve terminal (**Figure 3**) (Bazalakova and Blakely, 2006; Nestler, 2001). Within the cholinergic presynaptic terminal, the enzyme choline acetyltransferase (ChAT) synthesizes ACh from the precursors choline and acetyl coenzymeA (acetyl-CoA). Unlike the biosynthesis of catecholamines, ChAT is not believed to serve as the rate-limited step in the biosynthesis of ACh as the presynaptic concentrations of choline are much lower than the K_m for ChAT (Oda, 1999). Upon synthesis, ACh is packaged into synaptic vesicles by the vesicular ACh transporter (VAChT), and released into the synaptic cleft upon depolarization of the neuron by an action potential (Eiden, 2000). Once released into the synaptic cleft, ACh interacts with either nAChRs or muscarinic acetylcholine receptors (mAChRs). ACh then dissociates from the receptor and undergoes enzymatic inactivation by the enzyme acetylcholinesterase (AChE) which hydrolyzes ACh into acetate and choline

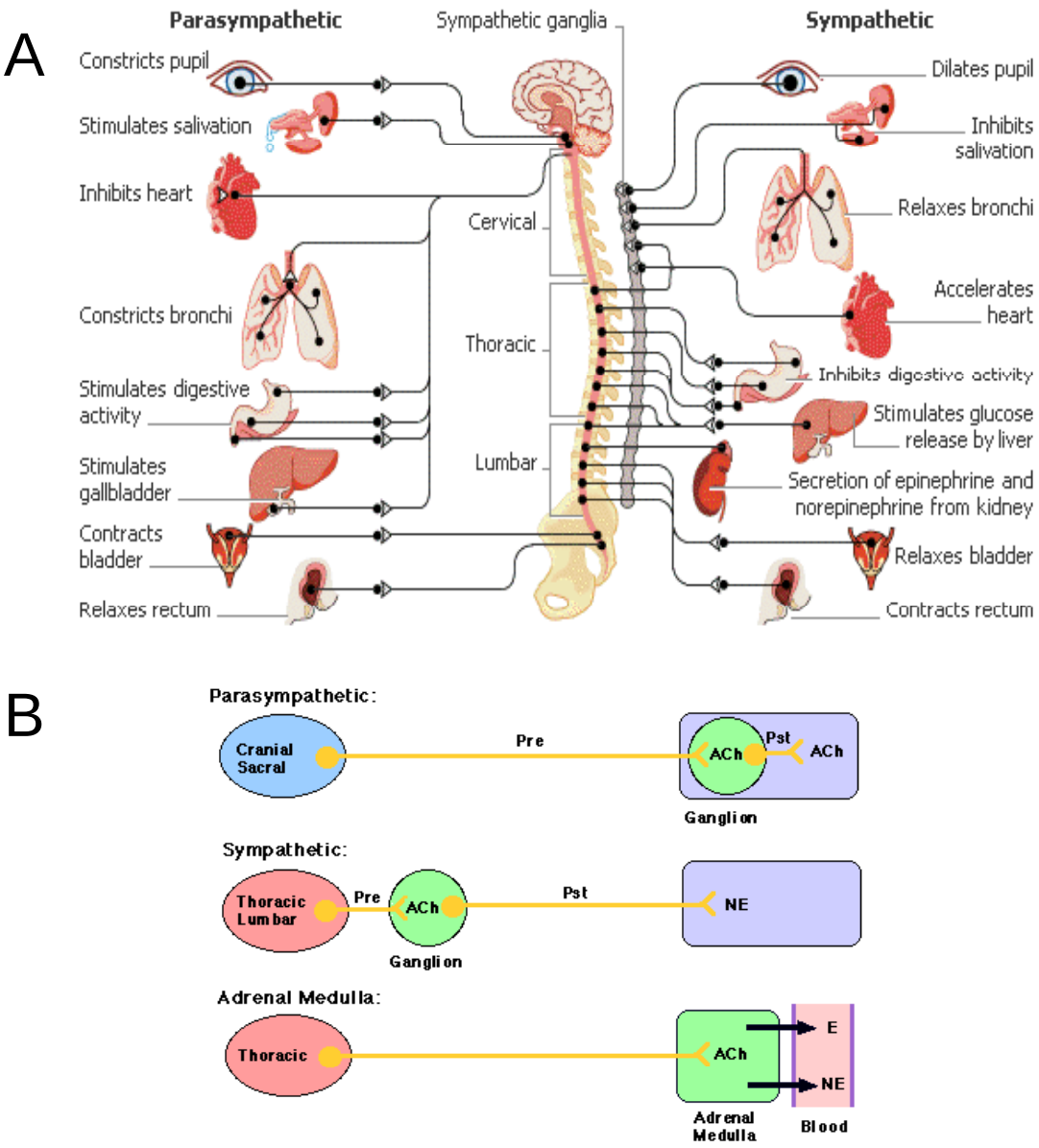

Figure 2. Autonomic Nervous System. (**A**) The PNS and the SNS form the branches of the ANS. The PNS comprises the craniosacral outflow with neurons originating in the lower brainstem and in the sacral portion of the spinal cord (S-2 to S-4). The SNS neurons originate from the lateral horns of the thoracic and lumbar spinal cord. These neurons synapse at either paravertebral or prevertebral columns and finally synapse at their target organ.. (**B**). Specific neurotransmitters at pre- and post-ganglionic synapses of the ANS. Parasympathetic nervous system, PNS; Sympathetic nervous system, SNS; Autonomic nervous system, ANS; Acetylcholine, ACh, Norepinephrine, NE; Epinephrine, E. Adapted from Piccioto et al. (2002) and www.CNSforum.com, 2002.

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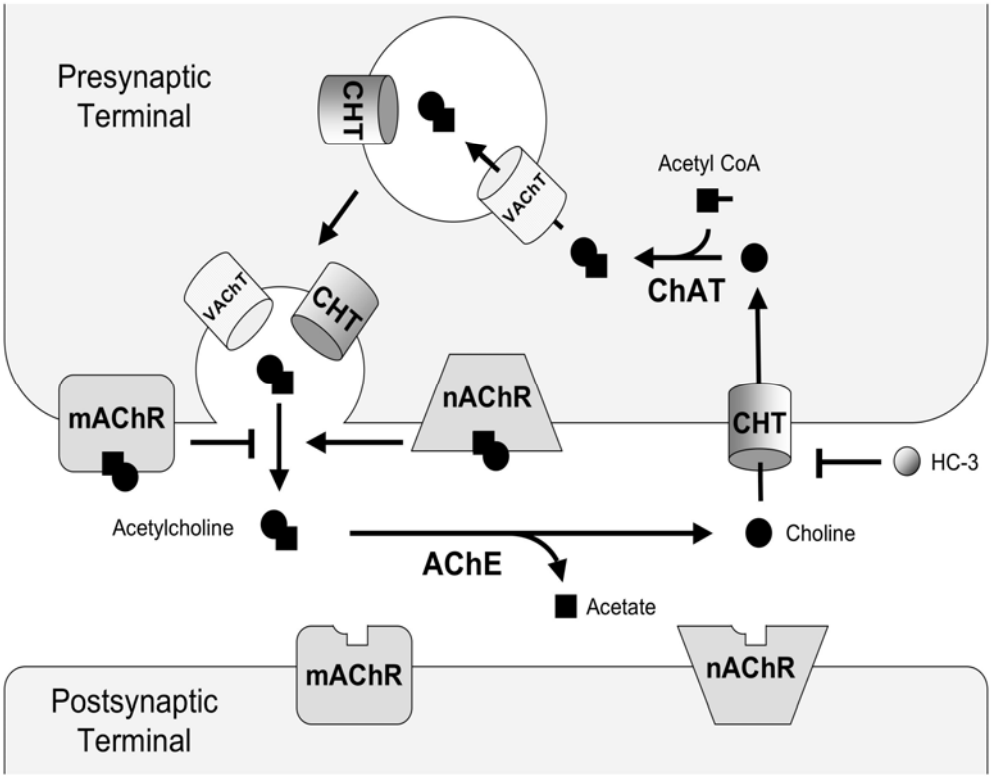


Figure 3. The cholinergic synapse. The enzyme choline acetyltransferase (ChAT) synthesizes ACh in the axoplasm from the precursors choline and acetyl coenzymeA (acetyl-CoA). ACh is then packaged into synaptic vesicles by the vesicular ACh transporter (VAChT), and released into the synaptic cleft upon neuronal depolarization. In the synaptic cleft, ACh interacts with pre- and post-synaptic nicotinic (nAChR) and muscarinic (mAChR) receptors. Presynaptic mAChRs exert an autoinhibitory effect on ACh release, whereas presynaptic nAChR activation increases ACh release. Synaptic ACh is hydrolyzed into acetate and choline by the enzyme acetylcholine esterase (AChE). Choline is then transported into the presynaptic terminal by the HC-3 sensitive, high-affinity choline transporter (CHT), in the rate-limiting step of subsequent ACh synthesis. Bazalakova M, et al. (2007).

(Soreq and Seidman, 2001). AChE operates at near diffusion-limited rates, thus this enzyme is not rate limiting in overall ACh homeostasis. The by-product of ACh hydrolysis, choline, is then recycled by the presynaptic terminal in a carrier-mediated mechanism of high-affinity choline uptake (HACU) process, which appears to be the critical step in modulating the rate and extent of ACh production (Ferguson and Blakely, 2004). Additionally, choline can be recycled by presynaptic neurons and other cells, via a low affinity choline uptake (LACU) process (Ferguson and Blakely, 2004; Ribeiro et al., 2006). Unlike transport proteins mediating HACU processes, low affinity choline uptake transporters are ubiquitously expressed in many cell types and are involved in choline transport for phosphatidylcholine synthesis in order to support cell membrane maintenance and repair (Inazu et al., 2005). Within cholinergic nerve terminals, choline is required for the biosynthesis of ACh since these neurons cannot synthesize *de novo*, hence the requirement for a HACU process (Birks and Macintosh, 1961; Ferguson and Blakely, 2004; Macintosh et al., 1956).

High Affinity Choline Uptake (HACU) and Regulation of CHT

High affinity choline uptake was originally described by Birks, et al. in 1961 and was subsequently shown to uptake choline specifically for the biosynthesis of ACh predominantly in presynaptic cholinergic nerve terminals (Atweh et al., 1975; Birks and Macintosh, 1961; Kuhar and Murrin, 1978). In contrast to LACU, which transports choline in a Na^+-independent manner and exhibits a lower affinity for choline (K_m = 50-100uM), HACU transports choline in a Na^+-dependent manner and exhibits a higher affinity for choline (K_m = 1-5uM) (Kuhar and Murrin, 1978; Simon et al., 1976). HACU

is also inhibited by the competitive antagonist hemicholinium-3 (HC-3) (K_{iHACU} = 10-100 nM), versus K_{iLACU} = 50 uM) (Simon et al., 1976; Yamamura and Snyder, 1972). HC-3 is a bicyclic, choline analog compound originally characterized as a respiratory paralytic agent (Macintosh et al., 1956). Unlike compounds such as botulinum toxin that inhibit synaptic vesicle fusion machinery preventing release of ACh, and that result in lethal respiratory paralysis, the respiratory effects of HC-3 can be relieved by artificial respiration or the administration of exogenous choline demonstrating similar targets at the high-affinity choline transporter (Ferguson and Blakely, 2004). The effects of respiratory impairment by HC-3 are consistent with cessation of ACh neurotransmission at the neuromuscular junction, and subsequent studies at the NMJ have shown that HC-3 suppresses stimulation-evoked ACh release (Van der Kloot et al., 2002). In addition to HC-3's physiologic effects, the radiolabeled form of HC-3 ([^{3}H]-HC-3) has proven a useful tool for identifying cholinergic terminals and studying CHT function (O'Regan, 1988).

Observations that the biosynthesis of ACh was dependent upon the uptake of extracellular choline in Na^+-dependent, HC-3 sensitive manner via a HACU process provided evidence that cholinergic nerve terminals possessed an alternative transporter (Ferguson and Blakely, 2004). The identification of the transporter responsible for HACU in cholinergic neurons remained elusive due to the low density of cholinergic terminals in cellular preparations and the low contribution of cholinergic-neuron specific high-affinity choline uptake relative to low-affinity choline uptake (Diamond, 1970). However, though many studies demonstrated that the majority of choline uptake in cholinergic nerve terminals was mediated by a high-affinity, Na^+-dependent, HC-3

sensitive mechanism, and that this process was linked to the availability of releasable ACh, none had identified the transporter responsible for mediating this process.

As noted above, several studies had observed that extracellular choline was required to sustain ACh release and that the process of choline uptake was saturable, and HC-3-sensitive indicating that a separate transport process existed in cholinergic neurons. Additionally, ACh synthesis from exogenous radiolabeled-choline requiring Na^+-containing buffers was demonstrated in synaptosomal preparations, demonstrating that a distinct high-affinity, Na^+-dependent choline uptake transport process existed in the CNS to support ACh synthesis (Yamamura and Snyder, 1972). These results were further supported by experiments demonstrating a significant decrease in high-affinity choline uptake activity in the hippocampus following denervation of the septal-hippocampal pathway (Kuhar and Murrin, 1978). Using a *Xenopus laevis* oocyte expression system combined with sequence information provided by the *C. elegans* Genome Project, a cDNA encoding the nematode and rat high-affinity choline transporter (CHT1) was isolated and found to demonstrate Na^+-dependent, HC-3 sensitive [^{3}H]-choline uptake (Okuda et al., 2000). These observations led to the subsequent cloning of CHT in mice (Apparsundaram et al., 2001) and humans, as well as the chromosomal localization of human CHT to chromosome 2q12 (Apparsundaram et al., 2000). Recently, the development of choline transporter (CHT) knockout mice (CHT-/-) provided *in vivo* evidence that CHT was responsible for mediating HACU in cholinergic neurons, and required for the biosynthesis of ACh (Ferguson et al., 2004).

Localization and Expression of CHT

Several studies have examined the neuroanatomical and subcellular distribution of CHT. The first anatomical visualization of CHT distribution was performed using [^{3}H]HC-3 autoradiography that was validated by lesioning of cholinergic pathways (Pascual et al., 1991; Rainbow et al., 1984; Rossner et al., 1995). This approach however, did not permit resolution of CHT at the synaptic level (Rainbow et al., 1984; Rossner et al., 1995). Although these early studies allowed for the visualization of specific regions of CHT expression, these results are somewhat difficult to interpret as substantial evidence exists that HC-3 binding to CHT has been shown to be dependent upon CHT availability and relative activity of cholinergic neurons (Lowenstein and Coyle, 1986). Studies by Blakely and colleagues, utilizing CHT-specific antibodies, confirmed the localization of CHT to cholinergic neurons in addition to its colocalization with vesicular acetylcholine transferase (VAChT) in rodent brain preparations (Ferguson et al., 2003), providing further support that CHT was involved in support of ACh synthesis. Additional studies conducted at the rodent NMJ and in primate brain supported these conclusions (Misawa et al., 2001) (Kus et al., 2003).

Examination of the anatomical distribution of CHT immunoreactivity identified in a number of brain regions known to have cholinergic inputs such as midbrain, brainstem, spinal cord, hippocampus, striatum and cortex (Kobayashi et al., 2002; Lips et al., 2002; Misawa et al., 2001). Northern blotting of various regions of mouse brain confirmed presence of CHT mRNA in brain regions with cholinergic projections, but that CHT mRNA was lacking in regions lacking cholinergic neurons such as the cerebellum (Apparsundaram et al., 2001). CHT has also been identified at the neuromuscular

junction (Nakata et al., 2004). Within the peripheral nervous system, CHT immunoreactivity has been identified within autonomic nervous system (ANS) projections of the parasympathetic nervous system branch (PNS) innervating the heart (Hoover et al., 2003; Mabe AM, 2006). These studies demonstrated that CHT expression was limited to cardiac nodal tissue, including the sinoatrial (SA) and atrioventricular (AV) nodes, regions shown to have PNS innervation via the vagus nerve (Hoover et al., 2003; Loffelholz and Pappano, 1985) (**Figure 4**).

Additionally CHT immunoreactivity has been identified in non-neuronal cell types including epithelial skin cells, tracheal epithelium and urinary bladder epithelium (Hanna-Mitchell et al., 2007; Pfeil et al., 2003a; Pfeil et al., 2003b). Using immunohistochemistry and CHT mRNA expression, CHT has been detected in tracheal epithelial cells by *in-situ* hybridization and has been suspected in regulating ACh after breakdown at the luminal service hence governing tracheal function of ciliated cells (Lips et al., 2007; Pfeil et al., 2003b). In the urinary bladder, CHT is expressed in epithelial cells in addition to ChAT, however VAChT could not be identified in these cells possibly due to sensitivity issues (Hanna-Mitchell et al., 2007). CHT was found that urinary bladder cells also express another plasma membrane transporter, organic cation transporter (OCT3) that may also be involved in the release of ACh from non-neuronal cells, however the interaction of CHT with the OCT transporter remains to be elucidated (Hanna-Mitchell et al., 2007). Similarly, CHT immunoreactivity and expression has been demonstrated in human keratinocytes, though a role of CHT in skin cells remains to be clarified (Pfeil et al., 2003a).

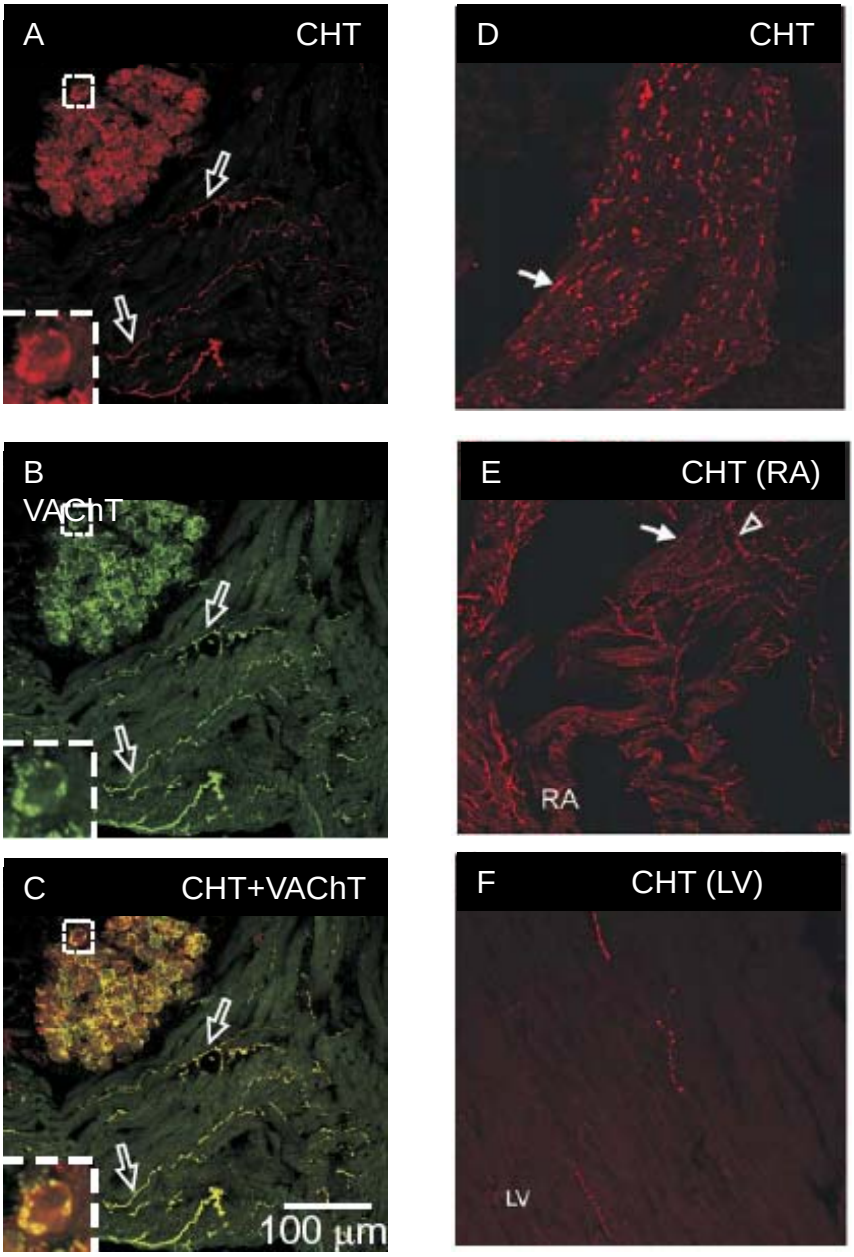


Figure 4. Immunoreactivy of CHT and VAChT in mouse heart. (A-E) Confocal images of intrinsic cardiac neurons (ICNs) double labeled for CHT (A), VAChT (B) and the overlay (C) show cholinergic localization in mouse nodal tissue. Open arrows indicate atrial nerve fibers. Colocalization of CHT and VAChT in atrial nerve fibers. **(D-E)** Immunoreactivity of CHT in sinus node, atrium and ventricle of mouse heart. Distribution of CHT is highest in nodal tissue, but diminishes in right atrial (RA) and left ventricular (LV) tissue. Adapted from Hoover et al. (2008, 2006).

Within the cardiovascular system, non-neuronal expression of CHT has been demonstrated in rat and human arterial vascular smooth muscle wall lending evidence to a role of a non-neuronal, intrinsic cholinergic system within the vasculature (Lips et al., 2003). Additional evidence for a non-neuronal, vascular cholinergic system are findings that the arterial and microvascular endothelial cells release ACh and have been shown to express ChAT and VAChT (Haberberger et al., 2000; Haberberger et al., 2002; Milner et al., 1989).

Lastly, the cholinergic nervous system has been implicated in the modulation of the immune response (Tayebati et al., 2002). Several studies have confirmed the presence of cholinergic markers within T-cell lymphocytes including mAChRs, nAChRs, ChAT, CHT and AChE (Kawashima and Fujii, 2004). Stimulation of T-cell activation has been shown to enhance lymphocytic cholinergic transmission by upregulation of ChAT, mediated by the nAChRs, thus implicating a role of cholinergic transmission modulating the immune response (Kawashima and Fujii, 2003b). Together, these findings reveal a broader role for CHT-supported cholinergic signaling than previously suspected.

To provide further resolution of the subcellular localization of CHT, immuno-EM microscopy analysis was performed in cholinergic neurons of the CNS and neuromuscular junction (NMJ) preparations. Significant CHT labeling was visualized at the presynaptic level in both CNS and the NMJ (Ferguson et al., 2003) (**Figure 5**). Surprisingly, there was a predominance of CHT labeling associated with the intracellular presynaptic vesicles (Ferguson et al., 2003). Immuno-EM labeling of CHT at cholinergic

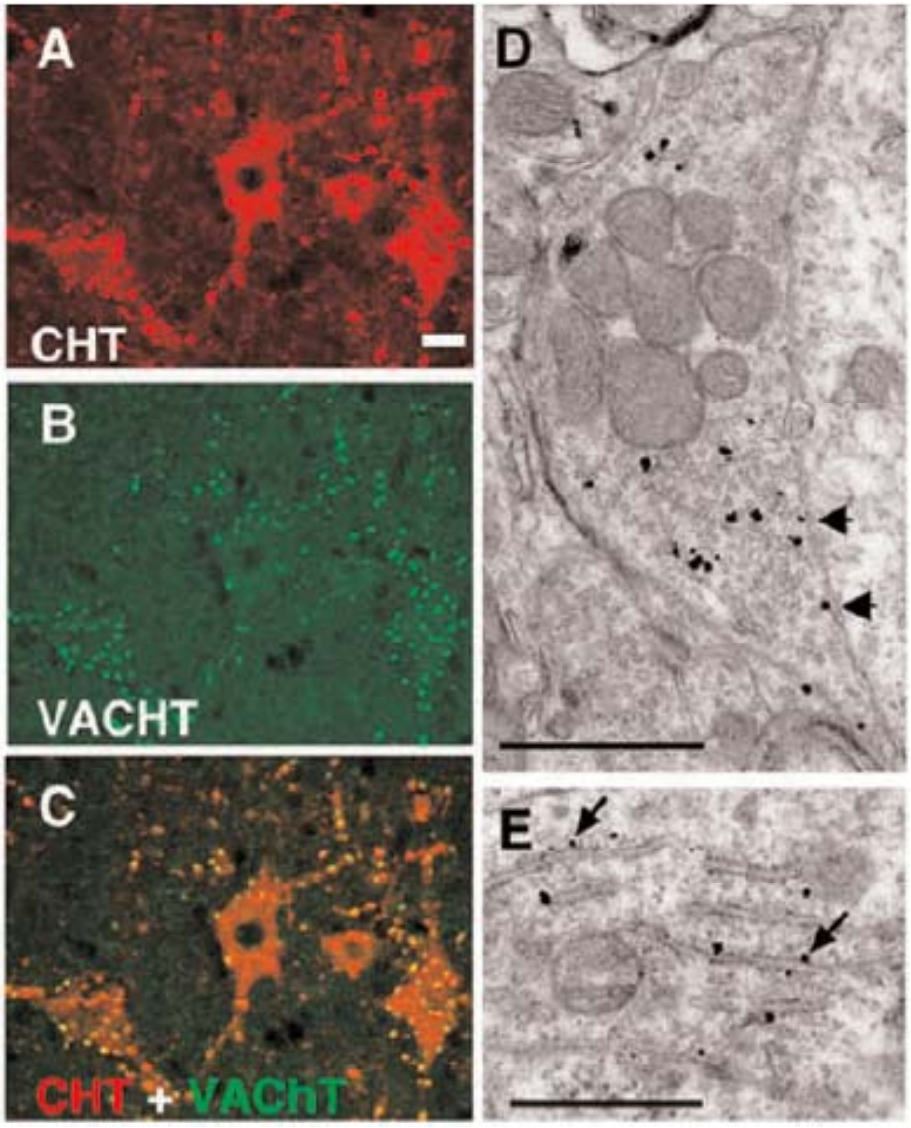


Figure. 5. Colocalization of CHT with VAChT at presynaptic terminals in cholinergic neurons. (A) Confocal imaging of CHT immunoreactivity in ventral horn of mouse spinal cord (scale bar = 20 μm). **(B)** VAChT immunoreactivity in similar cell bodies as CHT. **(C)** Overlapping distribution of CHT and VAChT confirming colocalization. **(D)** Immuno-EM with CHT-specific antibodies with arrows showing CHT is localized in the presynpatic neuron. **(E)** Cell body of cholinergic neuron with CHT immunoreactivity identified in the rough endoplasmic reticulum (scale = 1μm). Data adapted from Ferguson and Blakely (2004).

presynaptic terminals was found to be consistent with the known morphology of cholinergic terminals (Gilmor et al., 1996).

The identification of the presynaptic vesicular localization of CHT provided evidence that "pools" of CHT existed in cholinergic neurons and provided a mechanism by which CHT V_{max} can be increased following cholinergic neuron activation (Kuhar and Murrin, 1978). To confirm the localization of CHT in presynaptic cholinergic neurons, subcellular fractionation experiments of synaptosomal preparations from whole mouse brain, using high-speed gradient centrifugation was utilized. Similar to the EM studies, CHT was enriched in the P2 fraction with the majority of CHT enrichment found within the LP2 fractions along with other presynaptic vesicular proteins such as synaptophysin and VAChT (Eiden, 2000; Ferguson et al., 2003) (**Figure 6**). These subcellular localization studies along with synaptic vesicle immuno-isolation and EM vesicle analysis suggest a linkage of CHT to distinct vesicular pools containing VAChT and that fuse with the membrane during increased rates of cholinergic firing, therefore, CHT can possibly delivered to the plasma surface coupled to ACh release permitting activity-dependent choline recycling.

Activity Dependent Regulation of CHT

Multiple studies have demonstrated an intimate link between the V_{max} of CHT-mediated HACU and the B_{max} of [^{3}H]HC-3 binding coupled to changes in cholinergic activity (Atweh et al., 1975; Ferguson et al., 2004; Lowenstein and Coyle, 1986). Previous work showed that CHT-mediated HACU sustained ACh synthesis and release during persistent and prolonged stimulation (Birks and Macintosh, 1961; Maire and Wurtman, 1985), and as noted during steady state, the majority of CHT resides on

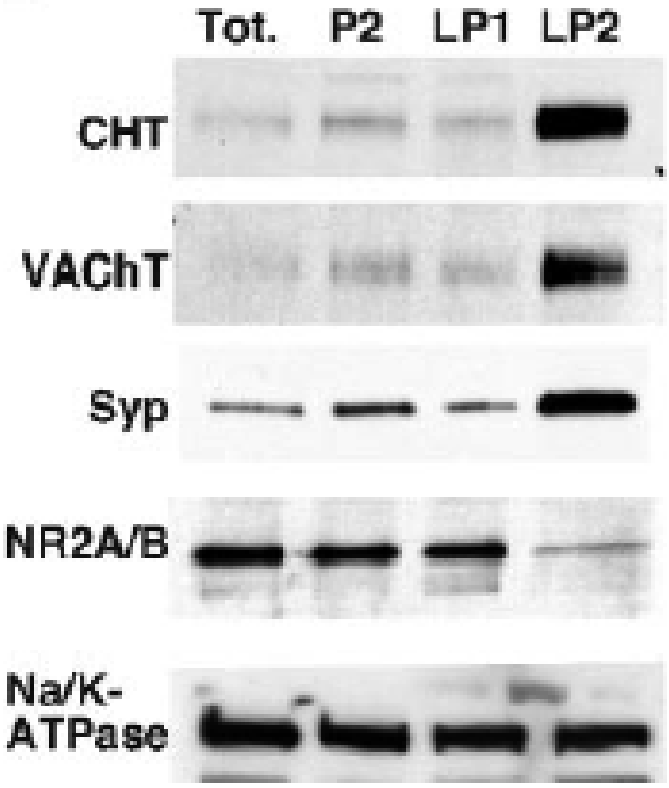


Figure 6. Subcellular fraction of whole mouse brain shows CHT localization to presynaptic vesicles. Whole mouse brain subcellular fractionation by differential centrifugation shows CHT enrichment in the LP2 fraction consistent with other presynaptic vesicle markers, VAChT and synaptophysin (Syp). Adapted from Ferguson et al. (2003).

VAChT containing presynaptic vesicles (Ferguson et al., 2003). The uptake of choline demonstrates saturation at low micromolar concentrations, therefore in order to increase choline uptake, an increased number of CHT's at the plasma surface would be required.

Depolarization of synaptosomes (e.g. high K^+) or electrical stimulation mimicking increased cholinergic firing rates have demonstrated an increase in the CHT-mediated uptake (V_{max}) (Collier et al., 1983; Simon and Kuhar, 1975) and that these changes can be blocked by pretreatment of hippocampal synaptosomes with botulinum toxin C, known to inhibit vesicular release of neurotransmitter (Blasi et al., 1993). Synaptosomal preparations made from rat hippocampus also showed that pharmacologic treatments leading to a decrease in ACh release resulted in a concomitant decrease in CHT V_{max} (Atweh et al., 1975). These changes observed in CHT V_{max} secondary to an increase in cholinergic activation have been shown to be coupled to an increase [^{3}H]HC-3 binding demonstrating that the increase in V_{max} was associated with an increase in the surface density of CHT (B_{max}) in stimulated membrane preparations and in striatal slices (Ferguson et al., 2003; Lowenstein and Coyle, 1986; Saltarelli et al., 1987). These findings have been eloquently replicated in *in vivo* studies whereby mice exposed to various learning paradigms exhibited an increase in CHT uptake and expression (Apparsundaram et al., 2005).

Whereas *in vitro* (Ferguson et al., 2003) and *in vivo* (Apparsundaram et al., 2005) studies demonstrated that CHT is inserted into the plasma membrane by a fraction of the same VAChT-positive vesicles that mediate ACh release, questions still arise as to the mechanisms regulating CHT sorting at the surface. Additionally, while depolarization of synaptosomes results in immediate increases in ACh release, changes in CHT function

require several minutes of stimulation indicating a temporal difference in CHT regulation upon insertion into the plasma membrane (Ferguson et al., 2003; Murrin et al., 1977). These studies demonstrate that the trafficking and activation of CHT is coupled to the activation state of cholinergic transmission but that other mechanisms exist regulating CHT function at the surface.

Less is known about the regulated recycling and plasma membrane recruitment of CHT. Since CHT and VAChT has been shown to be collocated on synaptic vesicles and both share a common acidic residue-flanked dileucine motif in their cytoplasmic C-terminal, it has been proposed that CHT recycling occurs via a clathrin-dependent pathway (Ribeiro et al., 2007). Thus, when COS-7 cells were cotransfected with a dominant-negative K44A dynamin I mutant, constituative endocytosis of CHT from the surface was inhibited (Tan et al., 1998). Similarly, Ribeiro, et al. demonstrated that the presence of CHT at the surface is limited by endocytosis of the transporter in clathrin-coated vesicles in a dileucine-dependent mechanism (Ribeiro et al., 2006). K^+-evoked depolarization studies using HEK-293 and SH-SY5Y cells demonstrated that the activity-dependent increase in CHT at the plasma membrane is regulated by two mechanisms involving increased externalization of the intracellular CHT pool and recruitment of additional intracellular CHT from the recycling pool, indicating two separate synaptic vesicular pools of CHT translocated to the surface during increased cholinergic demands (Ribeiro et al., 2007). The mechanisms regulating these various CHT-positive vesicular pools to the surface and their endocytosis by adaptor proteins and C-terminal motifs are a current area of investigation (Ruggiero AM, personal communication).

The Human Choline Transporter (CHT)

Genetics of the Choline Transporter

The human CHT (hCHT) is a member of the SLC5A gene family (designated *SLC5A7*) of the Na^+-dependent transporters and is similar in structure to the Na+-dependent glucose transporter (SGLT) that is the best characterized transporter in this family of ≈80 transporters (Wright et al., 1992). The human CHT gene, has been mapped to chromosome 2q12 (chromosome 17, mice), spanning 25kb of genomic sequence (**Figure 7A**) (Ferguson and Blakely, 2004; Okuda and Haga, 2000). Hydrophilicity analysis and topology prediction algorithms predict a protein with 13 transmembrane domains (TMDs) containing a short extracellular N-terminus and long intracellular C-terminus, that exhibit 93%, 98% and 52% amino acid sequence homology to murine, rat and *C. elegans* CHT proteins respectively (**Figure 7B**) (Ferguson et al., 2003; Okuda et al., 2000). Within the proposed topology of CHT, a consensus site for N-liked glycosylation has been identified at residue N^{301} in the fourth extracellular loop. The intracellular C-terminal tail possess several canonical motifs for serine and threonine phosphorylation sites that may be important for CHT regulation and trafficking of CHT to the cell surface (Ferguson et al., 2003; Gates et al., 2004).

Within the initiation of our studies, there had been few studies examining the polymorphic status of the CHT gene, though one report described a hypomorphic coding variant that might influence cholinergic traits or influence risk for cholinergic-mediated

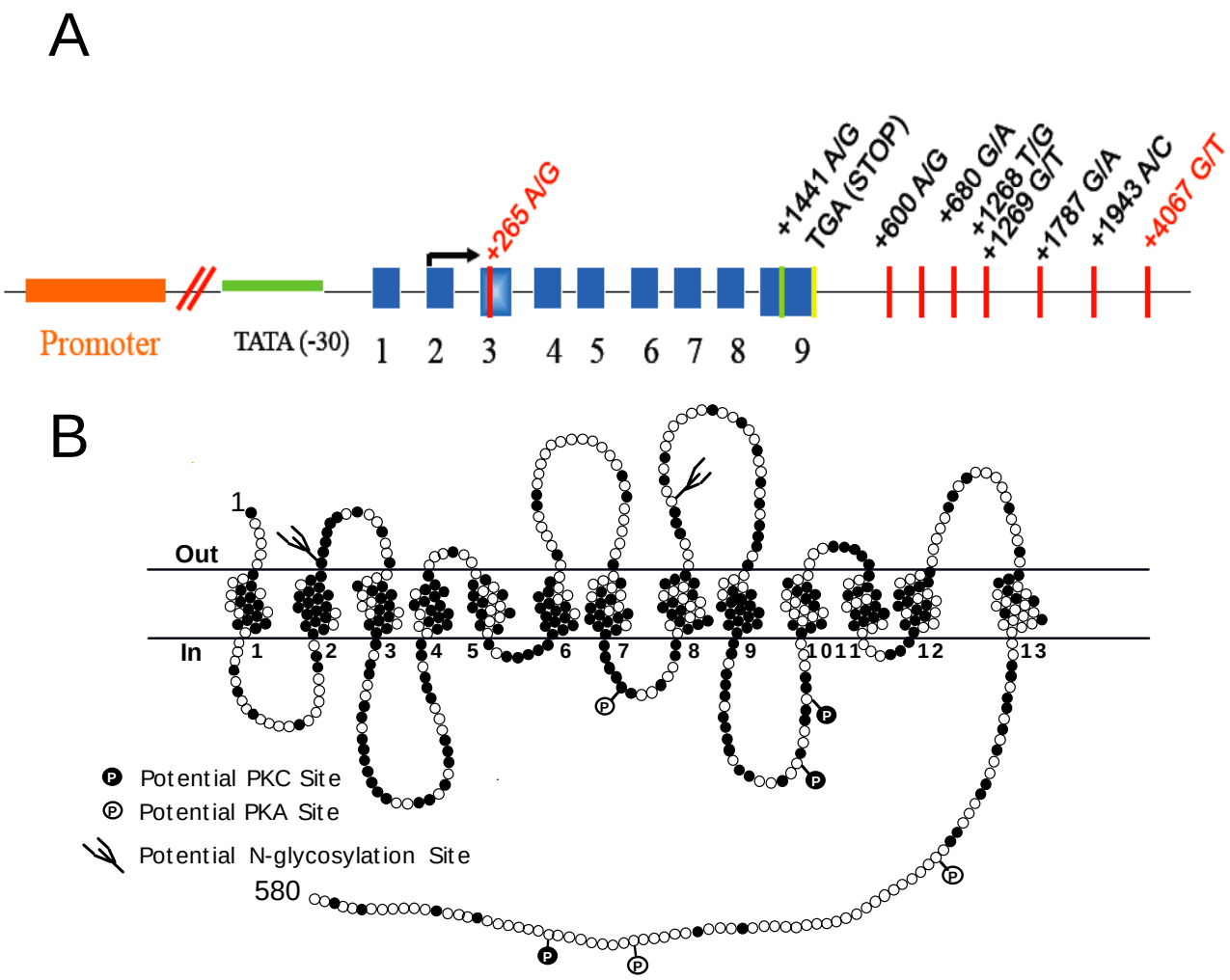


Figure 7. CHT gene and protein structures. (A) The human CHT gene is located on chromosome 2 and contains 9 exons. **(B)** The mCHT cDNA encodes a protein of 580 amino acids, with a molecular mass of approximately 63 kDa. Analysis of the amino acid sequence predicts a topology of thirteen transmembrane domains, an extracellular consensus site for N-linked glycosylation, and several cytoplasmic protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites (Apparsundaram et al. 2001a). Adapted from Ferguson and Blakely (2004).

disorders (Okuda et al., 2002). In their study, Okuda et al., identified a non-synonymous single nucleotide polymorphism (SNP) residing in exon 3 at nucleotide 265 (A265G, rs1013940) that results in an isoleucine to valine amino acid substitution at position 89 (Ile89Val) within TM3 of the expressed protein (Okuda et al., 2002) (**Figure 8A**). This study reported a frequency of the Ile89Val allele of 6% in a healthy Ashkenazi Jewish population (Okuda et al., 2002), findings that were subsequently validated in a larger panel by our group (Hahn et al., 2008)

Functional characterization of the Ile89Val variant in transfected COS-7 and HEK-293 cells demonstrated a ≈40-50% reduction in the transport velocity (V_{max}) for choline for the mutant (Val) versus the wildtype (Ile), whereas the affinity for choline was not changed (**Figure 8B**) (Okuda et al., 2002). While the reduction in V_{max} could be explained by a decrease in the cell surface expression of the CHT protein; Okuda et al., using both biotinylation and HC-3 surface binding, demonstrated that the reduction in V_{max} was not attributed to a reduction in the surface expression of CHT (Okuda et al., 2002). Although an Ile to Val represents a conservative change in amino acid substitution, this variant occurs in an exonic region displaying a high degree of sequence conservation among various species indicating that this region plays a significant role in the function of CHT (**Figure 9A**). Of the 82 reported SNPs within the CHT locus, including the Ile89Val variant, none within the initiation of this thesis had been explored for contributions to human disease.

Recently, another polymorphism originally designated as a component of the CHT 3'UTR (+4067 G/T; rs333229) was identified and found to be associated with altered cholinergic tone in healthy volunteers (Neumann et al., 2005). Our current

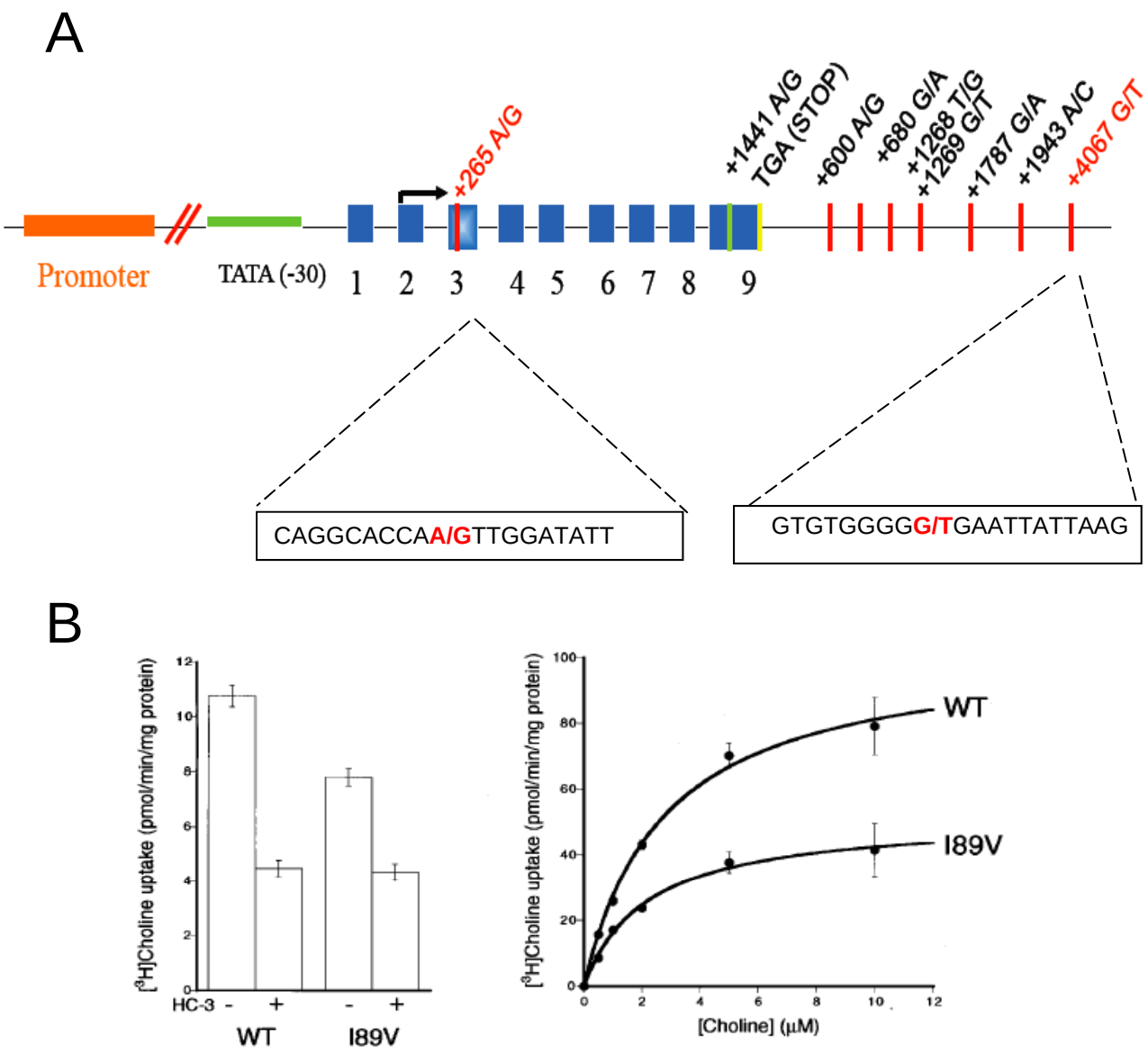


Figure 8. CHT gene SNPs and effects of Ile89Val on choline uptake in transfected cells. (A) The human CHT gene is located on chromosome 2 and contains 9 exons. Two SNPs (+265A/G and the 3'SNP) have been associated to altered CHT function. **(B)** [^{3}H]choline uptake (inhibited by 1μM HC-3) in transiently transfected COS-7 cells expressing WT or I89V tconstructs. Saturation kinetics of WT and I89V CHT in transfected cells shows normal affinity for choline (K_m), but diminished maximal uptake (V_{max}) (K_m: WT=2.8±0.1; I89V=3.0±0.3 and V_{max}: WT=102±9; I89V=55±6, *P*=0.01). Adapted from Okuda (2003).

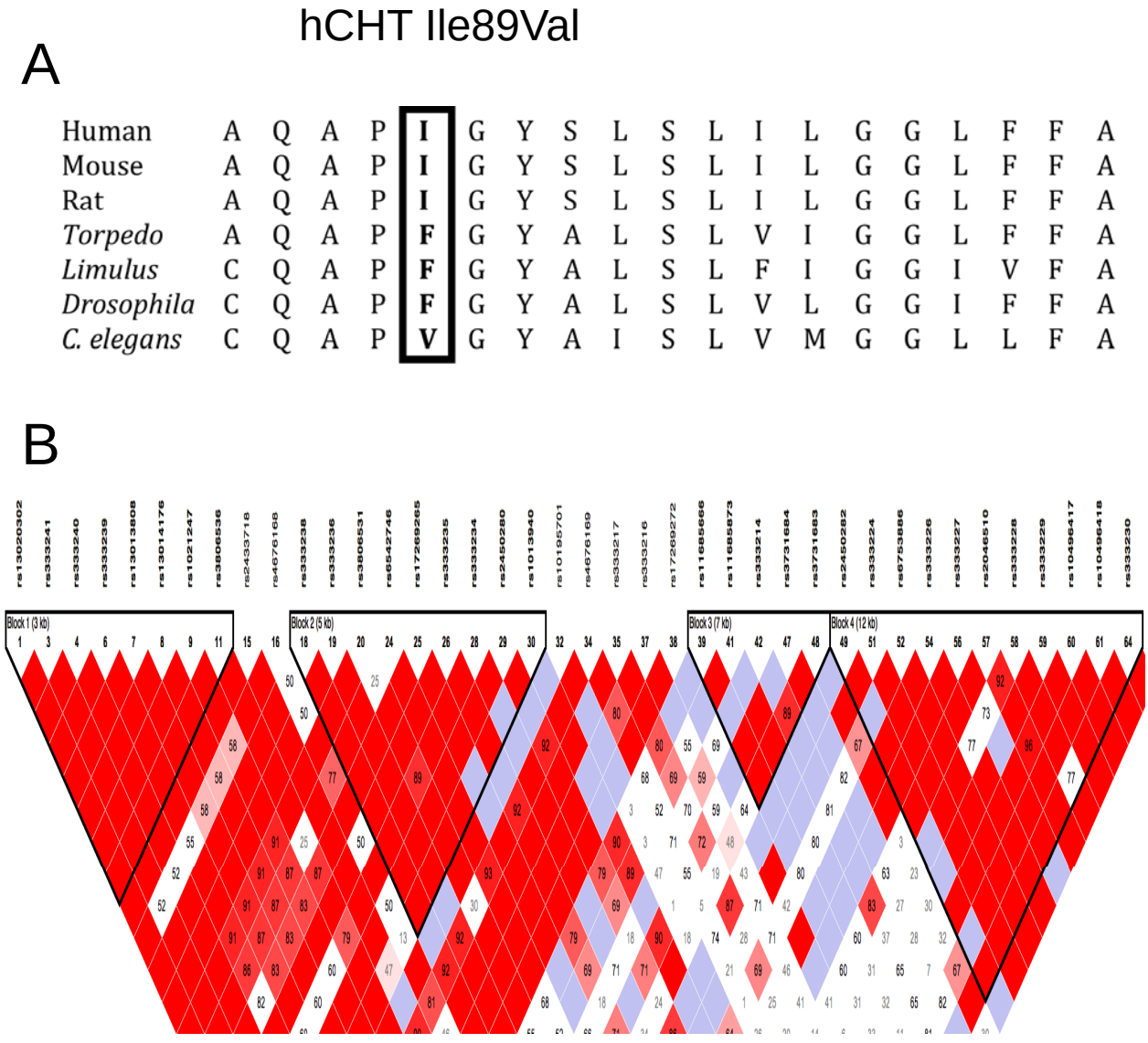


Figure 9. Sequence alignment of hCHT exon 3 (Ile89Val) and haplotype map of the hCHT gene. (A) Sequence alignment of CHT gene exon 3 shows significant sequence homology in mammalian CHT gene. **(B)** Linkage disequilibrium estimates for the reported SNPs within the hCHT gene identifies 4 distinct haplotypes. (Okuda et al. 2002). Adapted from International HapMap Project 2004.

genomic analysis place this variant 3' of the predicted polyadenylation site and could not be identified with deposited ESTs, and thus will be referred to as the CHT 3'SNP (**Figure 7A**). Although the functional consequence of this SNP remains unclear, this study found the 3'SNP minor allele to positively correlate with the high frequency (HF) component of heart rate variability (HRV), a measure of vagal regulation of heart rate, in healthy volunteers. Using fMRI and HRV measurements, the 3'SNP, was recently shown to be associated with a decrease in corticolimbic reactivity in Brodmann Areas 6, 9 and 46, which also correlated to HRV (Neumann et al., 2006). These studies suggest that the CHT 3'SNP has, or is linked to other variants functional effects associated with an increased cholinergic tone and contributes to the corticolimibic and autonomic circuitry mediating behavioral and physiologic arousal. Though 3'UTR variants are hypothesized to disrupt mRNA stability, further studies are required to determine the actual impact that this 3'SNP variant has on CHT expression. Additionally, further studies are required to determine the impact of this 3'SNP variant in relation to the non-synonymous CHT variant, Ile89Val. Importantly, these variants exist on distinct haplotypes. The high minor allele frequency of the 3'SNP variant (22%), allows determination of its impact in relatively modest size cohort studies **(Figure 9B**). To date, neither the Ile89Val nor the 3'SNP CHT variants have been examined in large numbers of subjects with various diseases which cholinergic dysregulation may have an impact on severity of is associated with overall symptom severity in a small study of patients with major depression disorder (MDD) (Hahn et al., 2008).

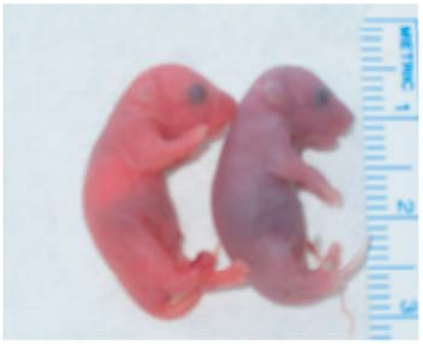
Role of CHT in Mammalian Physiology

Whereas the impact of disruptions in cholinergic function on mammalian physiology has been well studied, many of these studies have focused on presynaptic release of ACh or post-synaptic receptor effects. For example, exposure of animals to irreversible AChE inhibitors that results in a large increase of ACh in the synaptic cleft often results in lethality. In humans, disruption in cholinergic transmission has been implicated in many neurologic, cardiovascular, autoimmune and psychiatric disorders (Lefkowitz, 1996; Sarter and Parikh, 2005). A few studies utilizing HC-3 had demonstrated a physiologic lethality due to antagonism of CHT (Jones and Kwanbunbumpen, 1970). Until recently however, no studies had been conducted examining the role of the genetic loss of the presynaptic CHT. Given the role of the CHT in the biosynthesis of ACh and maintenance of cholinergic tone, disruptions in CHT function may contribute to these disorders.

To examine the role of a genetic loss of CHT on murine behavior and physiology, a homozygous CHT knockout mouse (CHT-/-) was developed (Bazalakova et al., 2003; Ferguson et al., 2004). CHT-/- mouse pups are smaller and appear cyanotic at birth (**Figure 10A)**, and are devoid of expressed CHT. As a consequence, these mice are deficient in functioning CHT, displaying loss of CHT-mediated HACU (**Figure 10B**) and exhibit a time-dependent loss of both spontaneous and evoked ACh induced responses at the NMJ (**Figure 10C**) (Ferguson et al., 2004). The loss of functioning CHT in CHT-/- mice results in postnatal lethality, possibly due to respiratory arrest as these mice become cyanotic, exhibit no alveolar inflation and die within 1 hour after birth (Ferguson et al., 2004). Additionally CHT-/- mice display histological changes at the NMJ as

Figure 10. CHT-/- mice demonstrate that CHT-mediated choline uptake is essential for postnatal viability. (A) While being equal in size, the CHT-/- pup is visibly cyanotic vs. the CHT+/+ pup (photo take 30 min post-birth). **(B)** Loss of HC-3 (1 μM) sensitive choline uptake in synaptosome from CHT-/- mice vs. CHT+/+ mice. Conversely, [^{3}H]GABA uptake was not significantly different (*P*=0.2). **(C)** Electrophysiologic tracings of ACh release from CHT+/+ and CHT+/- NMJs during evoked EPP (top panel) and spontaneous EPP (bottom panel) at 1hr and 4hrs. Notice loss of evoked and spontaneous EPPs in CHT-/- mice by 4 hours. **(D)** CHT protein expression is reduced in whole brain extracts from CHT+/- mice compared to CHT+/+ mice, although synaptosomal preparations **(E)** show CHT-mediated HACU kinetics (K_d and V_{max}) are similar between genotypes. Adapted from Ferguson, et al. (2004) and Bazalakova, et al. (2008)

A



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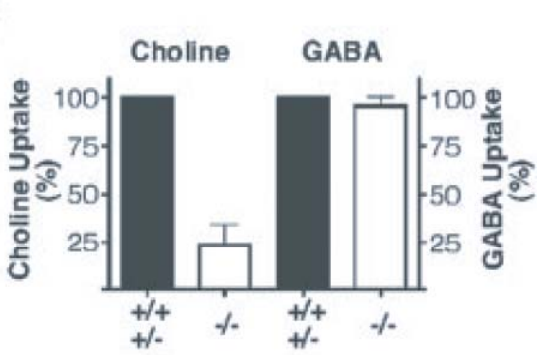
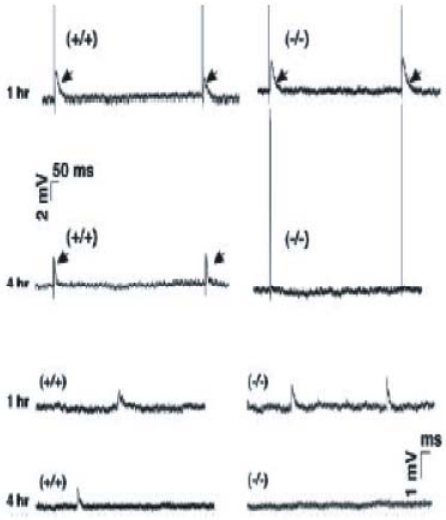

Choline
GABA
Choline Uptake (%)
GABA Uptake (%)
100
75
50
25
+/+
+/-
-/-

C


(+/+)
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1 hr
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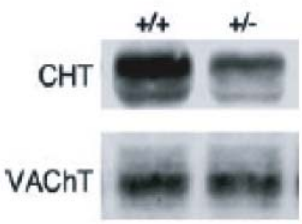
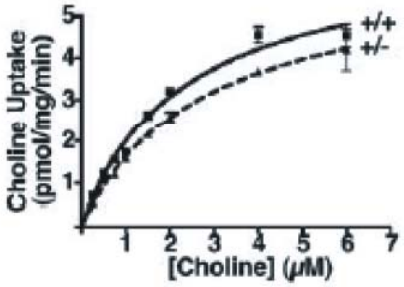
D


+/+
+/-
CHT
VAChT

E


Choline Uptake (pmol/mg/min)
[Choline] (μM)
+/+
+/-

demonstrated by changes in nAChR distribution with increased clusters of receptors, possibly to compensate for reductions in ACh availability (Ferguson et al., 2004).

The postnatal lethality phenotype of the CHT-/- mice limits further investigation into the genetic influences of CHT to cholinergic tone in other organ systems. Fortunately, heterozygous mice (CHT+/-) are both viable and seemingly healthy compared to CHT+/+ mice, yet express half the levels of CHT protein **(Figure 10D**). When compared to CHT+/+ mice, CHT+/- mice surprisingly exhibit equivalent choline uptake (V_{max}) accompanied by similar levels of [^{3}H]HC-3 binding (B_{max}) **(Figure 10E)** (Ferguson et al., 2004). These similarities in both carrier-mediated HACU and HC-3 binding densities between both genotypes corroborates an ability of cholinergic neurons to detect changes during ACh demand and mobilize reserves of CHT vesicular pools to the plasma membrane (Ferguson et al., 2004). Although the CHT+/- mice display similar CHT V_{max}, these mice display an increased sensitivity to sub-lethal i.p. doses of HC-3 **(Figure 11A**), demonstrating a lack of CHT reserves that cannot meet sustained, capable of meeting increases in cholinergic demand. Similarly, during sustained treadmill exercise, CHT+/- mice show impairments in duration and speed compared to CHT+/+ mice **(Figure 11B and C**) (Bazalakova et al., 2007). Further analysis is required to determine the impact this reduced CHT capacity has on functions requiring continuous cholinergic tone such as behavior, learning and autonomic regulation.

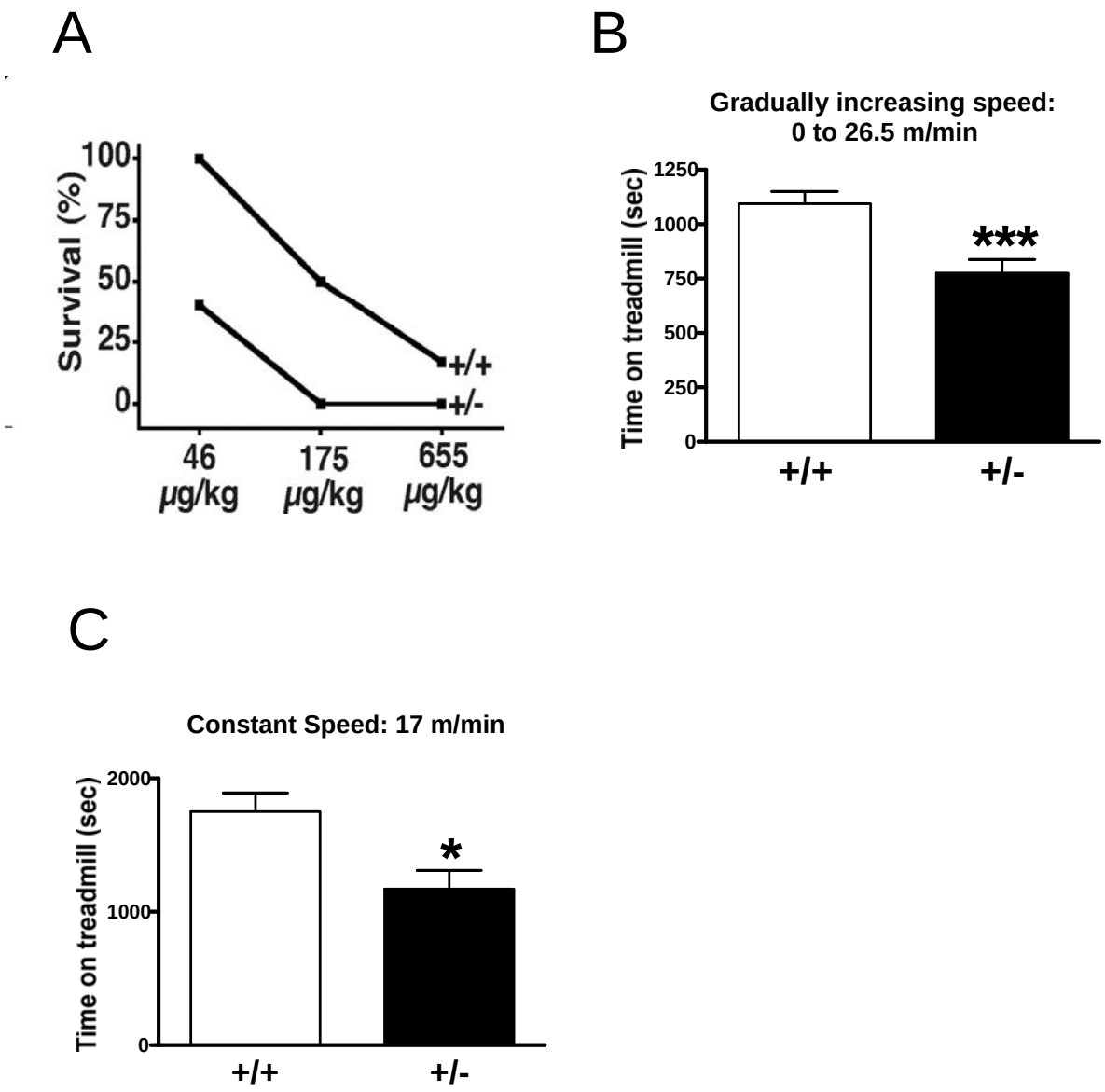


Figure 11. Genetic loss of CHT-mediated HACU produces a physiologic phenotype in CHT+/- mice. (A) CHT+/- mice increased sensitivity to sub-lethal doses of HC-3 vs. CHT+/+ mice. **(B)** CHT+/- show impaired exercise capacity with deficits in acute (speed) tolerance and endurance **(C)** on treadmill tests (**P*<0.01, ****P*<0.001). Values represent ±SEM. Adapted from Ferguson, et al. (2004) and Bazalakova, et al. (2008)

Cholinergic Transmission in Cardiovascular and Behavioral Function

Cholinergic Transmission and Cardiovascular Function

The regulation of many visceral physiological functions within the body is mediated by the autonomic nervous system (ANS). This system is responsible for the regulation of heart rate, blood pressure, gastrointestinal motility, secretion, body temperature and many other functions (Lefkowitz, 1996). To achieve dynamic control these functions, the ANS is subdivided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) **(Figure 2**). The ANS is predominantly activated by neural centers located within the spinal cord, brain stem and cerebral cortex (Guyton, 1992). The SNS, known colloquially as the "fight-or-flight" system as it mediates increases in those physiologic responses required by the organism to respond quickly such as heart rate and respirations (Lefkowitz et al., 2000). Conversely, the PNS is characterized as the "rest-and-digest" system and mediates more vegetative functions such as digestion, salivation and urination (Lefkowitz, 1996).

The autonomic nervous system plays a major role in regulating heart rate and the short-term regulation of blood pressure. The basic neurotransmitters, receptor systems and the effects mediated by the SNS and PNS are shown in **(Table 1**). Sympathetic regulation of the heart is mediated by the β_1 adrenergic receptor (β_1AR). The β_1 is a member of the G protein-coupled receptor (GPCR) family and is coupled to the Gs heterotrimeric protein family (Lefkowitz, 1996). Stimulation of β_1ARs results in an increase in adenylyl cyclase, activating the cAMP-PKA-dependent signaling pathways. Activation of cardiac β_1ARs results in an increase in chronotropic (heart rate) and ionotropic (contractile) properties of the heart (Lefkowitz, 1996). Conversely,

Table 1 Responses of effector organs to autonomic nervous system innervation

Organ	Sympathetic stimulation	Receptor	Parasympathetic stimulation	Receptor
Heart	Increased HR	β1	Decreased HR	M1
	Increased force contraction	β1	Decreased force contraction	M1
Arteries	Constriction	α1	Dilation	β2
	Dilation	β2		
Veins	Constriction	α1	n/a	
	Dilation	β2		
Lungs	Bronchial muscle relaxation	β2	Bronchial muscle contraction	M2
			Increased gland secretion	M2
GI	Decrease	α1/α2	Increase (constriction)	M2

parasympathetic regulation of the heart is primarily mediated by M_2-muscarininc acetylcholine receptor (M_2AChR) (Caulfield, 1993). The M_2AChR is also a member of the GPCR superfamily, and is coupled to the $G_{i/o}$ heterotrimeric protein family (Caulfield, 1993; Rouse et al., 1997). Agonist stimulation of the M_2AChR activates the Gi/o protein resulting in an inhibitory effect on adenylyl cyclase, counteracting cAMP-PKA-dependent signaling pathways (Wettschureck and Offermanns, 2005). Activation of the M_2AChR coupled $G_{i/o}$ protein also produces a direct increase in K^+-channel (I_K) activation, resulting in a hyperpolarization of both the SA and AV nodes (Szabo and Otero, 1989; Valenzuela et al., 1997). Additionally, the $G_{\beta\gamma}$ subunit of $G_{i/o}$ heterotrimeric protein can directly activate the muscarinic-gated-K^+-channels (I_{KACh}) within cardiac nodal tissue, allowing for vagal regulation of chronotropic effects of the heart (Logothetis et al., 1988; Zhu et al., 2001).

Few studies have examined the role of CHT-mediated HACU in cardiovascular regulation. Given the role of CHT in supporting cholinergic tone, it would be expected that alterations in CHT function contribute to diminished vagal tone to the heart resulting in cardiovascular dysfunction. As previously mentioned, CHT was found to be co-expressed with another protein marker, neurturin (NRTN), a marker required for the development of normal cholinergic innervation to the heart, in nodal tissue of murine hearts, demonstrating the presence of CHT in regions of the heart responsible for chronotropic effects (Mabe AM, 2006). Similarly, the finding of CHT expression in non-neuronal vascular endothelial tissue raises the possibility of cholinergic function mediated vascular tone (Haberberger et al., 2000).

In human cardiovascular disorders, though no specific disorder has been attributed to alterations in CHT function, the impact of diminished cholinergic tone in cardiovascular mortality and morbidity has been well documented (Barron and Lesh, 1996; Fox et al., 2007). Cholinergic innervation of the heart is localized to nodal areas and most commonly associated with reduction in HR, where it contributes to the overall autonomic tone of the heart, thereby influencing parameters such as acute variability of heart rate (HRV), baroreceptor sensitivity (BRS) and resting HR (Eckberg et al., 1971; Stein and Kleiger, 1999; Taylor, 1994). Depression of BRS and HRV due to diminished parasympathetic tone has been associated with increased mortality due to sudden cardiac death in both humans and animal models of myocardial infarction (MI) after ischemic episodes (Barron and Lesh, 1996; Vanoli et al., 1991). Using frequency-domain analysis, HRV can be shown to display two primary components, a low frequency (LF) and high frequency (HF) component, with the HF component of HRV in humans reflecting tonic vagal activity (Pieper, 1995). Frequency-domain analysis of HRV has been used in various clinical settings and has been shown to be a powerful predictor of adverse prognosis in patients with congestive heart failure (CHF), MI, coronary artery disease (CAD) and life threatening ventricular arrhythmias post-MI (Bigger, 1992; Binkley, 1991; Lombardi, 1996; Norris et al., 2006; Rich, 1988).

Unlike HRV, baroreceptor sensitivity (BRS) is a vagally-mediated response to an increase in peripheral vascular resistance and is responsible for acute regulation of HR and blood pressure (BP) (Taylor, 1994). In subjects with normal BRS, a rapid rise in arterial blood pressure elicits a vagally-mediated reduction in HR achieved by both an increase in direct parasympathetic tone and an indirect blunting of sympathetic activation

(Eckberg DL, 1992). Similar to HRV, BRS has also been shown to be a strong prognostic indicator of mortality post-MI, with patients having a reduced BRS demonstrating a higher mortality rate (La Rovere, 2001). The utility of monitoring BRS has been shown in both human and animal models of MI (Schwartz et al., 1988). Several cardiovascular disorders including hypertension (HTN), CAD, MI and HF, have been shown to be accompanied by decreased BRS (La Rovere et al., 2008). This imbalance in the sympathetic-vagal outflow to the heart results in a chronic increase in sympathetic tone and possibly contributes to progression of the cardiovascular disorder and end-organ damage (Eckberg DL, 1992).

Other cardiac parameters regulated by parasympathetic tone include resting HR and HR recovery following exercise. Several studies have shown that an elevated resting HR is associated with and increase in BP and can serve as a precursor to the development of chronic HTN, atherosclerosis and other cardiovascular events (Palatini and Julius, 1999). The Framingham Study showed that an increased resting HR was associated with an increase in mortality at 2 years post-diagnosis (Gillman et al., 1993). Tachycardia in hypertension is associated with decreased parasympathetic tone and increased sympathetic tone, contributing further dysfunction of cardiovascular physiology (Palatini and Julius, 2004). Regulation of HR by the administration of medications that either block an overactive sympathetic tone (e.g. β-blockers; propranolol) or increase vagal tone (e.g. angiotensin converting enzyme inhibitors) have become the mainstays of treatment in patients with MI and HF (Lanza et al., 2006; Smith et al., 2005). These medications have been shown to improve mortality associated with these conditions (Fox et al., 2007). Additionally, early recovery in HR after exercise has been shown to be mediated by

parasympathetic activation, and to be a predictor of ventricular susceptibility to fatal arrhythmias after MI (Pierpont et al., 2000; Smith et al., 2005). Similar to BRS and HRV, HR recovery (HRR) after exercise has also been shown to be an independent predictor of mortality in a wide range of patients (Cole et al., 1999; Curfman and Hillis, 2003).

In patients with chronically elevated resting HR and HF, studies have shown that the parasympathetic nervous system appears to be attenuated (La Rovere et al., 1994). Although most studies have not demonstrated changes in M_2AChR receptors or coupling to $G_{i/o}$ inhibitor G-proteins, in patients with HF, such changes may serve to be beneficial to the failing heart by promoting increased sympathetic tone preserving cardiac output (CO) (Brodde et al., 1998; Brodde and Leineweber, 2004). However in HF studies conducted in mongrel dogs, HF induced by rapid ventricular pacing resulted in an increase in M_2AChR and a decrease in AChE activity, thus demonstrating the cholinergic nervous systems attempt to reduce the damage secondary to sympathetic overactivation (Dunlap et al., 2003). Together, these data support the hypothesis that disruptions to CHT function may diminish ACh transmission of the vagal innervation to the heart and thus produce alterations in resting HR, HRV, BRS and HRR, lending to worsening of cardiac function and contribute to cardiovascular disorders.

Cholinergic Transmission and Behavioral Function

Within the mammalian CNS, cholinergic neurons sustain or modulate diverse and complex behaviors such as attention, arousal, memory and reward (Perry et al., 1999; Sarter and Parikh, 2005). Disorders such as Alzheimer's Disease (AD), a degenerative disorder of the basal forebrain cholinergic neurons, is thought to be the cause of the

dementia related cognitive deficits associated with the disorder (Whitehouse et al., 1982). Therapies such as acetylcholinesterase inhibitors (AChEI), that prevent the hydrolysis of ACh have been shown to provide improvement in cognitive decline associated with AD (Giacobini, 2000). Similar alterations in cholinergic function underlying the attentional and cognitive impairments associated with psychiatric disorders, such as attention deficit hyperactivity disorder (ADHD) (Beane and Marrocco, 2004), major depressive disorder (MDD) (Vakalopoulos, 2007) and delirium (Hshieh et al., 2008) have been hypothesized.

Attenuated cholinergic regulation of catecholaminergic transmission has been studied in a number of psychiatric disorders and novel therapeutics exhibiting pro-cholinergic effects have demonstrated efficacy in the treatment of several neuropsychiatric disorders. Dysfunctional activation of cholinergic inputs in dopamine regulation have been hypothesized to influence some of the movement deficits and negative or vegetative symptoms of psychiatric disorders such as schizophrenia and major depressive disorder (Laruelle et al., 2003; Lieberman et al., 2008). Modulation of cholinergic transmission by novel atypical antipsychotics have been implicated in the improvement of extrapyramidal symptoms, negative symptoms and cognitive impairment associated with schizophrenia (Gray and Roth, 2007). Additionally, several pro-cholinergic therapeutic agents increasing ACh transmission via inhibition of hydrolysis (Guillem et al., 2006) or direct acting agents targets specific muscarinic receptor subtypes (Langmead et al., 2008).

Murine Models of Cholinergic Dysfunction in Cardiovascular and Behavioral Disorders

Murine Models of Cholinergic Dysfunction in Cardiovascular Disorders

The murine cardiovascular system has received considerable attention due to the number of genetic manipulations and implantable telemetric devices available to study mammalian cardiovascular function *in vivo*. Several of the studies have examined the role of genetic manipulations on enzymes necessary for the biosynthesis of and degradation of neurotransmitters, transport proteins involved in uptake and recycling of neurotransmitters and receptor subtypes mediating the post-synaptic responses involved in autonomic function (Janssen and Smits, 2002). Other studies have been conducted examining the role of post-synaptic receptor signaling molecules or the ion channels mediating the autonomic stimulation (Gehrmann and Berul, 2000).

Within the sympathetic branch of the ANS, many of the studies examining the impact of nullizygous genotypes in enzymes involved in catecholamine biosynthetic pathways have provided limited cardiovascular information as many of these mice exhibited profound sympathetic deficits and die perinatally (Kobayashi et al., 1995; Thomas et al., 1998). Studies have also been conducted in mice deficient in catecholamine metabolism such as catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), however these mice have been evaluated primarily for their behavioral and neurological deficits due to elevated adrenergic tone (Cases et al., 1998; Grimsby et al., 1997). Although the cardiovascular phenotypes were not evaluated in these mice, there have been a number of studies linking stress response and cardiovascular disease (Farah et al., 2004; Keller et al., 2006).

The post-synaptic adrenergic receptors and their signaling molecules have been widely studied in the mouse. The adrenergic receptor family consists of three β-adrenergic receptor subtypes (β_1, β_2, β_3), three α_2-aderenergic subtypes (α_{2A}, α_{2B}, α_{2C}),

and three α_1-adrenergic subtypes (α_{1A}, α_{1B}, α_{1D}) (Lefkowitz, 1996). The α_1-receptors have been primarily studied with respect to the vasculature as these receptors mediate vasoconstriction (Lefkowitz, 1996). The α_2- and β-receptors have been studied within the CNS, renal vasculature and the heart (Janssen and Smits, 2002; Lefkowitz, 1996).

The primary receptors mediating the ANS regulation of the cardiovasculature are the β- and α-adrenergic receptors. Several transgenic mouse models of cardiovascular disease have been created from a variety of different mouse strains. The cardiovascular parameters of normal WT mice from the various mouse strains have been summarized in **Table 2**. Transgenic murine models of autonomic receptors have either included knockouts or overexpressors particularly focusing on β_1 and β_2, or α_2-adrenergic receptors in order to determine the impact of these receptor systems on the cardiovasculature. The α_{2A}-receptor subtype is highly expressed within the CNS and serves as the principle receptor mediating the central sympathoinhibitory effects of pharmacologic agonists such as clonidine resulting in hypotension and bradycardia (Hein et al., 1999; Makaritsis et al., 1999). Studies in α_{2A}-knockout mice, demonstrate elevated HR and increased plasma and tissue levels of NE indicating a hyperadrenergic state.

The β_1-adrenergic receptor subtype has been shown to mediate the chronotropic (heart rate) and ionotropic (contraction) of the heart, whereas the β_2-adrenergic receptor subtype has been shown to mediate vascular smooth muscle relaxation and to oppose the effects of β_1 over activation (Lefkowitz et al., 2000; Liggett et al., 2000). In studies conducted in β_1 and β_2 knockout mice, resting HR and BP were surprisingly similar to wildtype mice (Rohrer et al., 1999). In β_1-knockout mice, chronotropic activation with isoproterenol was blunted, however BRS reflex was preserved demonstrating that within

Table 2. Cardiovascular profiles in conscious, wild-type mouse strains.

Parameter	129/Sv	C57BL/6	FVB
HR (bpm)	571±13	692±5	736±5
MAP (mmHg)	145±6	110±2	76.9±2.5
SBP (mmHg)	115.7±8.7	120±2	110±4.9
DBP (mmHg)	72.5±6.1	99±2	70.8±4.5

Abbreviations for Table 2: Daytime (resting) heart rate (HR, measured beats/min (bpm)); mean arterial pressure (MAP, measured mmHg); systolic blood pressure (SBP); diastolic blood pressure (DBP). Adapted from Lopez O, et al. *BMC Physiology*, 2001.

the murine cardiovasculartur, the β_1-adrenergic receptor was responsible for mediating the chronotropic effects and that the parasympathetic nervous system was in tact as evidenced by unaltered BRS tone (Rohrer et al., 1998). However, in β_1/β_2- double knockout mice, mAChR receptor density was significantly reduced, possibly compensating for the loss of β_1-adrenergic tone to the heart (Rohrer et al., 1998) in an attempt to preserve cardiac output.

In contrast to β-adrenergic receptor nullizygous mice, overexpression of β-adrenergic receptors have produced some striking cardiovascular phenotypes. In mice with a 5-fold overexpression of the β_1-receptor, basal HR and contractile response was significantly enhanced, while HRV was significantly reduced (Engelhardt et al., 1999; Mansier et al., 1996) consistent with elevated sympathetic tone. However, as these mice aged (>10 weeks), β_1-ovexpressing mice exhibited marked deficits in cardiac function (reduced fractional shortening; evidence of reduced contractile efficiency) and associated ventricular hypertrophy (Engelhardt et al., 1999). Similarly, β_2-overexpressing mice exhibited increased basal cardiac function, however this effect appeared to be gene-dose related (Liggett et al., 2000). In mice with a 60-fold ovexpression of β_2, these mice exhibited an increase in cardiac function, however in mice with a 100-fold increase in β_2-expression, these mice develop a significant dilated cardiomyopathy and subsequent heart failure (Liggett et al., 2000). The role of β_2-adrenergic receptors mediating the inotropic support of the failing heart has also been identified in human models of heart failure (Bristow et al., 1986).

As noted above, the parasympathetic nervous system mediates the negative inotropic effects of heart rate regulation. The primary receptor subtypes mediating

cholinergic (parasympathetic) regulation of the cardiovasculature are the nicotinic acetylcholine (nAChR) and muscarinic acetylcholine (mAChR) receptors. Within the heart, the M_2AChR is the primary receptor and activation of this subtype results in a reduction in HR mediated by the muscarinic-gated potassium channel (I_{KACh}) (Gehrmann and Berul, 2000). Though the M_2AChR has been shown to be the principle muscarinic receptor subtype in the mammalian heart, and studies conducted in M_1AChR knockout mice show that these mice exhibit normal basal cardiovascular features (Hardouin et al., 2002). However, treatment of mice with a selective M_1 agonist (McN-A-343) failed to elicit increases in HR and MBP, indicating that the M_1AChR mediates sympathetic postganglionic neurons to release NE (Hardouin et al., 2002). Although the M_1AChR has not been shown to be expressed in the mammalian heart, this muscarinic receptor subtype has been shown to be involved in ANS regulation of cardiovascular function (Hardouin et al., 2002).

In M_2AChR knockout mice, basal HR and BP were similar to wildtype mice (LaCroix et al., 2008). However, bolus injection of the β-adrenergic agonist, isoproterenol (ISO) induced a significant increase in HR, and chronic administration of ISO resulted in significantly impaired ventricular function (LaCroix et al., 2008). Similarly, M_2AChR knockout mice were completely devoid of bradycardic activity during vagal nerve stimulation. Although reduced vagal tone has been associated with higher basal HR and contributes to the development of various life-threatening arrhythmias, M_2AChR knockout mice fail to demonstrate these effects (Fisher et al., 2004). This could be due the fact that mice tend to have lower vagal tone compared to larger mammals and are less susceptible to these abnormal cardiac conduction anomalies

(Fisher et al., 2004; Gehrmann and Berul, 2000). In addition to HF with chronic ISO stimulation, M_2AChR-/- mice also exhibited an increase in matrix metalloproteinase (MMP) activity, a protein associated with left ventricular dysfunction and hypertension (LaCroix et al., 2008). Excessive MMP causes loss of normal collagen function within the cardiac extracellular matrix and it has been hypothesized that in addition to lowering HR, M_2AChR stimulation also provides an inhibitory role on MMP via the ERK 1/2 signaling pathway (LaCroix et al., 2008).

Several transgenic mouse lines have been developed examining the role of the enzymes involved in the biosynthesis and degradation of ACh. In AChE-/- mice, a deficit in ACh metabolism produces a significant downregulation of M_1 and M_2AChRs within the CNS (Volpicelli-Daley et al., 2003). These changes are mirrored by a 60% increase in CHT, likely an effort to increase substrate availability for loss of post-synaptic cholinergic responses (Bazalakova et al., 2007; Volpicelli-Daley et al., 2003). Additionally, these mice also display reduced sensitivity to mAChR stimulation-induced behavior. However, none of these studies examined the impact of AChE loss on cardiovascular function. It could be hypothesized that if AChE nullizygous produces a downregulation of mAChRs within the CNS, loss of M_2AChRs within the heart would produce a hyperadrenergic state.

Within the ACh biosynthetic pathway, studies have been conducted in mice deficient for CHT, as well as the transporter responsible for providing substrate for ACh, (VAChT), and the enzyme choline acetyltransferase (ChAT), the enzyme responsible for conversion of choline and acetyl-CoA into ACh. ChAT-/- mice exhibit an inability to synthesize ACh and resulting in developmentally altered distribution of nAChRs at the

NMJ (Brandon et al., 2004). The post-synaptic nAChRs in ChAT-/- mice exhibit wider distribution in comparison to WT littermates, possibly in an effort increase cholinergic tone. The cardiovascular impact of a genetic loss of ChAT has not been examined in these transgenic mice (Brandon et al., 2004). Although cardiovascular function in ChAT-/- mice has not been directly examined, several studies have shown ChAT upregulation in the rostral ventrolateral medulla (RVLM) in animal models of hypertension (Lin and Li, 1990) indicating that a compensatory upregulation of the cholinergic system can occur during hypertension. These studies also showed an increase in central ACh content in hypertensive rats and has been hypothesized to contribute to the maintenance of hypertension in these rodent models (Kubo et al., 1995).

In both CHT-/- and CHT+/- mice, similar reductions in CNS levels of ACh have also been identified (Bazalakova et al., 2007; Ferguson et al., 2004). Similar to ChAT-/- mice, CHT-/- also show developmental alterations in nAChR distribution at the NMJ, however the overall pattern appears less severe (Ferguson et al., 2004). As noted above, CHT-/- mice have reduced stores of ACh and exhibit perinatal lethality, displaying cyanosis possibly due to respiratory failure, thus limiting further phenotype characterization (Ferguson et al., 2004). Thus, most of the studies examining the role of genetic loss of CHT have been conducted in CHT+/- mice (**Table 3**) (Bazalakova et al., 2007). Studies conducted in CHT+/- mice show reduced CNS tissue levels of ACh in addition to reductions in M_2AChRs (Bazalakova et al., 2007). The reduction in CNS M_2AChRs likely represents an attempt by cholinergic neurons to attenuate these autoinhibitory receptors to improve cholinergic tone. In the heart, reduction of M_2AChRs

Table 3. Biochemical and phenotypic characteristics of CHT+/- mice.

CHT expression	HACU-mediated choline uptake	ACh/Choline Levels	mAChRs	Phenotype	Reference
Reduced (50%) Whole brain Striatum Cortex Hippocampus Midbrain	Not changed	ACh (reduced) Cortex Striatum Hippocampus Choline (increased) Cortex Striatum	Increased Cortex Striatum	Hypersensitive to oxotremorine; HC-3 sensitive; Reduced treadmill performance	Ferguson et al. 2004; Bazalakova et al. 2004.
Reduced Atria	Reduced	ACh (reduced) Choline (reduced)	Increased	Tachycardic Hypertensive	English et al. submitted

Abbreviations for Table 3: High affinity choline uptake (HACU); acetylcholine (ACh); muscarinic acetylchoine receptors (mAChRs); hemicholinium-3 (HC-3). Adapted from Bazalakova M., et al. *Genes, Brain Behavior*, 2008.

would diminish the cholinergic tone within the heart, thus leading to unopposed sympathetic tone resulting in tachycardia and increased cardiac output.

Murine Models of Cholinergic Dysfunction in Behavioral Disorders

Cholinergic neurotransmission plays a significant role in mediating diverse and complex behavioral and neurologic functions within CNS and has been implicated in the cognitive impairments seen in schizophrenia and Alzheimer's disease, and to play a role in the pathology of other psychiatric disorders such as major depressive disorder and anxiety disorders (Coyle et al., 1983; File et al., 2000; Tandon, 1999; Vakalopoulos, 2007). Pharmacologic strategies to improve cognitive impairments in both schizophrenia and Alzheimer's disease have focused on increasing cholinergic neurotransmission (Friedman, 2004; Giacobini, 2000). Additionally, agents working to improve the attentional deficits in ADHD have also been shown to improve ACh transmission (Tzavara et al., 2006).

Utilizing various genetic approaches, numerous transgenic murine models with either overexpression or knockout of components regulating cholinergic transmission have been created to evaluate the impact on behavior and neurologic function. The coupling of changes in CHT-mediated HACU to increased turnover or release of ACh, in response to increased cholinergic demand, supports a critical role for CHT in cholinergically mediated behaviors (e.g.: attention, learning, memory) (Arnold et al., 2002). Additionally, the cellular localization of CHT to both an intracellular vesicular and plasma membrane pools permits the mobilization of CHT to meet increased demands for cholinergically mediated behaviors (Ferguson et al., 2003). And this may reveal itself under "challenge" such as sustained attention tasks.

Several genetically modified mouse models examining the impact of alterations in the ACh synthesis machinery have been examined behaviorally. Cholinergic signaling is terminated by the enzymatic hydrolysis of ACh by the enzyme AChE. Loss of AChE may produce enhanced ACh signaling which may lead to a hypercholinergic state possibly resulting in death. However, AChE-/- mice survive to adulthood, although they exhibit gastrointestinal difficulties and have shortened lifespans due to fatal seizures (Duysen et al., 2002; Xie et al., 2000). These mice also demonstrate resistance to pilocarpine-induced seizures during activation of M_1AChRs and resistance to oxotremorine-induced hypothermia and tremor compared to wild-type mice (Li et al., 2003). Although butyrylcholinesterase in AChE-/- mice was unchanged, [^{3}H]quinuclinyl benzilate ([^{3}H]-QNB) binding studies and immunoblotting for specific muscarinic AChR receptor subtypes demonstrated a 50 to 80% reduced expression of M_1, M_2 and M_4 receptors in cortex and hippocampal homogenates (Li et al., 2003; Volpicelli-Daley et al., 2003).

Interestingly, in addition to changes in post-synaptic muscarinic AChRs, immunoblotting for CHT reveals a 60% increase in CHT expression in striatal homogenates, without compensatory changes in ChAT activity or VAChT expression (Volpicelli-Daley et al., 2003). It has been hypothesized that the increase in CHT expression in AChE-/- mice may represent a compensatory change in the presynaptic neuron to recapture the choline-substrate in a cholinergic system that is lacking of ACh hydrolysis (Bazalakova and Blakely, 2006).

The observed behavioral phenotypes in AChE-/- mice compliment behavioral findings in CHT+/- mice, even though CHT+/- have a 50% reduction in CHT protein

compared to wildtype mice. CHT+/- mice exhibit a hyposensitivity to scopolamine challenge and are hyperresponsive to oxotremorine-induced seizures (Bazalakova et al., 2007). In addition, CHT+/- mice while exhibiting normal grooming and rearing behaviors, show significant exercise deficits on treadmill studies examining speed and endurance. These results point to both presynaptic and postsynaptic mechanisms that work synergistically to maintain extracellular ACh levels and regulate cholinergic signaling.

In contrast to AChE-/- mice, AChE transgenic (AChE-Tg) mice expressing human AChE show elevated AChE catalytic activity in synaptosomes from hippocampus, cortex and striatum, however the extracellular ACh concentration was similar between genotypes (Erb et al., 2001). Synaptosomal homogenates show similar increases in CHT-mediated HACU in the hippocampus, cortex and striatum of AChE-Tg mice (Erb et al., 2001). Similar to AChE-/- mice, AChE-Tg are resistant to muscarinic (oxotremorine)-induced hypothermia, but exhibit normal responses to scopolamine (Beeri et al., 1995). AChE-Tg mice also display normal motor behavior to familiar environments, but increased motor activity to novel environments and increased anxiety-related behaviors in elevated plus-maze tests, findings that are in contrast to those of CHT+/- mice (Bazalakova et al., 2007; Erb et al., 2001). These results show the compensatory increase of CHT-mediated HACU in the presence of a hypocholinergic state due to increased ACh hydrolysis in order to maintain cholinergic tone.

Other changes in CHT regulation have been shown in other models of cholinergic deficits in ChAT+/- and in the α3 nicotinic receptor knockout (α3-/-) mice showing CHT upregulation and downregulation respectively (Bazalakova et al., 2007; Krishnaswamy

and Cooper, 2009). The dynamic changes to CHT regulation due to changes in either pre- or post-synaptic cholinergic signaling mechanisms led to the characterization of CHT+/- mice. CHT knockout (CHT-/-) display a post-natal lethality phenotype and limit useful behavioral or physiologic characterization, however CHT heterozygous (CHT+/-) mice develop normally and exhibit normal lifespans compared to their wildtype littermates (Bazalakova et al., 2007; Ferguson et al., 2004). Initial behavioral characterization of CHT+/- mice showed similar performance to a variety of behavioral tasks including sensory-motor, motor coordination, overall locomotor activity, anxiety and spatial learning and memory tests (Bazalakova and Blakely, 2006; Bazalakova et al., 2007) (**Table 3**). These results led to the examination of behaviors, that require sustained cholinergic transmission with the hypothesis that CHT+/- mice would be unable to sustain releasable pools of ACh due to diminished capture of choline substrate. During physical challenge with treadmill, CHT+/- mice were unable to reach high speeds as those attained by CHT+/+ mice and also showed deficits in endurance (Bazalakova et al., 2007). CHT+/- mice also showed hyposensitivity to scopolamine-induced locomotion, reflected by a reduction in M_2AChRs in the striatum and cortex (Bazalakova et al., 2007). Although CHT+/- mice have 50% reduction in CHT protein and exhibit significantly reduced M_2AChR compared to wildtypes, ChAT and AChE activity was similar between both genotypes. These studies demonstrate that CHT heterozygosity results in adequate baseline stores of ACh capable of maintaining normal behavioral phenotypes, but that during sustained periods of increased cholinergic tone, CHT+/- mice are unable to maintain the pool of ACh and exhibit cholinergically-dependent phenotypes. Further studies in CHT+/- mice performing attentional or cognitive tasks dependent upon

sustained cholinergic tone are required to define the behavioral impact and may provide insights of the role of CHT in human cognitive disorders (Sarter and Parikh, 2005).

Significance

ACh was one of the first neurotransmitters discovered and plays a key role in modulating responses in both the central and peripheral nervous systems (Burnstock, 1979; Loewi, 1921). Within the CNS, ACh modulates complex behavioral and motor functions, although in the periphery, ACh modulates respiration, gastrointestinal motility and autonomic nervous system functions (Burnstock, 1979; Kasa, 1986). The choline transporter (CHT) serves as the rate-limiting step for the synthesis of ACh, and selective blockade of CHT by HC-3 reduces HACU and subsequent ACh synthesis and release (Apparsundaram et al., 2000; Maire and Wurtman, 1985). Studies conducted by our lab shows that CHT-mediated HACU is regulated by neuronal activity and that genetic disruptions of CHT produce phenotypic consequences *in vivo* (Ferguson et al., 2004; Simon et al., 1976).

The expirements conducted compliment previous studies conducted in the CHT+/- mice that focused primarily on CNS-mediated behaviors and extended them into the periphery examining the impact of CHT plasticity within the autonomic nervous system on the regulation of cardiac function. These studies provide an ability to examine cardiovascular dynamics and function in the intact organism and provide critical insights in the impact of cholinergic disruptions on cardiovascular health.

Additionally, the characterization of the CHT+/- mice may provide insight into a range of psychiatric disorders including cognitive disorders (ie. ADHD, Alzheimer's) (Potter et al., 2006; Tzavara et al., 2006), schizophrenia (Tandon, 1999) and Parkinsons's disease (Calabresi et al., 2006). Similarly these mice may also be useful in characterizing neurologic disorders such as multiple sclerosis (Nizri et al., 2007) and the non-neuronal

impacts of cholinergic neurotransmission on the immune system (Kawashima and Fujii, 2003a). A formal understanding of the regulators of cholinergic function can provide extremely useful insights into a wide range of physiologic functions and pathology.

Thesis Objectives

Our lab developed CHT-/- and CHT+/- mice, affording the opportunity to examine CHT contributions to physiology. The experiments conducted here examine CHT+/- and CHT+/+ animals utilizing biochemical, pharmacological, and surgical approaches to directly test the **central hypothesis: CHT is essential for ACh synthesis and release in response to sustained demands on cholinergic signaling, and in turn supports parasympathetic (vagal) regulation of the heart and CNS functions including cognition and behavior.** Three specific aims were pursued to test the central hypothesis:

Specific Aim 1. Determine the impact of genetic loss of CHT on cardiac HACU, CHT distribution, ACh levels and ACh/NE receptor expression and sensitivity. ***Hypothesis:*** *As the rate-limiting step of ACh synthesis, CHT is essential for cholinergically-supported parasympathetic (vagal) tone within the heart.* To test this hypothesis, I evaluated ACh/choline and NE/MHPG levels in cardiac tissues and used biochemical approaches to determine CHT activities, expression and muscarinic and adrenergic receptor levels in mouse heart.

Specific Aim 2. **Characterize the *in vivo* functional consequence and physiologic impact of genetic variation of CHT in mouse heart.** ***Hypothesis:*** *Reductions in the*

intracellular pool of CHT, the rate limiting step in the biosynthesis of ACh, will alter parasympathetic (vagal) tone of the heart, resulting in an inability to produce lower resting heart rates. To test this hypothesis, I surgically implanted telemetry devices to record HR and BP in conscious mice. To determine the impact on cardiac function, I used echocardiography and histology. I also performed exercise trials, baroreceptor reflex challenge and vagal nerve stimulation strategies to measure the impact of CHT loss on vagally driven bradycardic responses.

Specific Aim 3. **Determine the physiologic impact of known genetic variants within the human CHT gene in subjects with psychiatric and cardiovascular disorders.** ***Hypothesis:*** *Recently two variants within the choline transporter have been identified. One variant (Ile89Val) demonstrates a 50% reduction in* V_{max}*. Subjects carrying variants which affect CHT function may display symptom phenotypes associated with reduced cholinergic tone.* To test this hypothesis, I utilized the allelic-discrimination Taqman® genotyping assay to determine associations of the Ile89Val (rs1013940) and the 3'SNP (rs333999) variants in healthy controls and subjects diagnosed with various cardiovascular and psychiatric disorders.

CHAPTER II

CARDIOVASCULAR EFFECTS OF CHT HETEROZYGOSITY – MOLECULAR ALTERATIONS AND INITIAL PHENOTYPIC FINDINGS

Introduction

Acetylcholine (ACh) serves as an important neurotransmitter in the autonomic nervous system (ANS) serving as the sole neurotransmitter at the pre-ganglionic sites for both branches of the ANS, the sympathetic (SNS) and parasympathetic (PNS) nervous system activating nicotinic acetylcholine receptors (nAChRs) on the post-ganglionic neuron (Lefkowitz, 1996). Additionally, the parasympathetic nervous system uses ACh at the post-ganglionic site acting on multiple subtypes of muscarinic acetylcholine receptors (mAChRs) regulating such diverse functions such as heart rate, respiration, gastrointestinal motility and secretions and urinary functions (Caulfield, 1993; Lefkowitz, 1996). Within the heart, cholinergic terminals from the vagus nerve synapse directly onto nAChRs containing intrinsic cardiac ganglia and cause post-ganglionic ACh release, resulting in activation of mAChRs within the sinoatrial (SA) and atrioventricular (AV) nodal regions of the myocardium (Caulfield, 1993; Hancock et al., 1987; Szabo and Otero, 1989). Muscarinic antagonists such as atropine and scopolamine produce tachycardia by permitting sympathetic nervous system predominance at the SA and AV nodes, while muscarinic agonists such as bethanechol result in a bradycardic effect (Dhein et al., 2001; Loffelholz and Pappano, 1985).

The predominant mAChR in the mammalian heart is the muscarinic-2 subtype (M_2AChR), which exhibits regional differences with the majority of mAChRs being

localized to the atria and nodal tissue versus the ventricular myocardium (Brodde et al., 2001; Peralta et al., 1987). However recent evidence has suggested the presence of M_1- and M_3AChR receptor subtypes have been identified in ventricular cardiomyocytes, with M_1AChRs having been shown to enhance I_{Ca} currents and M_1AChRs demonstrating effects on I_{KM3} currents, producing both ionotropic and chronotropic effects on the myocardium (Dhein et al., 2001).

Although additional muscarinic ACh-receptor subtypes have been identified within cardiomyocytes, the M_2AChR serves as the predominant pre- and post-junctional muscarinic receptor subtype regulating the chrontopic effects of parasympathetic tone (Peralta et al., 1987). Several animal studies have shown the primary contribution of M_2AChRs in regulating the bradycardic effects of HR regulation. In atria from M_2AChR receptor knockout mice, stimulation with the M_2AChR agonist carbachol, failed to illicit a bradycardic effect compared to wildtype mice (Gomeza et al., 1999). Walker *et al*. investigating the role of cardiac role G-protein receptor kinase (GRK), found that GRK3 knockout mice demonstrated similar carbachol-induced bradycardia to wildtype mice, however the baroreceptor reflex and HR recovery was significantly enhanced in these mice (Walker et al., 1999).

It is well known that HRV diminishes with age and that this reduction in overall HRV is due to the reduction in the HF component mediated by the parasympathetic nervous system (Pieper, 1995; Stein and Kleiger, 1999). Studies examining the molecular mechanisms to this reduction in cholinergic tone have focused predominantly on the M_2AChR and its signaling mechanisms. In human studies, carbachol-induced inhibition of ACh release (as mediated by M_2AChR autoreceptors) decreases with age

(Oberhauser et al., 2001). Similarly, several studies have demonstrated an age-dependent association in the decrease in M_2AChR densities in heart atrium (Brodde et al., 1998; Poller et al., 1997). Conversely, in chronic heart failure, there is a substantial increase in the activity of G_i-protein activity and possible enhancement of M_2AChR density possibly contributing to a salvaging mechanism by the parasympathetic nervous system to prevent further damage in the failing heart (Brodde and Leineweber, 2004; LaCroix et al., 2008). Additionally, the enzyme responsible for ACh degradation AChE, was found to be reduced in patients with HF (Dunlap et al., 2003). These data support the importance of cholinergic signaling mechanisms in regulation of cardiovascular function.

Few studies have examined the role of HACU in the heart. Previous animal studies have identified a HC-3 sensitive process that limits vagal ACh production and that the administration of HC-3 demonstrated failure of vagal control of heart atrium (Lewartowski and Bielecki, 1963; Vincenzi and West, 1966). Lindmar, *et al*., demonstrated that electrical stimulation of chick heart explants led to a delayed increase in choline uptake (Lindmar et al., 1980). Similarly, Na^+-dependent, high-affinity, HC-3 sensitive [^{3}H]choline uptake was identified in isolated rat atrium, whereby chronic depolarization of the isolated atrium led to a compensatory upregulation of ACh synthesis, however whether this increase in ACh synthesis was coupled to an increase in CHT was not elucidated (Wetzel and Brown, 1983).

Since CHT-mediated HACU is believed to serve as the rate limiting step for the biosynthesis of ACh by providing critical precursor support, especially during high rates of cholinergic signaling, it is predicted that genetic perturbation of CHT function would limit cholinergic tone (Ferguson and Blakely, 2004; Simon and Kuhar, 1975). In the

heart, both sympathetic and parasympathetic preganglionic neurons utilize ACh as their key neurotransmitter and disruption of CHT could affect both autonomic branches. However since the parasympathetic system also uses ACh as its postganglionic neurotransmitter, we predict that disruption of CHT would produce a larger impact on parasympathetic tone via vagal innervation to the heart, leaving sympathetic tone unabated and possibly resulting in cardiovascular dysfunction.

In the experiments described below, I sought to determine the impact of CHT heterozygosity on the parasympathetic (vagal) regulation of the heart at the molecular level and to identify potential cardiovascular phenotypes with associated with reduced vagal tone.

Methods

Drugs

(±)-metoprolol (+)-tartrate (M-5391) was obtained from Sigma Aldrich (St. Louis, MO, USA) and dissolved in sterile saline (0.9% NaCl). Both drugs were injected intraperitoneally (i.p.) at a volume of 2 mg/kg. Isoflurane, USP (Terrell™) was obtained from RxElite (Meridian, ID, USA) and used as a general anesthetic mixed at 1-3% with 100% O_2.

Mice

All animal procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee (Protocol # M/04/075). Male mice (4-6 months old, young) were housed up to 5 per cage on a 12:12-h light/dark cycle (lights on at 0600h). Telemetry and cardiovascular experiments were performed during the light part of the cycle. Food (Purina Rodent Chow #5001) and water were provided *ad libitum*. All mice were back-crossed at least seven generations to the C57BL/6 background. In all cases, CHT+/+ littermates were used as controls. All cardiovascular experiments were performed in the laboratory of Dr. David Robertson of the Autonomic Dysfunction Center, Vanderbilt University.

Analysis of choline transporter activity

Crude atrial extracts from hearts of adult, male mice (n=4 mice/genotype) were prepared as previously described (Ferguson et al., 2003; Lindmar et al., 1980). Assays of choline transport activity in heart atrial tissue were performed in triplicate for 5 min at

37°C in Krebs Ringer's HEPES buffer (KRH: 130mM NaCl/3mM KCl/2.2 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM KH_2PO_4/10 mM glucose/10 mM HEPES, pH 7.4) with a final choline concentration of 100 nM (specific activity: 82 Ci/mmol, Amersham Pharmacia; 1 Ci = 37 GBq). HC-3 at 10 μM was used to define CHT-mediated choline uptake. Uptake assays were terminated by aspiration and washing onto polyethyleneimine-coated glass fiber filters with a Brandel (Gaithersburg, MD) cell harvester. The low yield of tissue from the heart precluded analysis of saturation kinetics in these samples.

Immunoblot analysis of CHT and mAChR expression

Freshly dissected heart atrial tissue (n=4 mice/genotype) were first homogenized in 0.32M sucrose+HEPES buffer, then vortexed at 3650 x g at 4°C for 20 min. The pellet was solubilized in 200 μl for 24 hours at 4°C in lysis buffer (1.0% Triton, 0.1% SDS, 50 mM Tris pH=7.4, 100 mM NaCl and protease inhibitors). Insoluble material was removed by centrifugation at 15,000*xg*. Protein concentration was measured and normalized using the Bradford method, and samples loaded onto SDS-PAGE 1X Laemmali buffer (1% SDS, Tris 31.25 mM, pH 6.8, 5% glycerol, 200 mM 2-mercaptoethanol). Samples were then removed and centrifuged at 13,000 rpm at 4°C for 20 min to remove cellular debris. Samples were then normalized for protein concentration using the Bradford method and resolved by standard SDS-PAGE, and transferred electrophoretically to polyvinylidene diflouride (PVDF) membranes (Amersham Biosciences, Arlington Heights, IL) followed standard procedures (Ferguson et al., 2003). Analysis of CHT, GAPDH and the M_2AChR proteins from a single PVDF

membrane was performed after stripping of blots between incubations with 2% SDS, Tris-HCl 62.5 mM, pH=8, and 2-mercaptoethanol 100 mM at 55°C for 20 min. After washing with PBS-T, blots were then blocked in 5% milk PBS-T before analysis with the next antibody.

ACh and choline levels

ACh levels in heart tissue were quantified by high-performance liquid chromatography using electrochemical detection (CHT+/+, n=11; CHT+/-, n=10) (Vanderbilt Neurochemistry Core Resource) as previously described (Damsma et al., 1985). Briefly, animals were decapitated and microwaved for 5 seconds to inactivate AChE degradation of ACh (Bertrand et al., 1994). Hearts were then quickly removed and placed onto dry ice. Heart samples were then homogenized in acetonitrile, and lipids removed using heptane and vacuum drying.

Catecholamine levels

Catecholamines (NE, Epi and metabolites) were measured in urine using spot collection from conscious adult male mice. Resting mice were immediately removed from their home cages and urine collected in microtubes. Urine was preserved in 6N HCl to prevent catechol breakdown and frozen at -80°C until analysis. Catecholamines were measured after alumina extraction by high-performance liquid chromatography with electrochemical detection (Keller et al., 2006).

Surgical placement of telemetric electrocardiogram transmitters

All cardiovascular studies were performed in collaboration with the laboratory of David Robertson, MD at Vanderbilt University, with murine surgical assistance provided by Martin Appalsamy and experimental support provided by Nancy Keller.

For long-term, ambulatory electrocardiogram (ECG) or blood pressure (BP) monitoring in conscious mice, telemetry devices (model TA10ETA-F20, ECG or TA10-C20, BP; DataSciences International, St. Paul, MN) (**Figure 12A**) were implanted using sterile technique. Mice were anesthetized with isoflurane 1% in 100% O_2 at 1.5 L/min and body temperature maintained at 36-37°C with an isothermal pad (Braintree Scientific, Inc., Braintree, MA, USA). Following antiseptic preparation, a midline incision was made subcutaneously along the back. For ECG determination, an implantable, radio-frequency transmitter (TA10ETA-F20; 3.9g) was inserted into the subcutaneous pocket with leads directed caudally. Using a trochar, the cathodal lead was placed over the scapula and anchored in place with permanent suture. Another incision was made subcutaneously over the apex of the heart, through which using the trochar, the anodal lead was tunneled underneath the left front paw and sutured in place over the heart apex. For BP determination, an implantable, transmitter (TA10-C20; 3.2g) was inserted into a subcutaneous pocket with the lead placed into the left carotid artery and advanced toward the bifurcation. Skin was sutured and secured with veterinary adhesive (Nexaband, Veterinary Products Laboratories, Phoenix, AZ, USA). Mice were allowed to recover for 5 days before use in experimental protocols.

<u>Electrocardiogram (ECG) and blood pressure (BP) recordings</u>

After a 5-day post-operative recovery period, 24-hour continuous ECG recordings of HR were performed in telemeterized mice (CHT+/+, n=8; CHT+/-, n=8) for a period of 5 days. Average HR and BP values were determined in CHT+/+ and CHT+/- mice during resting (light cycle) and awake (dark cycle) time periods. ECG and BP signals were recorded in 1-second intervals using flatbed radio-frequency receivers (DSI PhysioTel, Receiver RPC-1, DataScience International, St Paul, MN) and a digital acquisition system (Dataquest A.R.T., Data Sciences International, St Paul, MN). To determine the intrinsic HR, mice (n= 5/genotype) underwent challenge of metoprolol 6mg/kg i.p. and methscopolamine 2mg/kg s.q. simultaneously. Baseline HR was recorded 2 hours prior to administration of drug and for 2 hours post-administration of drugs. Pharmacologic studies were performed in conscious, telemeterized mice (CHT+/+, n=6; CHT+/-, n=6) by giving metoprolol 2 mg/kg by i.p. injection.

Statistical analysis

Data are expressed as mean ± S.E.M. Statistical comparisons were with one-tailed, unpaired Student's *t*-test with 95% confidence limits comparing transgenic values to controls, or one-way repeated measures ANOVA followed by the Bonferroni procedure for multiple group comparisons as indicated in the figure legends. The results were considered statistically significant if $P<0.05$. The specific statistical tests used are noted in the text and legends with respect to individual test design.

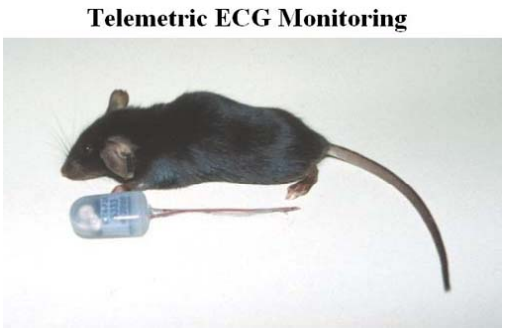
Results

CHT Hemizygosity Results in Elevated Heart Rate and Blood Pressure

Realizing that deficits in CHT could affect both branches of the autonomic nervous system given that both preganglionic neurons utilize ACh, we hypothesized that since the parasympathetic branch utilizes ACh at both pre-and postganglionic neurons, that deficits in CHT+/- mice would be due to reductions in PNS tone. Therefore, we predicted that CHT+/- mice would exhibit reduced vagal regulation of HR. Previous examination of subcutaneous heart rate recordings in anesthetized neonatal CHT+/- and CHT-/- mice demonstrated two distinct phenotypes (**Figure 12B**): 1. CHT+/- mice exhibited higher heart rates (220±22 bpm, n=4) when compared to WT neonatal mice. 2. CHT-/- mice exhibited significantly lower HR (80±15 bpm, n=14) compared to WT mice (Mihaela Bazalakova, unpublished data). The finding of tachycardia in CHT+/- was expected given our original hypothesis that loss of CHT could produce a reduction in vagal tone, however the finding of bradycardia in CHT-/- mice was unexpected. However, since CHT-/- mice appear anoxic at birth, many other factors impacting overall viability such as the reduction in respirations may impact HR (Ferguson et al., 2004).

In order to avoid complications of anoxia and neonatal lethality in CHT-/- mice, we focused on CHT+/- mice for the rest of our experiments. Earlier HR recordings in CHT+/- mice although showing elevated HRs compared to WT, were done under isoflurane anesthesia. While isoflurane is reported to have the least cardiogenic effects (Rottman et al., 2003), our early experiments showed that isoflurane produced a

A



B

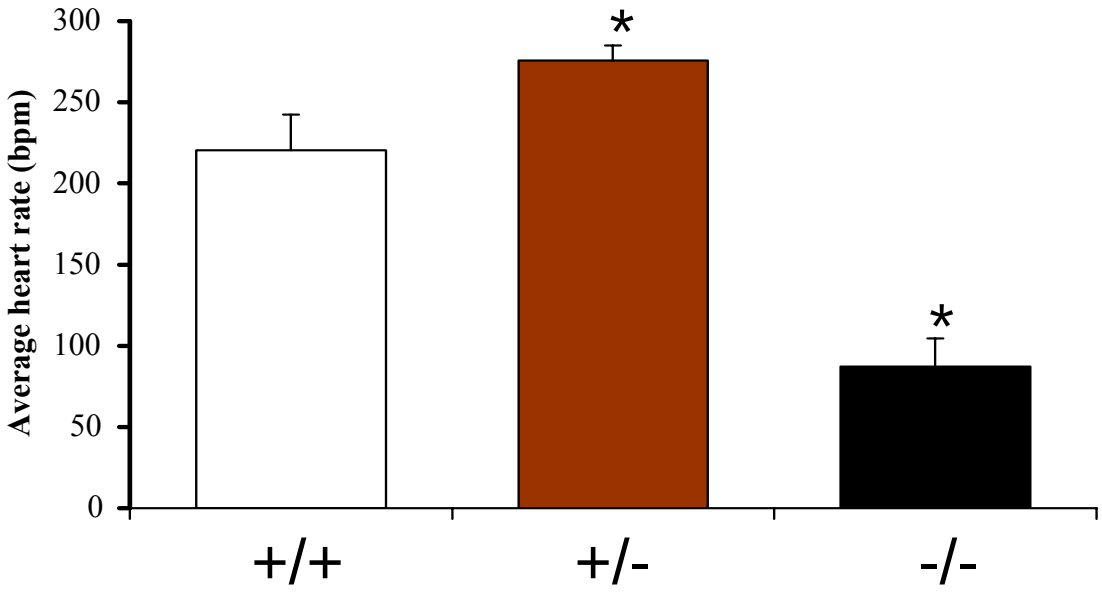


Figure 12. Initial HR recordings in CHT+/- and CHT-/- mice. (A) DSI telemetric device for ECG recordings in mice. **(B)** ECG recordings were obtained in unaesthetized newborns, using a lead II position Gould Amplifier and WinDaq Acquisition System. Mean heart rate values were 276±9 bpm in the CHT+/- (n=6), 220±22 bpm in the CHT+/+ (n=4), and 80±15 bpm in the CHT-/- (n=12) * $P<0.05$, two-tailed Student's t-test (values ±SEM). (Data courtesy of Mihaela Bazalakova, MD, PhD)

reduction in overall HR in our CHT+/- mice (Brett English; unpublished data). In order to characterize more accurate heart rate and blood pressure measurements, we implanted ECG (TA-10) and blood pressure (PA-20) telemetry devices into CHT+/+ and CHT+/- mice allowing us to characterize HR, mean, systolic and diastolic blood pressures in conscious, freely-moving animals.

Recording of resting and activity-dependent heart rates were measured in conscious, freely-moving, chronically telemeterized mice over a 24 hour period for 5 days. CHT+/- mice exhibited significantly higher mean resting (Day) HR (CHT+/+, 538.8±8.2; CHT+/-, 567±8.1; P=0.017) compared to CHT+/+ mice (**Table 4 and Figure 13A**). There were no significant genotype differences exhibited in HR during the active period (Nighttime) in either genotypes (**Figure 13A**). As a measure of the ability of the PNS to reduce the HR to a resting level, we measured the change in HR over a 2-hour period from the maximal HR achieved during increased motor activity, to its return during rest. Over the 2 hour monitoring period, both CHT+/+ and CHT+/- mice reduced HR by similar levels, however at the end of the 2 hour period, CHT+/- mice continued to have significantly elevated HR compared to CHT+/+ mice.

CHT+/- mice also exhibited significantly higher mean, resting (Day) blood pressures (CHT+/+, 98.20±1.2; CHT+/-, 104.9±2.1 mmHg; P<0.05) compared to CHT+/+ mice (**Figure 13B**). In CHT+/- mice, the elevation in mean resting BP was driven by significant increases in systolic BP between the two genotypes (**Figure 13C**). As with HR, activity-associated BP was similar between genotypes (data not shown). Evaluation of sympathetic tone using metoprolol (2mg/kg i.p.) showed that CHT+/- mice displayed significantly blunted response (CHT+/+, -166.0±36.5; CHT+/-, -66.8±22.23

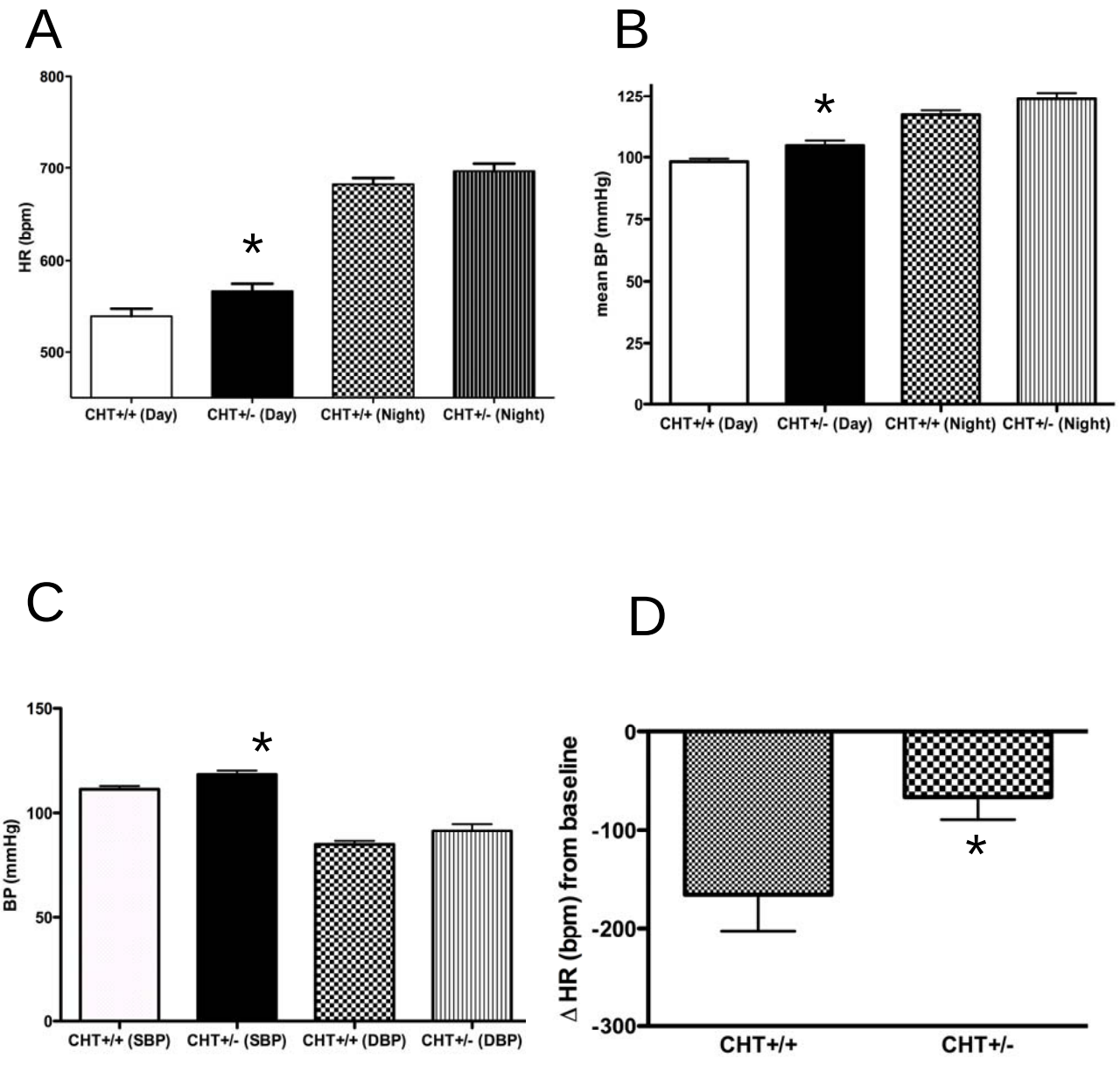


Fig. 13. CHT+/- mice show elevated HR and BP, and display reduced sensitivity to β-adrenergic receptor blockade. (A) CHT+/- mice display significantly elevated resting (day) HRs vs. CHT+/+ mice (**P*<0.01), while activity (night) HRs are similar. **(B)** Similarly, CHT+/- mice exhibit elevated resting mean BP (mBP) vs. CHT+/+ mice (**P*<0.05). **(C)** Increase in resting mBP is due to increased systolic BP (SBP) in CHT+/- mice (**P*<0.03). **(D)** Reduced sensitivity to metoprolol 2 mg/kg i.p. is exhibited in CHT+/- mice vs. CHT+/+ *(*P*<0.02). (CHT+/+, n=7-8; CHT+/-, n=7-8). Significance as determined by one-tailed, unpaired Student's *t*-test, Values represent ±SEM.

Table 4. Cardiovascular parameters in CHT+/+ and CHT+/- mice.

Parameter	CHT+/+	CHT+/-	*P*-value
HR (Day) (n=8)	538.8±8.16	567.0±8.12*	0.0172
HR (Night) (n=8)	682.6±6.50	696.6±8.50	0.1951
Mean BP (Day) (n=7)	98.20±1.18	104.9±2.06*	0.035
Systolic BP (n=7)	111.0±1.48	118.4±1.90*	0.035
Diastolic BP (n=7)	84.90±1.63	91.54±3.23	0.181
ΔHR w/metoprolol 2mg/kg i.p. (n=8)	-166.0±36.57	-66.83±22.23*	0.022

Abbreviations for Table 4: Heart rate (HR); blood pressure (BPM); intraperitoneal (i.p.). * denotes *P*-value CHT+/+ vs CHT+/- as shown in table.

mmHg, $P<0.05$) to β-adrenergic blockade (change in HR from baseline) compared to CHT+/+ mice (**Figure 13D)**.

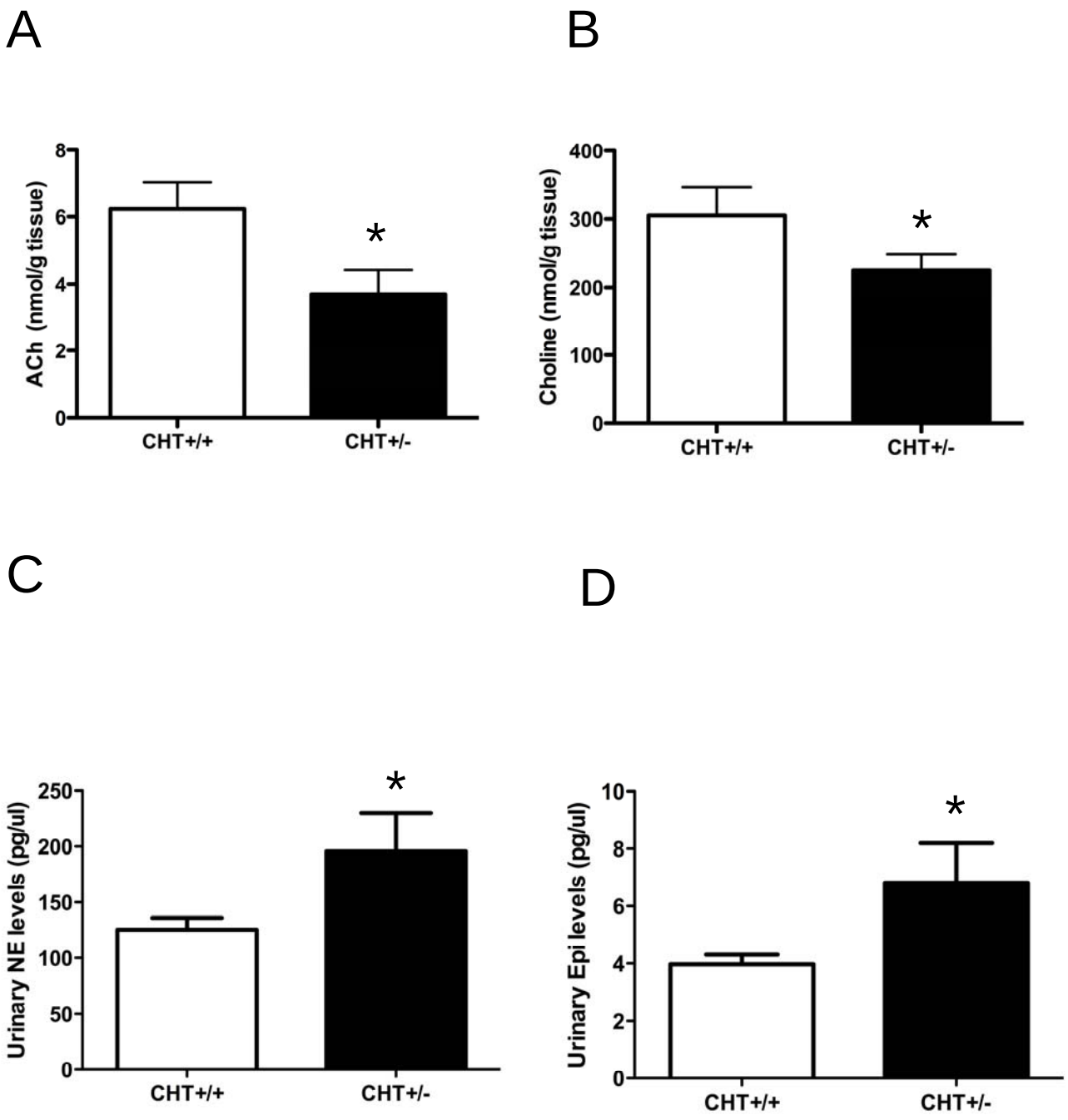
Loss of Cardiac CHT Results in Reduced Tissue Levels of ACh and Choline

Reductions in CHT-mediated choline uptake and expression of CHT could possibly result in decreases in availability of ACh within intracardiac ganglia. Previous studies had shown that CHT+/- mice exhibited a 50% reduction in the expression of CHT and subsequent reduction in tissue levels of ACh within mouse brain. Similar to observations in CHT+/- mouse brain, CHT+/- mice whole heart tissue levels of ACh were significantly lower (CHT+/+, 6.2±0.8; CHT+/-, 3.7±0.7 nmol/g wet tissue; $P=0.028$) compared to CHT+/+ mice (**Figure 14A**). Unlike that found in CHT+/- mouse brain tissue levels of choline were reduced to CHT+/+ mice (CHT+/+, 305.2±41.6; CHT+/-, 225.3±23.0 nmol/g wet tissue; $P=0.05$) (**Figure 14B**).

CHT+/- Mice Exhibit Increased Cardiac Tissue NE and MHPG Levels

Reduced parasympathetic tone has been shown to contribute to autonomic instability leading to an increased sympathetic tone (Palatini and Julius, 2004). To test this hypothesis, we collected urine from conscious, mice (CHT+/+, n=9; CHT+/-, n=9) during their resting period and measured catecholamine levels. CHT+/- mice displayed elevated urinary levels of norepinephrine (NE) (CHT+/+, 125±10.3; CHT+/-, 195.8±34.5 pg/μl; $P=0.05$) and epinephrine (EPI) (CHT+/+, 3.9±0.3; CHT+/- 6.8±1.3 pg/μl; $P=0.05$) compared to CHT+/+ mice (**Figure 14C-D**).

Fig. 14. Cardiac tissue levels of ACh and choline are reduced, and NE/Epi are elevated in CHT+/- mice. (A) Tissue ACh levels measured by high-performance liquid chromatography are significantly lower in whole hearts of CHT+/- mice. **(B)** Tissue levels of choline are lower in CHT+/- vs CHT+/+ mice. **(C)** Urinary levels of NE and Epi **(D)** are elevated in CHT+/- mice compared to CHT+/+. (CHT+/+, n=9; CHT+/-, n=9). Significance as determined by one-tailed, unpaired Student's *t*-test, **P*<0.05, n=9/genotype. Values represent ± S.E.M.


A
ACh (nmol/g tissue)
8
6
4
2
0
*
CHT+/+
CHT+/-
B
Choline (nmol/g tissue)
400
300
200
100
0
*
CHT+/+
CHT+/-
C
Urinary NE levels (pg/ul)
250
200
150
100
50
0
*
CHT+/+
CHT+/-
D
Urinary Epi levels (pg/ul)
10
8
6
4
2
0
*
CHT+/+
CHT+/-

CHT+/- Mice Exhibit Altered Cardiac CHT-mediated HACU, CHT and M_2AChR Expression in Atrial Tissue

To test whether altered CHT expression or function contributed to the reduced cardiac tissue levels of ACh, we measured CHT-mediated HACU and CHT expression in cardiac atrial tissue preparations. Isolated atria from CHT+/- mice displayed reduced HC-3 sensitive [^{3}H]choline transport rates compared to CHT+/+ atria **(Figure 15A)**. These reductions in HACU were supported by a reduction in CHT protein expression (**Figure 15B and C**) in CHT+/- mice compared to CHT+/+ mice. Quantitation of post-synaptic M_2AChRs in the same samples did not reveal significant differences in receptor expression between genotypes (**Figure 15D**).

Discussion

Previous studies have identified the HC-3-sensitive, Na^+/Cl^--dependent, high-affinity choline uptake mechanism as the rate-limiting step for ACh biosynthesis (Simon et al., 1976). Several murine models with genetically-modified cholinergic deficits have provided evidence for the critical role that CHT plays in modulating cholinergic neurotransmission. For example, in AChE-/- mice, expression of CHT is increased by 60%, which may reflect an attempt by the presynaptic terminal to increase capture of the choline substrate due to its lack of production by ACh hydrolysis (Volpicelli-Daley et al., 2003). Previous studies conducted by our lab have shown that availability of CHT is necessary for the production of ACh and that CHT-/- mice exhibit a perinatal lethality phenotype (Ferguson et al., 2004). In contrast, CHT+/- (hemizygous) mice appear normal compared to CHT+/+ mice, displaying normal lifespans, reproduction capability

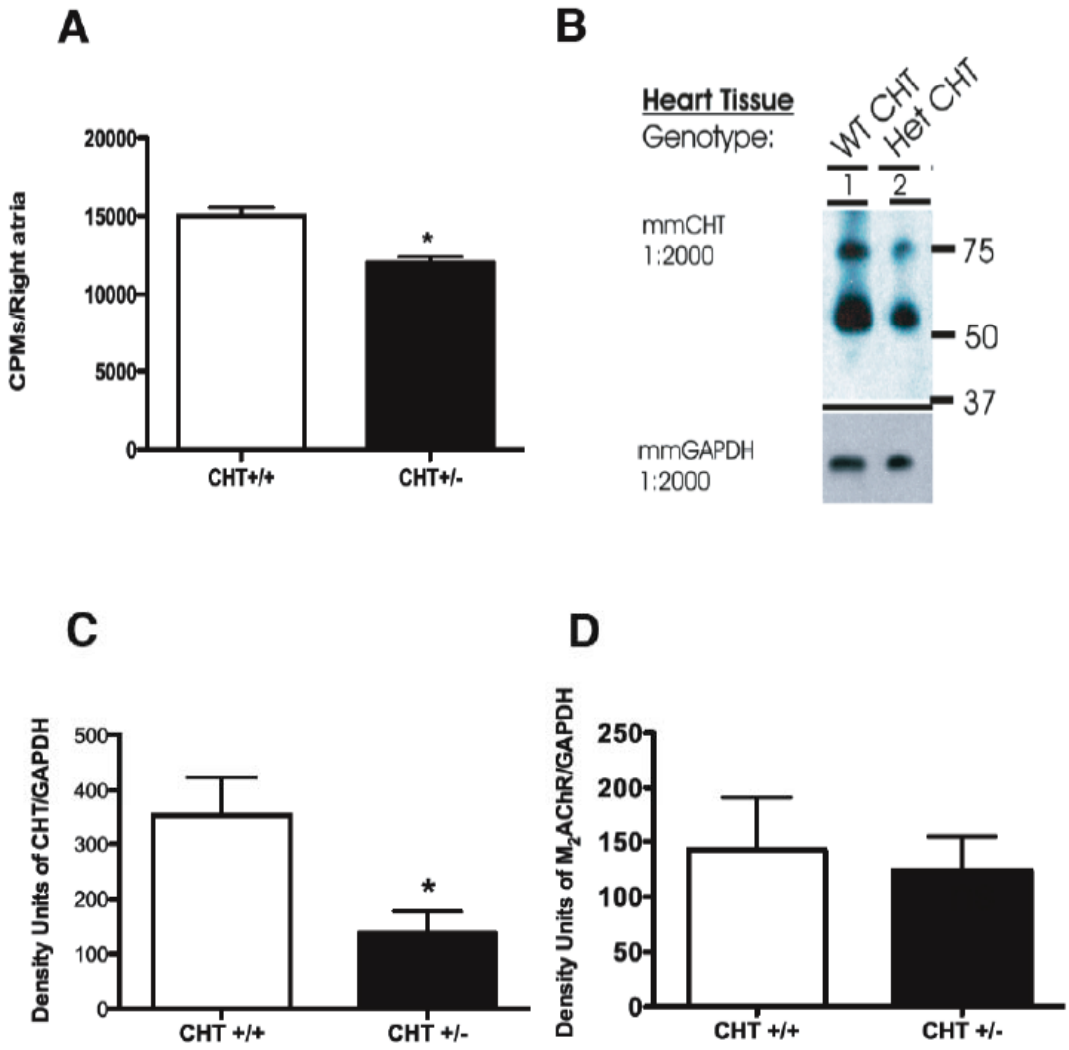


Fig. 15. CHT+/- mice display reduced cardiac CHT-mediated HACU and CHT expression. (A) In cardiac atrial preparations, CHT+/- show diminished HC-3 sensitive, [^{3}H]choline uptake compared to CHT+/+ atria. **(B)** CHT expression is significantly reduced in CHT+/- atria compared to CHT+/+, quantitated in **(C)**. **(D)** Expression of cardiac M_2AChRs is similar between genotypes. CHT protein quantitation is shown below. Significance as determined by one-tailed, unpaired Student's *t*-test, **P*<0.05, n=4/genotype. Values represent ± S.E.M.

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and sensory-motor behaviors (Bazalakova et al., 2007). However, while CHT+/- mice appear normal when compared to CHT+/+ mice, detailed examination of specific behaviors, which depend largely upon sustained cholinergic neurotransmission, CHT+/- mice show significant phenotypes consistent with attenuated cholinergic tone. For example, CHT+/- mice exhibit deficits in sustained treadmill motor tasks and pharmacologically-mediated hyperlocomotive behaviors (Bazalakova and Blakely, 2006; Bazalakova et al., 2007). Additional studies conducted in rats subjected to cognitive vigilance tasks, a cholinergically-mediated attention-performance task, exhibited increases in CHT-mediated HACU compared to controls (Apparsundaram et al., 2005). These results provided evidence that cholinergically-mediated behaviors requiring sustained ACh transmission depended upon availability of CHT and that disruptions of CHT-mediated HACU may possibly produce phenotypes consistent with attenuated cholinergic tone.

In the periphery, the parasympathetic nervous system plays a critical role in regulating many of the vegetative physiologic processes (Lefkowitz, 1996). Activation of parasympathetic inputs via vagal efferent projections to the heart results in bradycardia and contributes to resting HR (Fox et al., 2007; Lefkowitz, 1996). Genetically-modified mice with deficits in ACh biosynthesis machinery such as ChAT or AChE, exhibiting hypocholinergic states, have not been studied with respect to their cardiovascular phenotypes. In contrast, murine models featuring genetic deficits in postsynaptic cholinergic signaling mechanisms have been examined for their impact on cardiovascular regulation. For example, studies conducted in M_2AChR knockout mice show loss of vagally mediated bradycardia (Fisher et al., 2004). Additionally thought M_2AChR KO

mice display similar resting HRs compared to wildtype mice, chronic stimulation with isoproterenol in M_2AChR KO mice results in significantly impaired ventricular function (LaCroix et al., 2008). Given the results of increased CHT-mediated HACU in maintaining cholinergically-mediated behaviors, and that CHT+/- mice exhibited deficits in motor function tests, we hypothesized that CHT+/- may display altered vagal regulation of the cardiovasculature.

Unlike the diffuse cholinergic innervation within the CNS, cholinergic innervation within the heart is limited to the intrinsic cardiac ganglia (ICG) and contain the postganglionic parasympathetic neurons which provide cholinergic innervation to the cardiac myocytes (Hoard et al., 2008). These ganglia are localized within the atrial epicardium and receive cholinergic input from preganglionic vagal efferent neurons located in the medulla (Hoard et al., 2008; Parsons et al., 1987). Expression of CHT has been demonstrated within guinea pig and adult mouse heart, but localized primarily to the atrial nodal tissue and the atrium, with little expression within the ventricles (Hoover et al., 2003; Mabe AM, 2006).

Alterations in total ACh and choline levels have been observed in several human disorders and in transgenic murine models of Alzheimer's Disease (Bales et al., 2006). Similarly, CHT+/- mice show significantly reduced tissue levels of ACh within the cortex, hippocampus and striatum (Bazalakova et al., 2007). Additionally, CHT+/- mice show increased choline levels within the same brain regions and likely represent a compensatory attempt by additional choline uptake mechanisms to enrich choline supply for ACh synthesis (Bazalakova et al., 2007). Possible compensatory mechanisms could include upregulation of the low affinity, Na^+-independent choline uptake which has been

identified in rodent astrocytes (Inazu et al., 2005). Given these results and the expression patterns of CHT, we hypothesized that CHT+/- mice may have reduced tissue levels of ACh within cardiac atria. CHT+/- mice exhibited significantly reduced tissue ACh levels, consistent with similar observations found within various regions within CHT+/- brain **(Figure 14A**). However unlike that found in CNS, CHT+/- mouse heart had reduced tissue levels of choline compared to CHT+/+ mice (**Figure 14B**). These observed differences may reflect the limited innervation of cholinergic neurons into the heart, whereas the changes within the CNS may represent compensatory attempts by non-neuronal cells to increase tissue choline levels to help sustain ACh synthesis (Loffelholz and Pappano, 1985). Similarly, the differences in tissue levels of choline in the mouse heart may also reflect limited expression of additional choline uptake mechanisms that can support ACh synthesis, since lower order mammals have been shown to rely more upon elevated sympathetic tone in cardiovascular regulation (Fisher et al., 2004; Wickman et al., 1998). Lastly, the reduced ACh and choline levels may reflect changes in the redistribution of vesicular pools of ACh, where readily-releasable pools in CHT+/- mice are similar to CHT+/+, but that reserve pools are compromised (Birks and Macintosh, 1961). Preliminary studies measuring [^{3}H]ACh levels in brain slices after uptake show that CHT+/- mice have normal basal [^{3}H]ACh levels which may reflect stability of readily releasable vesicular pools (D. Lund, M. Bazalakova; unpublished data).

In the context of reduced cardiac tissue levels of ACh in CHT+/- mice and the role of the vagal feedback mechanisms on sympathetic tone, we sought to determine the level of circulating catecholamines in CHT+/- mice. In addition to the direct effects of

vagal signaling to the heart, activation of vagal efferents also results in a presynaptic blunting of sympathetic outflow, mediated by M_2AChR receptors (Lefkowitz, 1996). Reduced parasympathetic tone has also been shown to contribute to autonomic instability leading to an increased sympathetic tone (Palatini and Julius, 2004). We found that CHT+/- mice displayed elevated urinary levels of both NE and EPI compared to CHT+/+ mice (**Figure 14C, D**). Elevation in CHT+/- urinary catecholamines could reflect changes in M_2AChR expression, as activation of pre-synaptic cholinergic inputs to the medulla, blunts sympathetic outflow. Functional compensation within the CNS of CHT+/- mice have been shown, with decreased expression of M_2AChRs in the striatum and cortex (Bazalakova et al., 2007). A downregulation of M_2AChRs is consistent with compensatory changes in mAChR expression and function in AChE-/- mice, another model of cholinergic dysfunction (Volpicelli-Daley et al., 2003).

Deficits in cardiac CHT expression, cardiac tissue levels of ACh and elevated urinary catecholamines, pointed to reduced vagal regulation of the heart, thus we hypothesized that CHT+/- mice would exhibit a cardiac phenotype. To examine the impact of CHT loss on the cardiovasculature, we utilized implantable telemetry devices in freely moving mice. Our data indicate that CHT+/- mice exhibit a basal resting tachycardia and increased afterload compared to CHT+/+ mice (**Table 1**). Though the differences in resting HR and BP were statistically significant, the actual differences between both genotypes were not large. This could be explained by compensated CHT kinetic activity between the genotypes *in vivo*, providing for near normal cholinergic tone. As previously mentioned, vagal contributions to resting HR is lower in smaller mammals, thus the small differences in our CHT+/- mice may also reflect a lower

requirement of vagal contributions to resting HR in mice. The contributions of diminished CHT reserve may therefore be more pronounced during increased phasic demands on vagal tone, such as baroreceptor-mediated bradycardia or heart rate recovery after exercise. Similarly, since elevated resting HR has been shown to be a predictor of cardiovascular mortality, these small elevations may be more pathologic if experienced chronically (Fox et al., 2007; Palatini, 1999).

Long-term elevation in HR and BP has been shown in both humans and animal models to result in heart failure (Brodde et al., 2001; Brum et al., 2002). Though cholinergic of the vasculature is limited, CHT expression has been identified in rat and human arteries (Lips et al., 2002). The role of the parasympathetic cholinergic nerves in vasodilation was first identified in the cerebral circulation (Busija and Heistad, 1981). Recent studies have shown that vagally-mediated vasodilation is modulated by M_2AChRs and the nitric oxide (NO) interactions (Lepori et al., 2001; Sartori et al., 2005). Although the findings of elevated BP in CHT+/- mice (**Figure 13B and C**) may be reflective of reduced cholinergic activation of NO-mediated vasodilation, our findings of elevated systolic BP in the absence of elevated diastolic BP may be the result of increased CO due to increased sympathetic tone.

In conclusion, we have shown that CHT+/- have reduced CHT expression and HACU in intrinsic cardiac ganglia, representative of reduced vagal regulation of the heart. CHT+/- mice exhibit small, but significant increased HR and BP, that while not producing overt pathology, may predispose these mice to future cardiovascular risk if these parameters are elevated chronically. These finding in CHT+/- mice may have important clinical implications in human cardiovascular disorders as a hypomorphic

variant in the human CHT has been identified and found to have a minor allele frequency of 6% in a healthy population (Okuda et al., 2002).

CHAPTER III

IMPACT OF CHT HEMIZYGOSITY ON AUTONOMIC REGULATION OF CARDIAC FUNCTION

Introduction

Healthy cardiovascular function is reliant upon a balanced and responsive collaboration between sympathetic and parasympathetic innervation of the heart controlling inotropic and chronotropic responses. Elevated resting heart rate (HR) has been significantly associated with cardiovascular mortality in both healthy patients and in those with various cardiovascular disorders (Fox et al., 2007; Palatini, 1999). The parasympathetic branch (PNS) of the autonomic nervous system projects to the heart via the vagal nerve efferents to produce bradycardia through the release of ACh. ACh acts here on M_2AChRs, whereas vasodilation of the peripheral arteries through nitric oxide (NO) signaling pathways (Lefkowitz, 1996; Lepori et al., 2001). Disruptions in parasympathetic transmission may lead to sympathetic predominance resulting in elevated HR and BP, whereby long-term elevations in HR and BP have been shown in both human and animal models to produce heart failure (HF), myocardial infarction (MI) and sudden cardiac death (Brodde and Leineweber, 2004; Brum et al., 2002; Kannankeril and Goldberger, 2002). Increases in sympathetic tone can lead to autonomic instability and effect normal physiologic functions such as heart rate variability (HRV), baroreceptor sensitivity (BRS), and heart rate recovery (HRR) after exercise, which have both been shown to be independent risk predictors for increased mortality associated with cardiovascular disease (Cole et al., 1999; La Rovere, 2001).

Across a diverse patient population, heart rate recovery (HRR) after exercise has been shown to be an independent predictor of mortality and a large, prospective, multi-center study demonstrated that abnormal HRR after submaximal exercise predicted death (Cole et al., 2000; Smith et al., 2005). Several studies have shown that elevated PNS activity protected against ventricular fibrillation and may be protective against cardiac sudden death (Kannankeril and Goldberger, 2002; Smith et al., 2005). One potential mechanism is the direct effect of parasympathetic tone on cardiac electrophysiology, prolonging the sinus cycle length, AV conduction time and ventricular refractory period (Kannankeril and Goldberger, 2002). In addition to direct parasympathetic effects, exercise itself has also been shown to improve resting HR and parasympathetic tone (Freeman et al., 2006).

In addition to HRR, baroreceptor reflex sensitivity (BRS), a marker of cardiac vagal activity, was shown in a large prospective trial to be a strong independent predictor of mortality in patients with MI, with patients who exhibited a depressed BRS having an 18% mortality rate (La Rovere, 2001). The baroreceptors are stretch receptors located in the carotid sinuses and aortic arch, and detect acute changes in blood pressure. Upon acute increases in BP, these mechanoreceptors trigger vagal efferents resulting in a decrease in HR and reduction in cardiac output (CO). Experimentally, determination of the BRS index is performed by giving intravenous injections of a pressor agent, phenylephrine (PE), and a vasodilating agent, sodium nitroprusside (SNP) (Ma et al., 2002). The bradycardic effects elicited by the transient increase in SBP due to PE challenge have been shown to be primarily mediated by cholinergic projections of vagal efferents, activation of α_2-adrenergic autoreceptors have also been shown to augment the

bradycardic effects (Tank et al., 2004). These results were confirmed in mouse models where baroreceptor reflex is attenuated in α_2-adrenergic receptor knockout mice during challenge with PI (Niederhoffer et al., 2004). Nicotinic acetylcholine receptors (nAChRs) in autonomic ganglia have been shown to regulate autonomic functions affecting HR and BP. Studies conducted in α7 nAChR unit knockout mice show impaired sympathetic responses to vasodilation, but a supersensitivity to direct acting adrenergic agonists (Franceschini et al., 2000). Surprisingly, parasympathetic tone was normal in the α7 nAChR KO mice compared to controls (Franceschini et al., 2000). These results indicate that the α7 nAChRs in sympathetic ganglia participate in the maintenance of blood pressure during the autonomic reflex.

Our previous findings of a reduction in CHT expression, CHT-mediated HACU, elevated urinary catecholamines and the elevated HRs in CHT+/- mice pointed towards the hypothesis in CHT+/- mice exhibiting reduced parasympathetic tone in regulating HR. However, our phenotypic studies were conducted in the basal resting state and dependent upon tonic vagal activity. We therefore sought to determine the effects of CHT loss on regulating cardiovascular physiology dependent upon increased (phasic) vagal tone such as BRS and HRR.

In the experiments described below, I sought to determine the impact of CHT heterozygosity on the parasympathetic (vagal) regulation of the baroreceptor reflex (BRS), recovery of HR after exercise (HRR). Additionally I sought to determine the effects of direct acute and chronic vagal nerve stimulation (VNS) in eliciting a bradycardic response and the impact of a genetic loss of CHT in CHT+/- mice on their ability to sustain the bradycardia.

Methods

Drugs

l-Phenylephrine hydrochloride (P-6126) and sodium nitroprusside dihydrate (S-0501) were obtained from Sigma Aldrich (St. Louis, MO, USA) and dissolved in sterile saline (0.9% NaCl). Both drugs were injected intravenously at a volume of 10µl/kg. Isoflurane, USP (Terrell™) was obtained from RxElite (Meridian, ID, USA) and used as a general anesthetic mixed at 1-3% with 100% O_2.

Mice

All animal procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee (Protocol # M/04/075). Male mice (4-6 months old, young; 11-12 months old; aged) were housed up to 5 per cage on a 12:12-h light/dark cycle (lights on at 0600h). Telemetry and cardiovascular experiments were performed during the light part of the cycle. Food (Purina Rodent Chow #5001) and water were provided *ad libitum*. All mice were back-crossed at least seven generations to the C57BL/6 background. In all cases, CHT+/+ littermates were used as controls. All cardiovascular experiments were performed in the laboratory of Dr. David Robertson of the Autonomic Dysfunction Center, Vanderbilt University.

Surgical placement of telemetric blood pressure devices for acute studies

For short-term, acute studies of baroreceptor sensitivity, mice were anesthetized and temperature maintained as described in Chapter II (Methods). Following antiseptic

preparation, the left carotid artery was isolated, distal occlusion by suture of vessel was placed 8-10 mm below the bifurcation to occlude blood flow, and the lumen cut to allow insertion of the transmitter catheter (model TA10-C20; DataSciences International, St. Paul, MN, USA) to the point of bifurcation. For intravenous administration of drugs, the right jugular vein was then isolated, distal occlusion of the vessel by suture, and lumen cut to allow insertion of the venous catheter (Micro-Renathane, model MRE-025 0.025 O.D. x 0.12 I.D.; Braintree Scientific, Braintree, MA.). During the baroreceptor studies, mice were kept on 0.65% isoflurane on 100% O2 at 1.5 L/min. Mice were then sacrificed at the end of the study by intravenous urethane.

Baroreceptor reflex sensitivity studies

Determination of baroreceptor-mediated cardio-inhibitory response, mice (CHT+/+, n=8; CHT+/-, n=8) underwent a challenge with phenylephrine (PE) (5-30 μg/kg) and Na^+-nitroprusside (SNP) (5-30 μg/kg) given intravenously using a syringe pump (CMA 400 pump, CMA Microdialysis, Stockholm, Sweden) in a dose-response manner in anesthetized mice. Baseline ECG and BP was recorded for 1.0 min prior to administration of drug and post-drug response was recorded for 3.0 min after administration. The ratio of the maximal change in HR over the change of mean arterial blood pressure (MABP) was calculated and averaged at each dose in each animal. Baroreceptor sensitivity (BRS) was determined by the averaged ratio of HR change over MAPB change (ΔHR/ΔMABP) (Lin et al., 2007).

Heart rate recovery/treadmill studies

Mice (CHT+/+, n=9; CHT+/-, n=8) chronically telemeterized with BP transducers, (as described above) were run on a two-lane motorized treadmill (Columbus Instruments, Columbus, OH, USA) equipped with an adjustable-speed belt (0-90 m/min) and an electric shock grid at one end. On Day-1 (training-1) the mice were exposed to the treadmill for 10 min without shock. Mice were then exposed to two timed runs (5 min duration) at 5 and 10 m/min with 10-minute recovery periods between runs. On Day-2 (training-2), mice were then exposed to the treadmill in the presence of shock (2 mA, 4 min-1 frequency) activated by physical contact with the grid. The mice were then run on the treadmill starting at 5m/min and gradually increased 2m/min every 2 minutes until exhaustion. Exhaustion was defined as resting on the electric grid >15 sec/min or falling back onto the grid >15 times/min (Bazalakova et al., 2007). On Day-3 (fixed speed/time), mice were run on the treadmill for 13m/min for 5 min. HR and BP were collected in home cage 30 min prior to exercise challenge and during the 60 min recovery period after the 5 min treadmill run.

Vagal nerve stimulation (VNS) studies

Mice (CHT+/+, n=9; CHT+/-, n=9) were anesthetized as described above and a cervical midline incision was performed and right vagus nerve isolated from surrounding tissues. The nerve was then placed on a pair of platinum hook electrodes (PT101 (25mm), World Precision Instruments, Inc, Sarasota, FL, USA) and covered with silicone gel for insulation and immobilization (Tsutsumi et al., 2008). To determine cardiac sensitivity to VNS, two protocols were utilized. In protocol-1 (frequency-response), the vagus nerve was stimulated with rectangular wave pulses of 1-ms duration, in

randomized frequencies 1-50Hz, 20 sec duration. Baseline HR was recorded for 1 min prior to the stimulus and 5 min post-stimulus. Mice were given 10 min recovery periods between stimulations. In protocol-2 (saturation), the vagus nerve was stimulated at 20 Hz for 5 min continuously in order to determine the duration of bradycardia with constant vagal stimulation.

Chronic exercise/swim studies

CHT+/+ and CHT+/- mice (ages 4-7 mo) were randomly divided into 4 groups. The control group (CHT+/+, n=4, CHT+/-, n=4) did not perform any exercise. The exercise group (CHT+/+, n=4; CHT+/- n=3) were trained two-times per day for 5 min duration for days 1 thru 6, and two-times per day for 10 min for days 7-10. After successful training, the exercise group were subjected to the chronic swim/exercise protocol consisting of 10-minute swims, two-times per day for 30 days. The chronic swim protocol established by Evangelista, et al. (Evangelista et al., 2003) consisted of a Plexiglas tank, 225 cm^2, water depth of 15-20 cm, water temperature of 30-35°C. To determine changes in cardiovascular function, 2-D targeted, M-mode echocardiography was performed at baseline and at the end of the chronic exercise period. Echocardiographically defined measurements of LV function were calculated as previously described (Rottman et al., 2003).

2-D and M-mode echocardiography (Echo)

Transthoracic echocardiography was performed using a system (Sonos 5500, Agilent, Andover, Mass.) with a 15-MHz high-frequency linear transducer at a frame rate

of 100 frames/sec in young (4-6 months old) (CHT+/+, n=8; CHT+/-, n=8) and aged (10-12 months old) CHT+/+, n=9; CHT/, n=8). All images were acquired at a depth setting of 20 mm. Echocardiography was performed in conscious mice. Before the study, mice were trained by holding mice by the nape of the neck, holding it in one hand in the prone position for at least 5 minutes. During the procedure, mice were held in the position required for echocardiographic imaging. The mouse chest was not shaved; ultrasound-coupling gel heated to 34C was applied to the precordium. Optimal parasternal long- and short-axis views were obtained by visualization of endo- and epicardial walls. Two-dimensional targeted, M-mode echocardiographic images were obtained to determine LV systolic (LVIDs) and diastolic (LVIDd) internal dimensions as previously described (Rottman et al., 2003; Syed et al., 2005). These parameters allowed determination of LV fractional shortening (FS %) by the equation: %FS=[(LVIDd-LVIDs)/LVIDd]x100%. Additional echocardiographic parameters were calculated using M-mode data as previously described (Collins, 2003; Syed et al., 2005).

Histologic analysis

Mice (CHT+/+, n=6; CHT+/-, n=6) were weighed and hearts dissected at 2 and 12 months of age. Dissected mouse hearts were rinsed and weighed in PBS. Hearts were cut in cross-section just below the papillary muscle, and the top half fixed in formalin and embedded in paraffin. Sections (5 μm) were prepared at 200 μm intervals and fixed with hematoxylin and eosin (H&E) for gross examination, Masson's Trichrome (MT) for quantification of fibrosis, and Periodic Acid-Schiff, counterstained with hematoxylin (PAS-H) to determine cardiomyocyte size (Barrick et al., 2007).

Results

CHT Deficiency Leads to Impaired Heart Rate Recovery after Exercise.

Blood pressure tracings during exercise were of good quality for calculation of HR and mean BP (mBP). As expected, resting baseline HRs in CHT+/- mice were significantly higher compared to CHT+/+ mice ($P<0.001$; **Figure 16A**). During the 5-min treadmill exercise run, both CHT+/+ and CHT+/- mice exhibited similar onset and maximal HR increases (**Figure 16A**). With cessation of exercise, both CHT+/+ and CHT+/- mice achieved a resting heart rate equivalent to their pre-exercise levels, though CHT +/- mice required 27% longer to achieve full recovery (CHT+/+, 24±3.4 min; CHT+/-, 33±2.4 min, $P<0.05$). When changes in HR from peak values were calculated during the recovery period (**Figure 16B**), CHT+/- mice displayed overall deficits in HR recovery ($P<0.05$, CHT+/+, n=9; CHT+/-, n=8; **Figure 16B**). Moreover, three periods of differential HR adjustment became evident. Although CHT +/- animals generated consistently smaller reductions in HR during the first ~10 minutes of recovery, these differences were not statistically significant. In contrast, over the next 10 minutes, the change in HR from peak values was significantly blunted for the CHT+/- animals. Finally, over the final 40 min of recording, genotype differences in HR recovery were lost.

CHT Hemizygosity Results in Impaired Baroreceptor Sensitivity.

The increase in resting HR and the inability to return from an activity-dependent HR after exercise suggested a decrease in the vagal tone in regulating the chronotropic

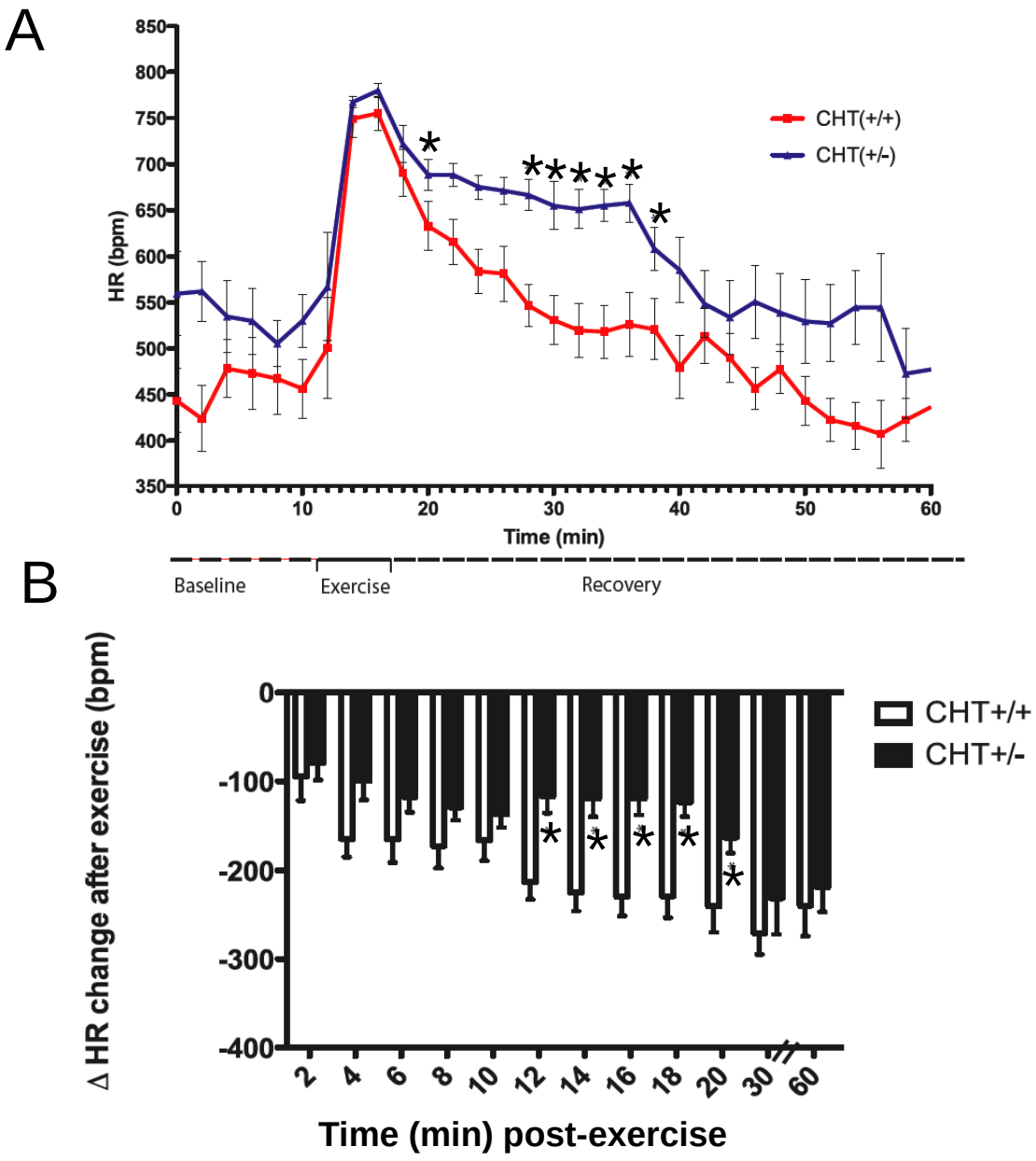


Fig. 16. Reduced HR recovery after moderate exercise in CHT+/- mice. **(A)** Averaged HR recordings in CHT+/+ and CHT+/- mice undergoing moderate exercise (13m/min) for 5 min. Baseline HR recorded in home cage for 10 min; 5-min exercise run; then 40-min post-exercise HR recovery monitoring. **(B)** Change in exercise HR from maximal HR obtained during exercise at 2-minute intervals. CHT+/+, n=9; CHT+/-, n=8. Mean (±SEM) values are indicated (*P<0.05; one-tailed, paired, Student's t-test).

control of the heart. To further elucidate the vagal contribution to HR response in CHT+/- mice, we measured baroreceptor-mediated changes in HR response following intravenous administration of phenylephrine (PE) in anesthetized CHT+/+ and +/- mice. CHT+/+ and +/- mice exhibited similar anesthetized baseline mean arterial pressure, however CHT+/- higher baseline HR compared to CHT+/+ mice. While both genotypes exhibited similar increases in BP to PE challenge, CHT+/- mice demonstrated a marked reduction in bradycardic response (**Figure 17A**). The ratio of HR change to BP change during exposure to pressor agents has been used as an index of baroreceptor sensitivity (Lin et al., 2007). CHT+/- mice also demonstrated a significant reduction in the ratio of the mean change in HR to the maximal mean BP change in response to 20 μg/kg PE challenge (P<0.03; **Figure 17B**). These results indicate that CHT+/- mice exhibit impaired vagal regulation of HR response to acute changes in blood pressure and reduced baroreceptor sensitivity.

<u>CHT+/- Show an Enhanced Bradycardic Response to VNS, but Inability to Sustain Bradycardia to Chronic Stimulation.</u>

To directly determine the impact of CHT heterzygosity on vagal regulation of HR, the effect of stimulation of vagal efferents on HR was determined using two separate vagal nerve stimulation protocols. In protocol 1, we examined the frequency-dependent change in the RR interval (RRI) in anesthetized mice. CHT+/- mice exhibited significantly higher baseline heart rates. Stimulation of the right vagus nerve significantly increased RR interval in a frequency-dependent manner in CHT+/- mice indicating a hypersensitivity of vagal regulation of HR (**Figure 18A**). Given the importance of CHT

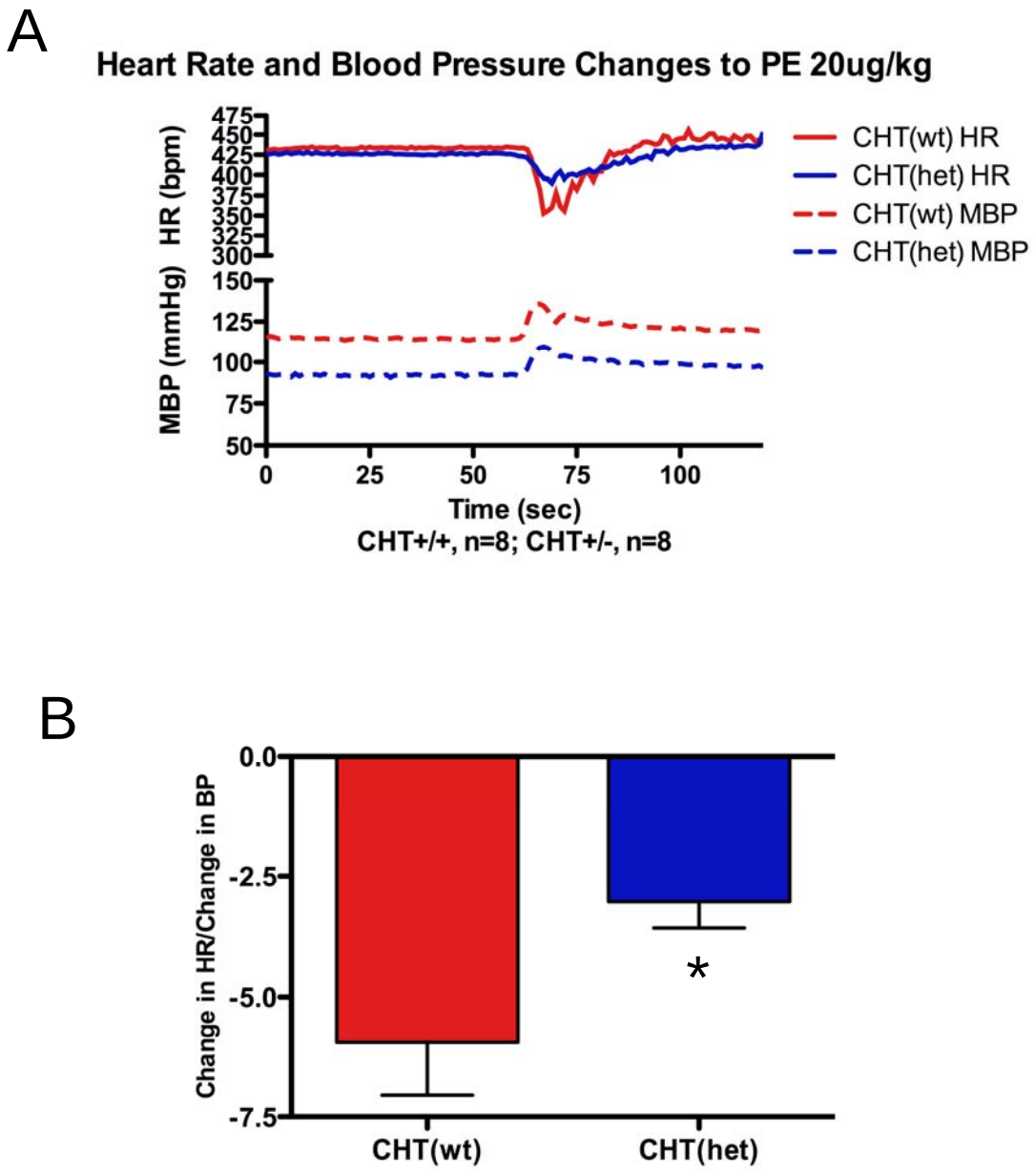
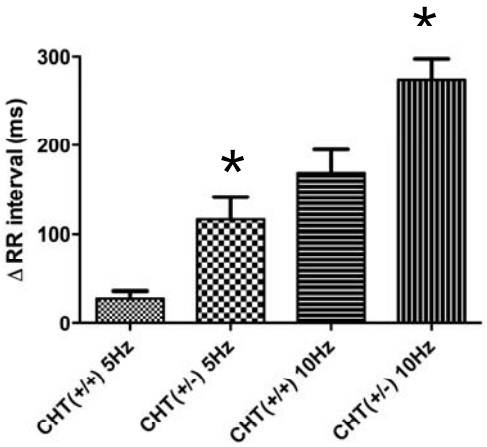


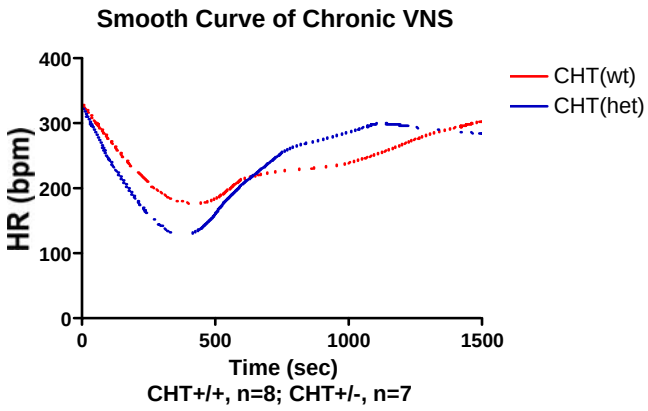
Fig. 17. Blunted baroreceptor sensitivity (BRS) in CHT+/- mice. (A) Averaged HR (bpm) (solid line) and BP (mmHg) (dotted line) recording in anesthetized CHT+/+ (red) and CHT+/- (blue) mice during intravenous infusions of phenylephrine 20 μg/kg. CHT+/- mice show similar changes in BP, however fail to exhibit compensatory changes in HR similar to CHT+/+ mice. **(B)** Estimation of BRS sensitivity index (Δ HR/Δ MBP) ratio. CHT+/+, n=8; CHT+/-, n=8. Mean (±SEM) values are indicated (**P*<0.03; one-tailed, paired, Student's t-test).

Fig. 18. CHT+/- mice show altered HR responses to acute and chronic VNS. (A) Change in RR-interval during frequency-response VNS stimulation (5 and 10 Hz) at 30 sec. intervals. CHT+/- show significant increase in RR-interval (reduced HR) at both 5 and 10 Hz frequencies vs. CHT+/+ mice (CHT+/+, n=9; CHT+/-, n=8 $P<0.004$). **(B)** Smoothed HR (bpm) tracings during chronic VNS (40Hz) over 3 min. CHT+/- mice exhibit hypersensitive bradycardic response initially, but lose this effect more rapidly vs. CHT+/+ mice. **(C)** Time (sec) to 50% baseline HR recovery is also shorter in CHT+/- mice vs CHT+/+ mice ($P=0.01$). **(D)** Percent (%) HR recovery at 1.0 min time interval is greater in CHT+/- vs. CHT+/+ mice ($P<0.05$). Mean (**±**SEM) values are indicated (one-tailed, paired, Student's t-test).

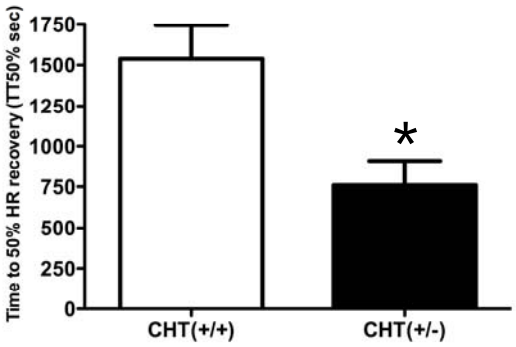
A


Δ RR interval (ms)
300
200
100
0
*
*
CHT(+/+) 5Hz
CHT(+/-) 5Hz
CHT(+/+) 10Hz
CHT(+/-) 10Hz

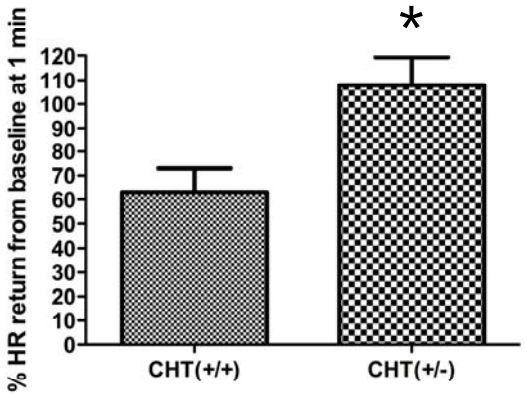
B


Smooth Curve of Chronic VNS
HR (bpm)
400
300
200
100
0
0
500
1000
1500
Time (sec)
CHT+/+, n=8; CHT+/-, n=7
CHT(wt)
CHT(het)

C


Time to 50% HR recovery (TT50% sec)
1750
1500
1250
1000
750
500
250
0
*
CHT(+/+)
CHT(+/-)

D


% HR return from baseline at 1 min
120
110
100
90
80
70
60
50
40
30
20
10
0
*
CHT(+/+)
CHT(+/-)

in maintaining the presynaptic pool of available ACh, we hypothesized that CHT+/- would be unable to maintain the bradycardic response to chronic, continuous vagal nerve stimulation. Therefore in protocol 2, during chronic vagal stimulation (5 min) at 40Hz, CHT+/- exhibited a more pronounced decrease in HR, however (**Figure 18B**), the average rate of return of the HR in response to VNS (measured as the $TT_{50\%}$) was significantly faster in CHT+/- mice compared to CHT+/+ (**Figure 18C and D**) indicating an inability of CHT+/- mice to sustain prolonged vagal stimulation of HR.

CHT+/- Mice Show Impairments in Cardiac Function.

Ultrasonic echocardiography allows non-invasive determination of both cardiac function and structure throughout the entire cardiac cycle. Chronic tachycardia exhibited by β1-adrenergic overexpressing mice display a significant decrease in echocardiographic determinations of cardiac function by 35 weeks (Engelhardt et al., 1999). Whereas body weights of CHT+/- and CHT+/+ mice did not differ, CHT+/- mice exhibited significantly increased heart/body weight ratios ($P<0.05$; **Figure 19A**). To explore this further, we utilized 2D- and M-mode echocardiography to determine the impact of chronic tachycardia in both young (4-6 month old) and aged (10-12 month old) CHT+/- mice. Both young and aged CHT+/- mice (**Table 5**) show significantly diminished loss of cardiac contractility as shown by a reduction in left ventricular function (fractional shortening, %FS) ($P<0.01$; **Figure 19B**). Although CHT+/- mice exhibited reduced left ventricular function, cardiac output (CO) was slightly higher in young CHT+/- mice, but similar in older CHT+/+ mice. It is possible that this difference in CO seen in the young mice was due to increased heart rate, however these studies were

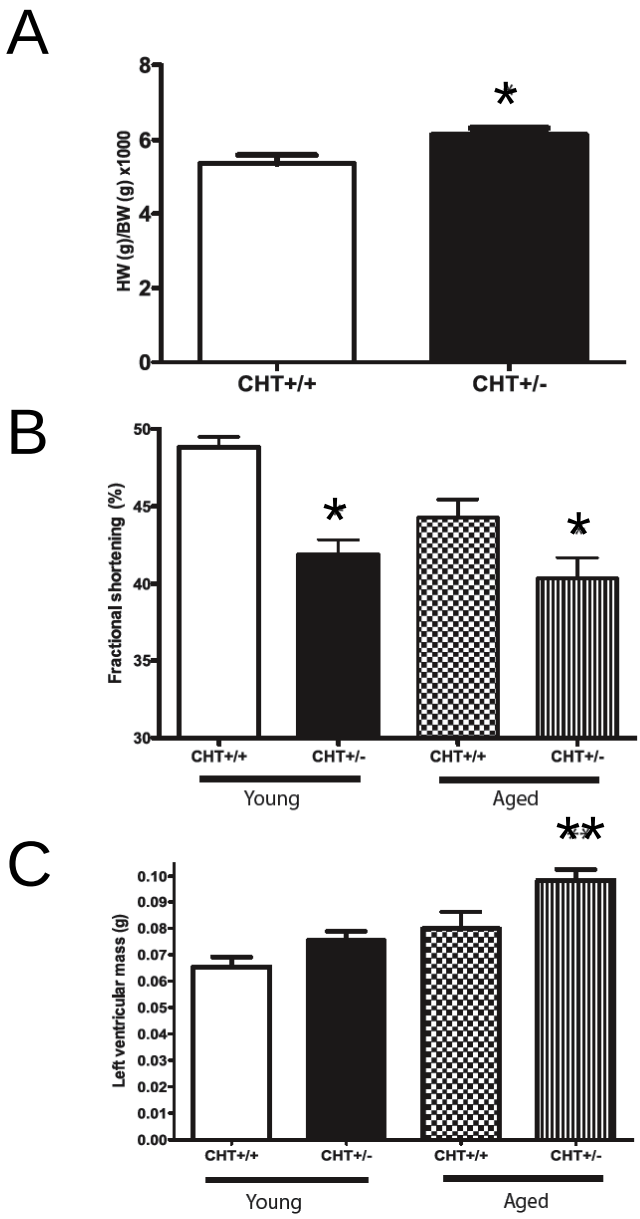


Fig. 19. Histologic and echocardiographic detection of cardiac hypertrophy and diminished cardiac contractility in CHT+/- mice. (A) The ratio of heart weight (HW; g) vs. body weight (BW; g) x1000. Values are means (±SEM); CHT+/+, n=7; CHT+/-, n=9. *P<0.05. **(B)** 2-D guided, M-mode derived echocardiography shows CHT+/- mice with reduced left ventricular fractional shortening (FS%) in both young (4-6 mo) and aged (10-12 mo) mice. Mean (±SEM) values are indicated (*P<0.01; one-tailed, paired, t-test). **(C)** LVM (g) is slightly elevated in young CHT+/- mice vs young CHT+/+ mice. Aged CHT+/- show significantly elevated LVM compared to aged CHT+/+ mice. Mean (±SEM) values are indicated (**P<0.03; one-tailed, paired, Student's t-test). Young CHT+/+, n=8; CHT+/-, n=8. Aged CHT+/+, n=9; CHT+/-, n=8.

Table 5. Echocardiographic findings in both young and aged CHT+/+ and CHT+/- mice.

Parameter	CHT+/+ young (n=8)	CHT+/- young (n=9)	CHT+/+ aged (n=8)	CHT+/- aged (n=8)
LVIDd	0.292 ± 0.005	0.330 ± 0.006*	0.342 ± 0.008	0.333 ± 0.009
LVIDs	0.149 ± 0.004	0.191 ± 0.006*	0.186 ± 0.008	0.198 ± 0.002
CO (ml/min)	15.36 ± 0.7310	19.59 ± 1.221	23.86 ± 1.08	20.66 ± 1.38**
SV (ml)	0.021 ± 0.001	0.029 ± 0.001*	0.031 ± 0.002	0.029 ± 0.002
HR (beats/min)	704.5 ± 5.61	675.8 ± 16.59	692.2 ± 7.4	705 ± 7.55
FS%	48.83 ± 0.667	41.89 ± 0.9484*+	44.29 ± 1.16	39.26 ± 0.853**+
LVM (g)	0.065 ± 0.004	0.075 ± 0.004	0.079 ± 0.006	0.098 ± 0.004**

Abbreviations for Table 5: Left ventricular internal dimension-diastole (LVIDs); left ventricular internal dimension-systole (LVIDs); cardiac output (CO); stroke volume (SV); heart rate (HR); fractional shortening (FS%); left ventricular mass (LVM). One-tailed, unpaired Student's t-test. *P<0.05 in young CHT+/- vs CHT+/+. **P<0.05 in aged CHT+/- vs. CHT+/+, +P<0.05 in young vs. aged CHT+/-. Values are ±S.E.M.

conducted in trained, conscious mice exhibiting similar "stressed" heart rates between genotypes. Left ventricular mass (LVM) a sign of heart failure was also determined in CHT+/- mice. Young CHT+/- showed a trend toward elevated LVM compared to CHT+/+ mice, however this did not achieve statistical significance. However, in older CHT+/- mice, LVM was significantly increased compared to CHT+/+ mice ($P<0.03$; **Figure 19C**). These results are consistent with other transgenic models displaying tachycardia and increased afterload (Engelhardt et al., 1999).

CHT+/- Mice Display Aged-Related Cardiac Histologic Changes Consistent with Progressive Heart Failure**.**

A typical pathologic response to chronic adrenergic stimulation of the heart often results in cardiac muscle hypertrophy characterized by fibrosis (Weber et al., 1991). Using quantitative analysis of Masson's Trichrome stained sections, revealed increased interstitial fibrosis in aged, but not young CHT+/- mice as compared to CHT+/+ littermates ($P<0.004$; **Figure 20A-E**). Histologic examining of cardiac myocyte area using PAS-H revealed similar age-dependent increases in mean myocyte are in CHT+/- mice ($P<0.01$; **Figure 21A-E**). These results indicate that the sustained resting tachycardia seen in aged CHT+/- mice is sufficient to produce overall histologic pathology consistent with fulminate heart failure.

Chronic Exercise Leads to a Trend of Improved Cardiac Function in CHT+/- Mice.

Although CHT+/- mice showed reduced vagal regulation of cardiovascular function, as evidenced by chronic resting tachycardia, hypertension and

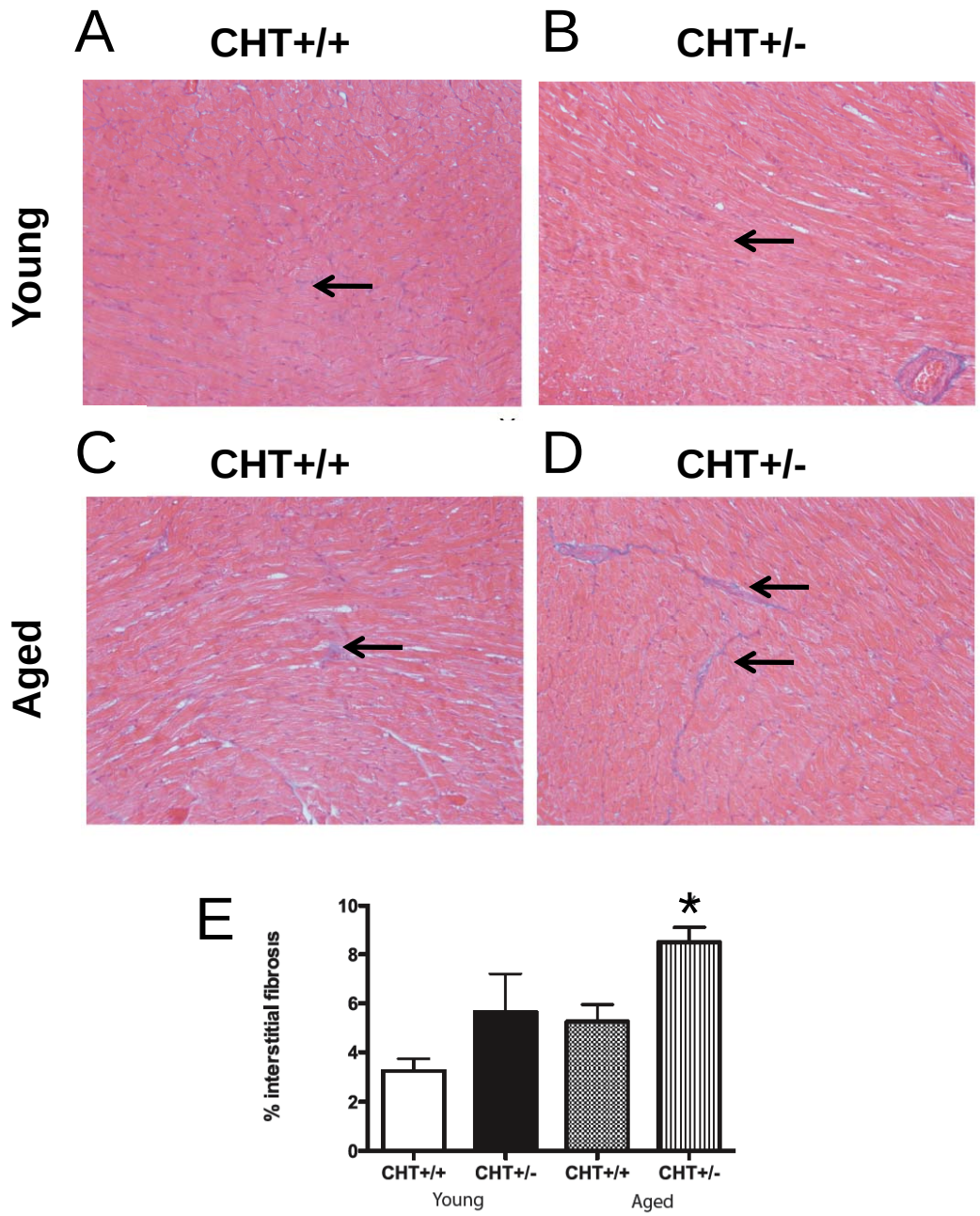


Fig. 20. Cardiac interstitial fibrosis alterations in CHT+/+ and CHT+/- mice. Masson's Trichrome stained 5-um sections of paraffin-embedded left ventricular myocardium from young (2-3 mo) and aged (10-12 mo) CHT+/+ **(A, C)** and CHT+/- **(B, D)** mice. Arrows indicate areas of fibrosis. ***(E)*** Morphometrical analysis of myocyte fibrosis shows that aged CHT+/- mice exhibit significantly higher left ventricular fibrosis compared to CHT+/+ mice. Significance as determined by one-tailed, unpaired Student's *t*-test (genotype x same age group), **P*<0.004, n=5/genotype. Values represent ± S.E.M.

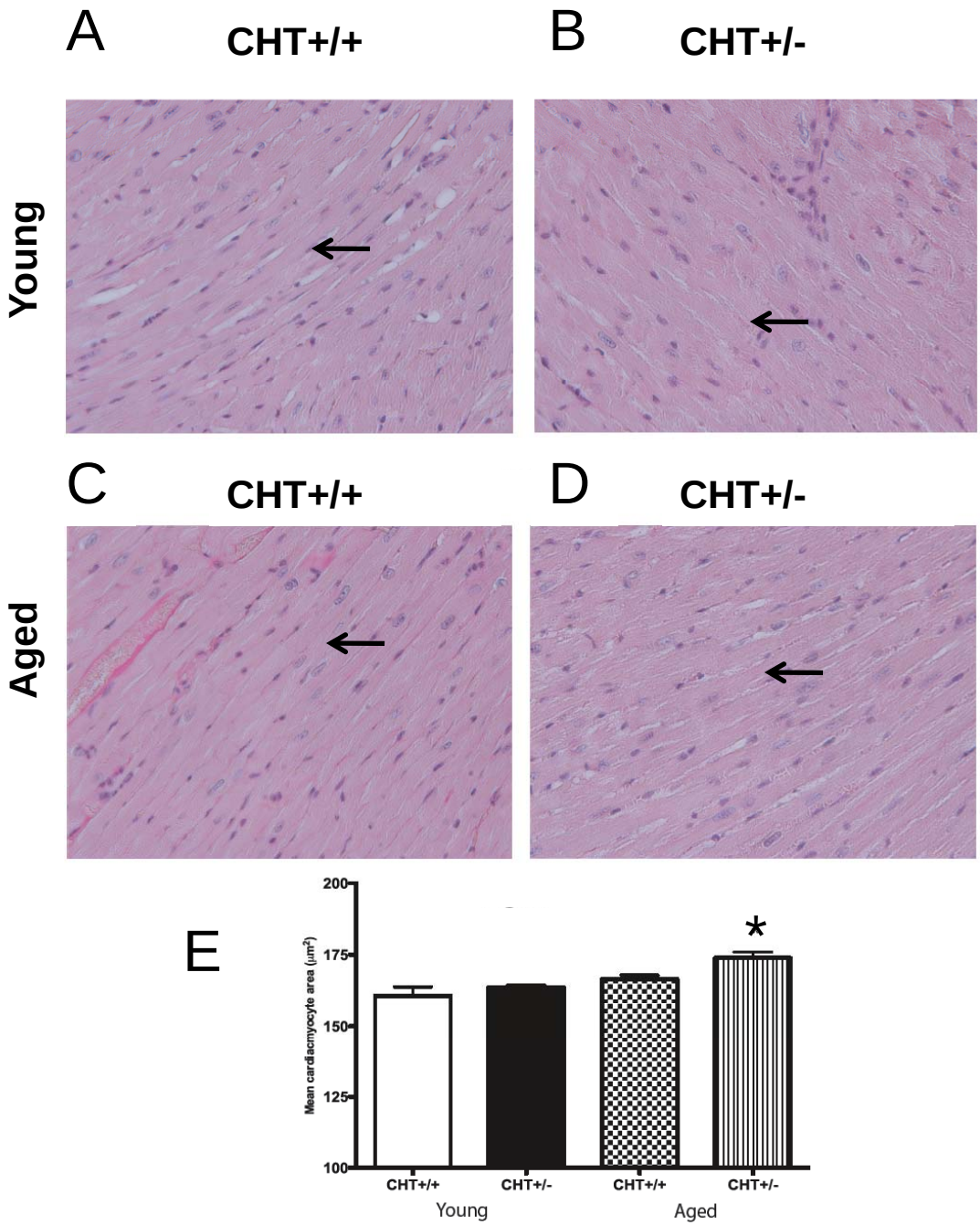


Fig. 21. Comparison of cardiomyocyte alterations in CHT+/+ and CHT+/- mice. Periodic-Acid Schiff stained 5-um sections of paraffin-embedded left ventricular myocardium from young (2-3 mo) and aged (10-12 mo) CHT+/+ **(A, C)** and CHT+/- **(B, D)** mice. Arrows indicate areas of myocyte hypertrophy. **(E)** Morphometrical analysis of mean cardiomyocyte area shows similar cardiomyocyte area in young CHT+/+ and CHT+/- mice. However, aged CHT+/- mice display significantly increased cardiomyocyte area versus CHT+/+. Significance as determined by one-tailed, unpaired Student's *t*-test (genotype x same age group), **P*<0.01, n=5/genotype. Values represent ± S.E.M

echocardiographic and histologic changes consistent with mild cardiac hypertrophy, they do not exhibit overt physical symptoms of heart failure. Thus CHT hemizygosity may be sufficient to sustain adequate baseline ACh stores capable of delaying heart failure. Cardiac hypertrophy is a normal adaptive response to chronic exercise, however the hypertrophic response can be associated with cardiac disease and heart failure in the presence of reduced vagal tone or sympathetic hyperactivity (LaCroix et al., 2008; Medeiros et al., 2008). Conversely, chronic moderate exercise has also been shown to improve autonomic balance in mice and to delay cardiac dysfunction (De Angelis et al., 2004; Medeiros et al., 2008). Therefore, we conducted chronic exercise studies to examine the impact of CHT hemizygosity on development of cardiac hypertrophy. Consistent with earlier findings, CHT+/- mice in both the control and exercise-swim group showed significantly reduced fractional shortening (%FS) compared to CHT+/+ mice in both control and exercise groups (CHT+/+ control, n=4, 50.38±0.8; CHT+/-, n=4, 38.55±0.5; CHT+/+ exercise, n=4, 49.83±0.2; CHT+/- exercise, n=3, 41.73±1.3) (**Figure 22A**). Baseline HRs were not significantly different between groups or between genotypes. Derived calculations for baseline cardiac output (CO) showed no significant differences between groups or genotypes at baseline, although there was a trend of the LV mass (LVM) to be increased in both groups of CHT+/- mice (**Figure 22B**). At the end of the 30-day chronic swim exercise cardiac function indices (FS%, CO and LVmass) in both CHT+/+ mice control and swim groups were unchanged from baseline (**Figure 22A and B**). In the CHT+/- mice, both the control and swim groups surprisingly showed an improvement in FS% (**Figure 22A**), however CO and SV were similar to baseline values in the CHT+/- control group compared to the CHT+/- swim group. These

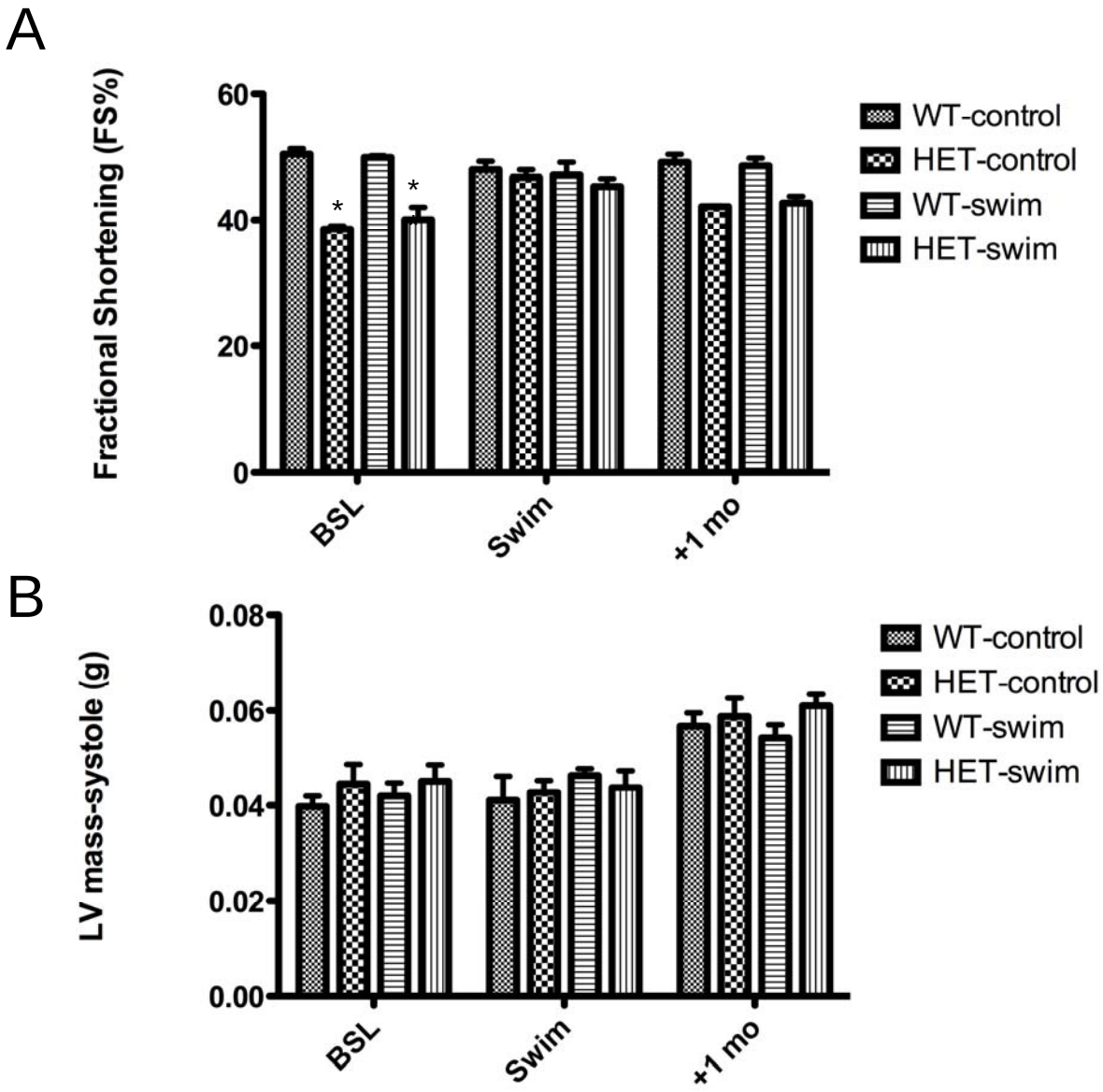


Fig. 22. Chronic swim exercise and its impact on cardiac function in CHT+/- mice. 2-D guided, M-mode derived echocardiography measurement of (**A)** fractional shortening (%FS) and **(B)** left ventricular mass (LVM) in 4-6 mo CHT+/+ and CHT+/- mice at baseline, during a 30-day chronic swim and 1 month post-exercise. (**P*<0.001 CHT+/+ vs CHT+/- control and ***P*<0.01 CHT+/+ vs CHT+/- swim at baseline (BSL); 2-way repeated measures ANOVA with Bonferroni post-test.

results however were not significant when compared between groups at the end of the exercise period or change from baseline to exercise. To determine if these changes observed in CHT+/- mice were exercise dependent, we repeated echocardiography in the same set of mice 1-month after the exercise training. Quite surprisingly, FS% in both CHT+/- groups had reverted back to baseline values, while LV mass was slightly elevated compared to CHT+/+ mice. While our experimental group size was small (N=3 or 4/group), these results raise the possibility that the basal phenotypes observed in CHT+/- mice are not irreversible, however due to the short duration and small sample size further studies are needed to characterize the impact of exercise on cholinergic tone in CHT+/- mice.

Discussion

Cholinergically Mediated Regulation of Cardiovascular Function

To determine the impact of CHT hemizygosity on vagally mediated cholinergic regulation of HR, we examined HR recovery (HRR) and baroreceptor sensitivity (BRS) in CHT+/- mice. The physiologic mechanisms of HRR and BRS are dependent upon the coordination of vagal-cholinergic and sympathetic efferents to the heart and vasculature. The overall importance of vagal regulation of HRR and BRS in cardiovascular disorders has been shown in several studies wither both HRR and BRS have been proven in human patients to be independent risk factors of mortality associated with cardiovascular disease (Cole et al., 2000; La Rovere, 2001).

To examine HRR, telemeterized CHT+/+ and CHT+/- mice were placed onto a treadmill and required to exercise at a constant speed (13m/min) for 5 min. The speed and duration was chosen due to the motor deficits previously established in CHT+/- mice (Bazalakova et al., 2007), in addition to running difficulties due to the weight and placement of the implantable telemetry devices. As previously seen, baseline resting HR was statistically elevated in CHT+/- mice compared to wild-type littermates. During the 5-min treadmill exercise run, both CHT+/+ and CHT+/- mice exhibited similar maximal HR increases (**Figure 16A**). During the early phase of HRR immediately post-exercise, CHT+/- mice show an initial HRR similar to CHT+/+ mice, which plateaued 5-minutes into the HR recovery period, and stayed elevated 20 minutes into the recovery period (Figure 16A). During the later phase of HRR (30-min post-exercise), CHT+/- mice slowly began to return back to baseline HR levels. The initial HR recovery within the first 10 minutes post-exercise was similar between genotypes and may be reflective of a combination of similarities in withdrawal of sympathetic tone and increase in vagal tone, however studies using antagonists of both branches of the ANS would be required to full elucidate the contributions of the either SNS or PNS in early HRR. Additionally, initial cardio-deceleration could be due to changes in venous return, since decreases in cardiac filling pressures have been shown to result in reduced HR (Chen et al., 1995). From 12 to 20 minutes post-exercise, CHT+/- exhibited a significant blunting of their HRR. The HRR plateau seen in CHT+/- mice may be reflective of the lack of sustainable ACh due to insufficient intracellular CHT pools to mobilize to meet increased cholinergic demands. Significant controversy exists as to the temporal contributions of the sympathetic and parasympathetic nervous system in regulation of HRR (Cole et al.,

1999). Although the majority of the data supports parasympathetic activation in the early phase of HR recovery after exercise in human and canine models, these data are lacking in rodent models. One study conducted in rats demonstrated that parasympathetic contributions to HR recovery in hypertensive rats occurred within 20 minutes after exercise, similar to our findings in CHT+/- mice (Chen et al., 1995). Additionally, attenuation of cardio-deceleration during HRR may be complicated by heightened sympathetic tone in our CHT+/- as our previous results demonstrated elevated cardiac tissue levels of catecholamines and blunted HR responses to the β-adrenergic receptor antagonist, metoprolol.

Baroreceptors are stretch receptors located in the endothelium of the carotid sinus and aortic arch. These receptors detect transient increases in blood pressure, which result in increased vagal transmission to the heart producing decreased HR and venodilation. Therefore, we hypothesized that CHT+/- mice may exhibit blunted BRS upon eliciting the vagal reflex due to transient increases in BP upon infusion with phenylephrine (PE). Both CHT+/+ and CHT+/- mice (CHT+/+, n=8; CHT+/-, n=8) exhibited similar anesthetized baseline mean arterial pressure (MAP), however CHT+/- mice had higher baseline HR compared to CHT+/+ mice. Both CHT+/+ and CHT+/- mice showed similar increases in BP upon intravenous infusion of PE (CHT+/+, 22.50±2.2 mmHg; CHT+/-, 19.43±1.9; p=0.359), however CHT+/- mice failed to elicit a robust decrease in HR compared to CHT+/+ mice (**Figure 17**). Comparison of the BRS index showed that CHT+/- mice demonstrated a significant reduction in the ratio of the mean change in HR to the maximal mean blood pressure change in response to PE challenge (**Figure 17**). These results indicate that CHT+/- mice exhibit impaired vagal regulation of HR

response to acute changes in blood pressure and reduced baroreceptor sensitivity. Thus it was possible that these physiologic findings represented the inability of sensory afferents projecting to regulatory centers within the CNS to elicit a decrease in HR with changes in BP. Similarly the inability of CHT+/- mice to return to resting HR as quickly as CHT+/+ mice may have been due to hyperactive sympathetic tone and not due to diminished vagal efferent regulation.

Although HR recovery and BRS allowed us to examine inducible vagal tone, changes in HR due to either exercise cessation or pharmacologic manipulation could be influenced by feedback mechanisms and influences by sympathetic tone allowing only transient examination of vagal tone. Therefore, we utilized vagal nerve stimulation (VNS) to directly examine the vagal efferent projection onto the heart and the impact of CHT hemizygosity on bradycardic responses to stimulation.

CHAPTER IV

ASSOCIATION OF MUTATIONS IN THE HUMAN CHOLINE TRANSPORTER TO HUMAN DISEASE

Introduction

Single nucleotide polymorphisms (SNPs) identified in various neurotransmitter transporters and having a functional impact on activity or trafficking, have been found to be associated with a number of human diseases (Garland et al., 2002; Hahn et al., 2008; Hahn and Blakely, 2002). CHT-mediated HACU serves as the rate-limiting step for the synthesis of the neurotransmitter ACh, which functions to regulate a wide variety of physiologic and behavioral processes. Thus, discoveries of genetic variants impacting the synthesis or release of ACh would be predicted to have a significant impact on both physiologic and behavior functions. While evidence does not exist for CHT haploinsufficiency in human disease, Okuda, *et al*., identified a missense mutation occurring within an exonic region of the human CHT gene (Okuda et al., 2002) resulting in a hypomorphic transporter (<50% V_{max}) with a minor allele frequency of 6%. The presence of a hypomorphic variant in the human CHT may have significant impact on cholinergically-mediated physiologic and behavior functions. Studies performed in CHT-/- and CHT+/- mice demonstrate the critical significance of CHT in survival (Ferguson et al., 2004), motor and behavioral functions (Bazalakova et al., 2007).

An additional SNP has also been identified within the purported 3'UTR region of the human CHT gene (Neumann et al., 2005). Although no functional data currently exists on this variant, the presence of the major allele has been associated with reduced

HRV (Neumann et al., 2005) and increased corticolimibic activation with physiologic arousal (Neumann et al., 2006) in healthy controls. These studies indicate that the presence of the major allele SNP within the CHT 3' region may attenuate cholinergic tone regulating HR variability and negative feedback control of the corticolimbic projections.

Characterization of CHT+/- mice may provide invaluable information on the impact of CHT deficiency and its impact on behavioral or physiologic phenotypes, which may be extrapolated to human disease. These early studies identifying SNPs within the human CHT in the context to our behavioral and cardiovascular findings in CHT+/- provide clues as to the role of CHT on cholinergic transmission regulating psychiatric or cardiovascular disorders.

Human genetics of CHT

The human CHT is a member of the SLC5 (SLC5A7) family of mammalian Na+-dependent transporters (Ferguson and Blakely, 2004). The CHT gene is located on chromosome 2 and consists of 9 highly conserved exons spanning 25kb of genomic sequence **(Figure 7)** (Ferguson and Blakely, 2004). Within CHT coding regions, only one SNP has been identified to reside within the exonic region of CHT, resulting in a non-synonymous coding variant +265 A/G (rs1013940), encoding an Ile to Val at amino acid position 89 exhibiting functional transport deficiency (Okuda et al., 2002). This SNP has been shown to have a minor allele frequency of 6% in health controls (Okuda et al., 2002). Another non-coding polymorphism, originally identified as a component of the CHT 3' transcription stop site (+4037 G/T; rs333229), here designated the 3'SNP

(see Methods) has been associated with altered cholinergic tone, as measured by heart rate variability (HRV), and to altered corticolimbic reactivity to stress (Neumann et al., 2006; Neumann et al., 2005). Haplotype determination for both CHT Ile89Val and 3' SNPs, shows that neither of these SNPs are in LD, with each SNP residing within its own haplotype block (**Figure 9**) (International HapMap Project, 2005).

Cholinergic genes involved in neuropsychiatric disorders

Given the role of CHT function in maintaining the releasable pool of ACh, especially during high states of cholinergic tone, the possibility exists that polymorphisms having functional consequences on CHT may contribute to various psychiatric or cardiovascular diseases. Alterations in cholinergic function have been shown to underlie various neuropsychiatric disorders such as schizophrenia, major depressive disorder, various anxiety disorders (ie. posttraumatic stress disorder, PTSD), Alzheimer's disease (AD), attention deficit hyperactivity disorder (ADHD) and myasthenic syndromes (Coyle et al., 1983; Sarter and Parikh, 2005; Tandon, 1999; Vakalopoulos, 2007). Disruptions in cholinergic transmission have been implicated in the cognitive impairments that are commonly seen in patients suffering from schizophrenia and several pharmacologic treatments which enhance cholinergic tone have been studied in various clinical trials to alleviate these impairments (Friedman, 2004; Tzavara et al., 2004). Additionally, nAChR agonists have been shown in animal models to ameliorate anxiogenic effects by increasing serotonin (5-HT) release from neurons within raphe nuclei (File et al., 2000).

Genes regulating cholinergic neurotransmission have received considerable attention in disorders of cognition and memory such as ADHD and AD (Sarter and Parikh, 2005). In AD, degeneration in basal forebrain cholinergic circuits contributes to cognitive impairments in the disorder. Single nucleotide polymorphisms in several genes including nAChR and ChAT, mediating cholinergic neurotransmission have been found to be associated with AD (Cook et al., 2004; Cook et al., 2005). Similarly, in ADHD, SNPs within subunits of nAChR (CHRNA4 and CHNRA7) have been found to be associated with the disease (Kent et al., 2001; Todd et al., 2003).

Given the role of CHT in the maintenance of ACh biosynthesis, we hypothesize that diseases that are modified by alterations in cholinergic tone may be influenced by deficits in CHT function. Thus, these CHT variants may be enriched in patients suffering from disorders due to cholinergic disruptions.

Cholinergic genes involved in cardiovascular disorders

Acetylcholine is an important regulator of cardiac function, mediating processes such as heart rate and to a lesser degree, blood pressure. Few studies have examined the role of SNPs in genes regulating cholinergic neurotransmission and the impact on cardiovascular health. Most studies have focused on post-synaptic receptors (nicotinic or muscarinic) and their roles in facilitating cholinergic transmission and its regulation of the cardiovasculature.

Regulation of blood pressure is critically important to the overall physiology of the heart and is tightly regulated predominantly by the sympathetic branch of the autonomic nervous system. Nicotinic receptors mediate the post-synaptic transmission of

both the parasympathetic and sympathetic branches of the ANS. Recently a SNP within the gamma subunit of the nicotinic receptor (CHRNG; rs2099489), encoding a synonymous SNP (Arg_{474} ->Arg_{474}) was found to be associated with higher systolic BP in subjects from the Amish Family Diabetes Study (AFDS) (McArdle et al., 2008). These results were also confirmed in a panel of subjects from the Framingham Heart Study (McArdle et al., 2008). Though this SNP does not result in an amino acid substitution, it has been hypothesized to influence expression by altering pre-mRNA splicing, mRNA stability and efficiency of translation (Parmley et al., 2006). Alternatively, it may not result in a functional consequence by be in linkage disequilibrium with a causative SNP, yet to be identified (Parmley et al., 2006).

The negative chronotropic effects of ACh on the heart after exercise and its temporal regulation of HR recovery are not well known. The effects of ACh in promoting the negative chronotropic effects of the heart are mediated by the muscarinic-2 acetylcholine receptor (M_2AChR) function to slow the heart rate down and diminish sympathetic tone to the heart via negative feedback mechanisms. In a study examining the association of M_2AChR SNPs with heart rate recovery, Hautala, et al., found an association of two M_2AChR SNPs (rs324640 and rs8191992) were associated with HR and BP differences in the exercise group vs. controls (Hautala et al., 2006). Subjects homozygous for the rs8191992 SNP exhibited higher LF-to-HF ratios indicative of reduced HRV post-exercise, a measurement of reduced vagal regulation of HR (Hautala et al., 2006). Additionally, subjects with the rs324640 SNP exhibited higher resting diastolic BP (Hautala et al., 2006). Though no functional data exists on these two SNPs

within M_2AChRs, this study showed that genetic variants within the muscarinic receptor effect HRR and HRV and may predispose patients to increased risk of fatal arrhythmias.

Our phenotypic characterization of the CHT+/- mice identified potential human phenotypes that may similarly be found in patients suffering from a number of disorders where loss of cholinergic tone has been identified as either causing or promoting pathology. The behavioral and cardiovascular phenotypes observed in CHT+/- mice demonstrate the impact of a genetic loss of CHT and its role in regulating a number of cholinergically-mediated behaviors and physiology. Thus patients carrying the hypomorphic CHT allele (Ile89Val) may suffer from a number of neuropsychiatric and cardiovascular disorders where loss of CHT function contributes to possible morbidity or mortality.

In the experiments described below, I sought to identify the association and allelic transmission of the human hypomorphic CHT allele (Ile89Val) in subjects with primary Axis I psychiatric disorders, major depressive disorder and attention-deficit hyperactivity of which deficits in cholinergic function has been reported to affect neuropsychiatric physiology. I also sought to examine a small group of subjects undergoing treadmill studies subjected to the QT prolonging drug, ibutilide to determine the role of the CHT Ile89Val hypomorph on resting and exercise HR.

Methods

Allelic Discrimination genotyping assay (TaqMan®)

An allelic discrimination assay was performed in the Vanderbilt Center for Human Genetics Research DNA Resources Core using TaqMan® SNP Genotyping Assay reagents (Applied Biosystems, Inc). Four nanograms (ng) of whole genome amplified DNA were used as template in a reaction containing 900 nM of the following primer sets for CHT (Ile89Val, rs1013940); forward (5-TGTACCAGGTTATGGCCTAGCTT-3') and reverse (5'-ACTGAGATTTGCACTTTCACTTACCT-3') amplification primers, 200 nM VIC® (5'-CAGGCACCAATTGGATA-3') and FAM® (5'-AGGCACCAGTTGGATA-3') and for the CHT 3'SNP (rs333999); forward (5'-GTGGACACACTTCTGGAGATTATACATTT-3') and reverse (5'-GTCCACGGGCCCTAATATTATATTCT-3') and 200 nM VIC® (5'-CTCTTAATAATTCCCCCCCACACT-3') and FAM® (5'-CTCTTAATAATTCACCCCACACT-3') dye-labeled probes, and 1X TaqMan® Universal PCR Master Mix. The 3'SNP has been previously described as the 3'UTR SNP (Neumann et al., 2006; Neumann et al., 2005), but our current genomic analysis place the variant 3' of the predicted polyadenylation sites and could not be identified with deposited ESTs. Thermal cycling (95°C for 10 min, followed by 50 cycles of 92°C for 15 sec and 60°C for 1 min) and product detection was accomplished using the ABI 7900HT Sequence Detection System (ABI).

Following PCR and sample scanning, genotype calls were performed using the autocall feature of the SDS 2.2 software (Applied Biosystems, Inc, Foster City, CA).

The SDS software utilizes an advanced multi-component algorithm to calculate distinct allele/marker signal contributions from the fluorescence measurements for each sample on a 384-well plate. The SNP auto-caller was set at 95% confidence interval for each sample plate and genotypes cluster plot generated for each sample set **(Figure 23A and B)** .

Selection for subjects with Major Depressive Disorder (MDD)

The clinical research protocol was approved by the Vanderbilt University Institutional Review Board. Written informed consent was obtained from all research participants prior to any research procedures. Subjects were recruited from the Adult Psychiatry Outpatient Clinic at the Vanderbilt University Medical Center. Patients were overall physically healthy and were excluded if diagnosed with any of the following Axis I and II conditions: schizophrenia, bipolar disorder, psychotic disorder; borderline, schizotypal or antisocial disorder. Additionally, subjects were excluded if having a substance abuse diagnosis within 6 months prior to the assessment.

Patients (n=110) were evaluated using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorder (DSM IV) and the Structured Clinical Interview for Axis II Personality Disorders (SCID). Patient assessments of psychiatric conditions were evaluated using the 17-item Hamilton Rating Scale for Depression (HAM-D). The mean age of the sample was 42.7 years (SD=10.7), consisting of 61% female and 39% male subjects. Race was reported at 87% Caucasian, 6.5% African-American, 4.6% Asian, 1% Hispanic and 1% other. Major depressive disorder subtype was classified as 18% atypical, 35% melancholic and 47% no subtype.

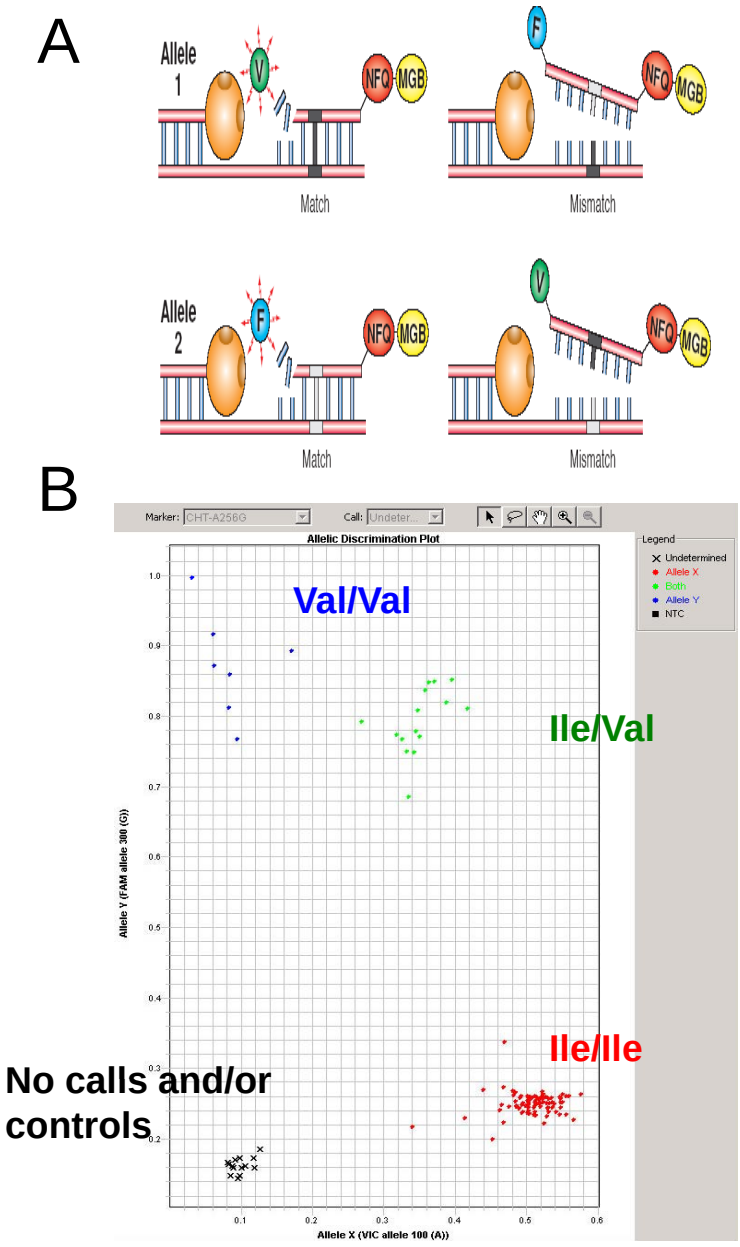


Fig. 23. ABI TaqMan® methodology and representative data plot. (A) ABI TaqMan® assay involves the use of two TaqMan® minor groove binder (MGB) probes labeled with the VIC® and FAM® dyes detecting alleles 1 and 2 respectively. During PCR, each TaqMan® MGB probe hybridizes to their respective SNP. DNA polymerase cleavage separates the reporter dye from the quencher dye resulting in a fluorescence signal thus indicating which alleles are present in the sample. **(B)** Representative data plot produces by the ABI Prism® allelic discrimination detection system. Individual genotypes are plotted based upon fluorescent intensities. **Black**, controls; **Red,** homozygous major allele; **Green**, heterozygous; **Blue**, homozygous minor allele.

Sample selection of subjects with attention deficit hyperactivity disorder (ADHD)

Subjects for the ADHD association study were recruited from Emory University and the University of Arizona with the assistance of Irwin Waldman, Ph.D. and Ian Gizer, Ph.D., Department of Psychology, Emory University, Atlanta, GA. Subjects (n=403) were recruited from 251 families at the two sites from clinics, which specialize in the assessment of childhood oppositional defiant disorders. Parents of children 4-18 were provided the Emory Diagnostic Rating Scale (EDRS), a symptom checklist developed to assess symptoms of major DSM-IV childhood psychiatric disorders. Parents were asked to rate their children's symptoms as exhibited while off medication. Parents rated the symptom on a 0-4 scale, with 0 indicating that the symptom is "not characteristic" of the child, and 4 indicating that the symptom is "very much characteristic" of the child. ADHD subtype was determined by obtaining the sum of the symptom checklist based upon parental responses.

Sample selection of subjects enrolled in the Vanderbilt exercise study

Study design and study recruitment was performed by Prince Kannakeril, MD, MSCI with the Vanderbilt University Department of Medicine, Division of Cardiovascular Medicine. Subjects (N=151) enrolled for this study were healthy human volunteers, aged 18-40. Subjects performed two submaximal recumbent bicycle exercise tests, one drug-free and a second after 10 μg/kg intravenous ibutilide. Continuous ECG monitoring is collected during both tests. Analysis of beat-to-beat changes (heart rate variability) in RR interval and QT interval during exercise and recovery is assessed. Variability in baseline or drug-induced changes will be analyzed as a function of specific

polymorphism in CHT gene expected to affect the QT interval. During the exercise periods, target HR was 120 bpm and continuous ECG monitoring and evaluation of RR interval and QT intervals were determined for HR at 90 bpm and 120 bpm. Analysis of ECG measurements occurred at rest, during exercise (HR = 90 and 120; Ex90, Ex120) and recovery (HR = 90 and 120; Rec90, Rec120). Subjects were genotyped for the hypomorphic CHT variant (Ile89Val; rs1013940) and the 3'SNP (rs333229).

Statistical analyses used in the genetic association study of Major Depressive Disorder (Vanderbilt Panel)

The multivariate permutation test (MPT) was used to estimate statistical significance of association of genotype with clinical variables (Troendle, 1996). This statistical approach was utilized because the conduction of multiple significance tests incrementally increases the family wise error (FWE), with Type 1 errors contributing to the failure of these studies. Likewise, correction methods such as the Bonferroni method are too conservative and reduce power in these studies. The MPT method does not assume independence and uses correlations among the outcome variables, resulting in increased statistical power. The MPT method tests for statistical significance by comparison of the observed statistic to an empirical distribution of the test statistic, instead of the standard distribution typically used. Further description of the utility of this statistical method can be found elsewhere (Hahn et al., 2008; Troendle, 1996)

The SAS statistical software package (SAS Institute, Inc., Cary, NC, USA) was used for all analyses with α=0.05. PROC MULTITEST was used to perform stepwise MPTs of mean differences using a trend contrast with 20,000 permutations.

Statistical analyses used for genetic association study of ADHD (Emory Panel)

Family-based analysis of association and linkage using extensions of the Transmission Disequilibrium Test (TDT), and crosstab analysis using SPSS, were conducted by Irwin Waldman and Ian Gizer, Department of Psychology, Emory University, Atlanta, GA. For the quality control group, crosstab analysis using SPSS v15 (SPSS, Inc., Chicago, IL) were conducted for quality control analysis including measures of genotype reliability. Call rates, Mendelian error rates and exact HWE tests and p-values were estimated using PEDSTATS software (Wigginton et al., 2005).

We conducted family-based analyses of association and linkage using extensions of the TDT applicable to both categorical and continuous variables and tests of moderation (Dudbridge, 2003; Waldman et al., 1999). Derived from the TDT, the family-based association test (FBAT) and pedigree-based association test (PBAT) software allow for the contribution of unaffected control subjects in the calculation of the FBAT statistic (Lange et al., 2003).

Statistical analyses used for genetic association study of ADHD (Emory Panel)

Logistic regression analysis was used to test the null hypothesis of no association between our candidate CHT gene and RR-interval and QT-interval responses when the dependent variable has a bimodal distribution with a discrete cutoff point. Assistance with newer statistical techniques for genetic analysis were provided by Marylyn Ritchie, PhD with the Vanderbilt Center for Human Genetics Research. Significance tests will be

two-tailed, with a *P*-value of <0.05 considered significance. Data was analyzed using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL).

Results and Discussion

The CHT hypomorphic allele (Val) is associated with overall symptom severity in patients with MDD

The call rate in our sample set was 98% and confirmed using a previously reported RFLP PCR assay. Allele frequency determination of the hypomorphic CHT Ile89Val in healthy controls has been reported to be 6% for the Val allele (Okuda et al., 2002). This minor allele frequency for the CHT Ile89Val variant was also confirmed in our Coriell control sample set (n=100), estimated at 6%. Interestingly, in subjects suffering from MDD (n=122) the frequency for the Val allele was calculated to be 12% **(Table 6**). For the 3'SNP, the estimated allele frequencies in subjects for MDD were similar to those reported in healthy controls (**Table 6**). There were no significant Hardy-Weinberg equilibrium (HWE) departures for the estimated genotypes.

Subphenotypic characteristics were also evaluated using MPT methods to determine association of the CHT Ile89Val alleles with symptom subtype and severity. Analysis of the MDD sample set with subphenotypic characteristics (n=110), showed a significant association of the Val allele with HAM-D-17 total score at intake or overall depression severity (*P*=0.04; OR=2.74 (1.05-7.18); **Table 7**) (Hahn et al., 2008). There appeared to be a gene dose effect as HAMD-D-17 total intake scores for homozygous major allele (Ile) and heterozygous groups (Ile/Val) were lower compared to the homozygous minor group.

Disruptions in cholinergic signaling have been implicated in mood disorders (Janowsky et al., 1983). Several genetic association studies and clinical trials of

Table 6. Genotype and allele frequencies in CHT polymorphisms in subjects with major depressive disorder (MDD) (n=110).

Polymorphism	Genotype	Frequency	Allele	Frequency
CHT Ile89Val (rs1013940)	Ile/Ile	88 (0.80)	Ile	193 (0.83)
	Ile/Val	17 (0.16)	Val	27 (0.12)
	Val/Val	5 (0.05)		
CHT 3'SNP (rs333229)	G/G	63 (0.57)	G	167 (0.77)
	G/T	41 (0.37)	T	51 (0.23)
	T/T	5 (0.05)		

Table 7. Summary of significant dose-response relationships between polymorphic Ile89Val variants and clinical functioning variables in major depressive disorder.

Gene	Polymorphic Variant	Clinical Functioning Variable	Uncorrected *P*-value	Exact *P*-value	Dose response to minor allele
CHT	Ile89Val	HAM-D 17 total score, intake	0.00018	**0.0381**	**Increase**
		HAM-D Item 11; Somatic anxiety	0.00756	0.30085	Increase
		HAM-D Item 8; Motor retardation	0.0095	0.3366	Increase

Abbreviations for Table 7: Hamilton depression rating scale (HAM-D). Adapted from Hahn MK, et al. *Genes, Brain, Behavior*, 2007.

antidepressant medications provide evidence for the role of cholinergic signaling in MDD (Furey and Drevets, 2006; Wang et al., 2004). The findings of the association of the hypomorphic CHT (Val) allele with total HAM-D-17 score and overall depression severity supports the hypothesis that altered cholinergic tone regulates multiple traits within MDD.

CHT Val allele is selectively overtransmitted with the Combined-subtype of ADHD

Subjects (Vandy sample, n=110; Emory sample, n=403) were genotyped for the hypomorphic CHT Ile89Val and the 3'SNP allele. The call rate in our sample set was 93% for the Ile89Val and 94% for the 3'SNP. Allele frequency determination of the CHT Ile89Val and the 3'SNP in healthy controls has been reported to be 6% and 23% respectively (Neumann et al., 2005; Okuda et al., 2002). The minor allele frequency for the both the CHT Ile89Val and 3'SNP variant was also confirmed in our Coriell control sample set (n=100), estimated at 6% and 24% respectively. Interestingly, in subjects suffering from ADHD (n=110) the frequency for the Val allele was calculated to be 12% **(Table 8**). There was no significant departure from HWE calculated in either sample set.

In our family-based trio analysis (n=403) of association of the ADHD diagnosis with the CHT Ile89Val and 3'SNP variants using FBAT, we found no evidence for association with either SNP across additive, dominant or recessive genetic models regardless of either analysis of affected or contrast with controls were determined. However, there was an association of the CHT Val variant with the Combined subtype of ADHD using the additive and dominant models (additive: Z=2.19, one-tailed P=0.014, R^2=0.09, OR=3.16; dominant: Z=1.97, one-tailed P=0.024, R^2=0.07, OR=2.79)

Table 8. Allele frequencies of Ile89Val and 3'SNP CHT SNPs in ADHD.

Group	Subgroup	Gender (%)		Ethnicity (%)		Genotype (n)			Allele Frequency (%)	
Ile89Val (rs1013940)		**Male**	**Female**	**White**	**Black**	**A/A**	**A/G**	**G/G**	**A**	**G**
Control panel (n=290)		121 (42%)	169 (58%)	127 (44%)	163 (56%)	260	27	3	94	6
	Caucasian (n=127)	65 (51%)	62 (49%)	127 (100%)	-	111	15	1	93	7
	Caucasian male (n=65)	65 (100%)	-	65 (100%)	-	59	5	1	95	5
ADHD panel (n=100)		76 (76%)	24 (24%)	81 (81%)	19 (19%)	78	21	1	88	12
Caucasian only (n=81)		61 (75%)	20 (25%)	81 (100%)	-	60	20	1	86	14
	Hyperactive	4 (5%)	0	4 (5%)	-	4	0	0	100	0
	Combined	41 (51%)	10 (12%)	51 (63%)	-	39	13	1	86	14
	Inattentive	13 (16%)	10 (12%)	23 (28%)	-	17	7	0	85	15
Caucasian male only (n=61)		61 (100%)	-	61	-	44	16	1	85	15
	Hyperactive	5 (9%)	-	5	-	4	1	0	90	10
	Combined	41 (67%)	-	41	-	30	10	1	85	15
	Inattentive	15 (25%)	-	15	-	10	5	0	83	17
3'SNP (rs333229)										
Control panel (n=233)		109 (47%)	118 (51%)	127 (55%)	91 (39%)	121	87	19	71	27
	Caucasian	64 (27%)	63 (27%)	127 (55%)	-	73	46	8	76	24
	Caucasian male	64 (27%)	-	64 (27%)	-	39	23	2	79	21
ADHD panel (n=100)		76 (76%)	24 (24%)	81 (81%)	19 (19%)	64	30	6	79	21
Diagnosis subtype (n=96)	Hyperactive	4 (4%)	1 (1%)	4 (4%)	1 (1%)	3	2	0	80	20
	Combined	50 (52%)	11 (11%)	52 (54%)	9 (9%)	41	18	2	82	18
	Inattentive	18 (19%)	12 (13%)	23 (24%)	4 94%)	19	7	4	75	25
Caucasian only (n=80)		60 (75%)	20 (25%)	80 (100%)	-	57	20	2	84	16
	Hyperactive	4 (5%)	0	4 (5%)	-	3	1	0	88	12
	Combined	41 (51%)	10 (13%)	51 (64%)	-	36	14	1	84	16
	Inattentive	13 (16%)	10 (13%)	23 (29%)		18	4	1	87	13
Caucasian male only (n=60)		60 (100%)	-	60 (100%)	-	46	12	2	87	13
	Hyperactive	4 (7%)	-	4 (7%)	-	3	1	0	88	12
	Combined	41 (68%)	-	41 (68%)	-	31	9	1	87	13
	Inattentive	13 (22%)	-	13 (22%)	-	11	1	1	88	12

(Table 9). Interestingly, the CHT Ile89Val SNP was also associated with the Inattentive subtype, however this association was with the major allele (Ile) and was strongest under the additive and recessive models **(Table 9).** The 3'SNP variant was not found to be associated with either overall ADHD diagnosis, nor subtype (**Table 10**).

For the overall diagnosis of ADHD, as well as the Combined and Inattentive subtypes, we conducted an omnibus test of association for all haplotypes and followed this up by determination of specific haplotypes for the observed transmission in ADHD cases versus unaffected controls or transmissions in the diagnostic subtypes versus unaffected controls and all other subtypes. All of the haplotype tests for either the ADHD diagnosis or the Inattentive subtype were non-significant (**Table 11**). In contrast, the omnibus tests yielded a significant association with the Combined subtype under an additive model (Z=8.48, P=0.037) and a trend towards an association under a dominant model (Z=8.16, P=0.086) with the haplotype comprising both Ile89Val and 3'SNP minor alleles showing the strongest associations (additive model: Z=2.65, P=0.008, R^2=0.048, OR=2.25; dominant model: Z=2.65, P=0.008, R^2=0.069, OR=2.68) (**Table 11**).

In this study, the best fit model appeared to be the additive model vs. the dominant or recessive model, indicating that having 1 or 2 copies of the risk allele increased the likelihood of possessing the Combined subtype of ADHD in an additive fashion. The preferential transmission of the CHT (Val) hypomorphic allele to the Combined subtype in the additive model may reflect the importance of cholinergic transmission in modulating both motor and attentional components of ADHD, such that the inheritance of the Val allele disrupts cholinergic tone to an extent to produce both attentional and motor deficits. Disruptions in cholinergic transmission have been

Table 9. FBAT results for association of the CHT Ile89Val and ADHD and the combined and inattentive subtypes.

Diagnosis		ADHD (offset = .14)*				Combined (offset=.06)				Inattentive (offset=.08)		
	N	Z	p	R^2	OR	Z	p	R^2	OR	Z	P	R^2
Model												
Additive	51	-.903	.817	.004	0.79	0.802	.211	.012	1.50	2.406	.016	.11
*with unaffecteds		-.004	.502	.00	1.00	2.189	.014	.092	3.16	0.834	.404	.013
Dominant	51	-.842	.800	.014	0.65	0.574	.283	.006	1.33	0.990	.322	.16
*with unaffecteds		0.090	.464	.00	1.05	1.969	.024	.074	2.79	0.736	.461	.088
Recessive	6	-.428	.666	.03	0.53	0.938	.174	.143	4.39	2.314	.021	.105
*with unaffecteds		-.285	.612	.013	0.66	1.262	.103	.26	8.54	0.659	.510	.009

Abbreviations for Table 9: Family based association test (FBAT); attention-deficit, hyperactivity disorder (ADHD). N= number of informative families. * Models in which ADHD cases were contrasted with unaffected individuals, or in which individuals with the target ADHD subtype were contrasted with both unaffected individuals and individuals diagnosed with the other ADHD subtypes. Note that as predicted the minor allele was over-transmitted to children with the Combined subtype, but that the major allele was over-transmitted to children with the Inattentive subtype and the overall diagnosis of ADHD. Reference: English BA, et al. *J Neurodevelopmental Disorder*, 2009.

Table 10. FBAT results for association of the CHT 3'SNP and ADHD and the combined and inattentive subtypes.

Diagnosis		ADHD (offset = .14)*				Combined (offset=.06)				Inattentive (offset=.08)			
	N	Z	P	R^2	OR	Z	P	R^2	OR	Z	P	R^2	OR
Model													
Additive	125	0.470	.319	.002	1.16	1.004	.158	.008	1.38	-.411	.659	.001	0.88
*with unaffecteds		0.478	.316	.002	1.17	1.001	.158	.008	1.38	-.285	.612	.001	0.91
Dominant	114	0.661	.254	.004	1.25	1.235	.108	.014	1.52	-.157	.562	.00	0.91
*with unaffecteds		0.797	.213	.006	1.32	1.366	.086	.016	1.59	0.130	.897	.045	2.19
Recessive	35	-.179	.571	.00	0.90	-.049	.520	.00	0.97	-.408	.658	.001	0.87
*with unaffecteds		-.393	.653	.004	0.79	-.314	.623	.003	0.83	-.422	.663	.002	0.87

Abbreviations for Table 7: Family based association test (FBAT); attention-deficit, hyperactivity disorder (ADHD). N = number of informative families;. * Models in which ADHD cases were contrasted with unaffected individuals, or in which individuals with the target ADHD subtype were contrasted with both unaffected individuals and individuals diagnosed with the other ADHD subtypes. Note that as predicted the minor allele was over-transmitted to children with the Combined subtype, but that the major allele was over-transmitted to children with the Inattentive subtype and the overall ADHD diagnosis. Reference: English BA, et al. *J Neurodevelopmental Disorder*, 2009

Table 11. Association of the CHT Ile89Val and 3'SNP haplotypes with ADHD and the combined and inattentive subtypes.

Diagnosis	ADHD (offset = .14)*				Combined (offset=.06)				Inattentive (offset=.08)			
	χ^2 / Z	p	R^2	OR	χ^2 / Z	p	R^2	OR	χ^2 / Z	P	R^2	OR
Model												
Additive [Multimarker]	0.457	.324				.059			-.233	.592		
Omnibus χ^2 Test	0.855	.836			8.482	.037			2.396	.494		
Test of G-C haplotype	0.742	.229	.034	1.96	2.653	.004	.425	22.41	0.779	.436	.005	1.28
Dominant [Multimarker]	0.777	.219			0.184	.427			0.861	.195		
Omnibus χ^2 Test	1.063	.900			8.159	.043			3.812	.432		
Test of G-C haplotype	0.742	.229	.034	1.96	2.653	.004	.425	22.41	1.154	.248	.029	1.87
Recessive [Multimarker]	-0.362	.641			-1.771	.962			-.558	.712		
Omnibus χ^2 Test	0.661	.882			4.764	.190			1.563	.668		
Test of G-C haplotype^	NA	NA	NA	NA	NA	NA	NA	NA	0.236	.813	.00	1.08

Legend for Table 11: N= number of informative families; NA indicates that there were too few informative families to test the target haplotype. * In all models, ADHD cases were contrasted with unaffected individuals, or individuals with the target ADHD subtype were contrasted with both unaffected individuals and individuals diagnosed with the other ADHD subtypes. Omnibus C^2 tests of all 4 haplotypes had 3 degrees-of-freedom and were evaluated using a two-tailed p-value., whereas Z tests of the hypothesized high-risk haplotype (i.e., the G-C haplotype comprising the minor alleles for both CHT SNPs) had 1 degree-of-freedom and were evaluated using a one –tailed P-value. ^For the inattentive subtype, the high-risk haplotype tested was the A-T halpotype comprising the common alleles for both SNPs. Reference: English BA, et al. *J Neurodevelopmental Disorder*, 2009

hypothesized to underlie deficits seen in animal models of both attention and hyperactivity (Gerber et al., 2001; Sarter and Parikh, 2005). The finding of the CHT Ile major allele associated with the Inattentive subtype may reflect different genetic mechanisms modulating this subtype. Although ACh plays a significant role in modulating attention, studies have also shown that the neurotransmitter NE has long been associated with modulating attention and there is strong evidence demonstrating that NE plays a role in the Inattentive subtype of ADHD (Beane and Marrocco, 2004; Biederman and Spencer, 1999).

CHT Val allele is associated with impaired HR recovery after exercise

Genotyping was accomplished in 149 of 151 subjects revealing 2 distinct genotyping groups for the hypomorphic CHT allele, Ile89Val: 124 (Ile/Ile); and 25 (Ile/Val). There were no homozygous (Val/Val) for the CHT variant. There was no difference between groups in resting HR or QTc (QT interval corrected for HR), either at baseline or after drug (**Table 12**). Prior to ibutilide, there was no difference in QT Ex or QT Rec. However, after ibutilide QT Ex was similar, but QT Rec was significantly shorter in the Ile/Val group. In this study, the CHT Ile89Val polymorphism affected the QT interval during recovery from exercise when QT has been prolonged by drug. Thus subjects carrying the hypomorphic CHT allele exhibit reduced cholinergic tone on ventricular repolarization in an exercise-dependent fashion.

These findings have significant implications for sudden death during exercise and recovery, as well as QT-prolongation by drug. For instance, the Autonomic Tone and

Table 12. CHT Ile89Val is associated with reduced HR recovery after moderate exercise in the presence of a QT-prolonging drug.

Absolute QT (ms) at HR 90 during exercise and recovery				
	Pre-Ibutilide	**Pre-Ibutilide**	**Post-Ibutilide**	**Post-Ibutilide**
	Ex90	**Rec90**	**Ex90**	**Rec90**
Ile/Ile	351±18	337±15	379±29	355±24
Ile/Val	346±14	334±14	379±27	340±17
P-Value	0.195	0.295	0.944	**0.006**

Abbreviations for Table 12: Heart rate (HR); exercise (EX); recovery (Rec).

Reflexes After Myocardial Infarction trial (ATRAMI) established that autonomic tone after an MI was a strong predictor of mortality (La Rovere, 2001). Another large study of 5,234 subjects examining HR recovery after submaximal exercise demonstrated that abnormal HR recovery strongly predicted death due to ventricular arrhythmia (Cole et al., 2000). Kannankeril et al. showed that subjects with impaired ventricular function exhibited diminished parasympathetic tone compared to subjects with normal ventricular function, and that diminished parasympathetic tone failed to produce prolonged ventricular refractoriness shown to be anti-arrhythmic (Kannankeril and Goldberger, 2002). While subjects carrying the 89Val variant failed to show statistically elevated HRs compared to controls, these subjects failed to exhibit a QT-prolonging effect with ibutilide demonstrating a possible failure of the parasympathetic contribution to changes in action potential duration and increasing the risk of fatal arrhythmias by promoting raid repolarization of ventricular myocytes. These data suggest that subjects carrying the hypomorphic 89Val allele may exhibit impaired parasympathetic tone or inability of parasympathetic tone to recover after exercise may result in an increased risk of sudden cardiac death.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

CHT Hemizygosity Produces Deficits in Cholinergically Mediated Physiology

Previous work conducted by Ferguson, et al. in CHT-/- mice demonstrated the requirement of CHT for post-natal viability. While CHT-/- mice were born at expected Mendelian ratios, CHT-/- pups appeared smaller, were largely immobile and exhibited hypoxia resulting in death within 1 hour (Ferguson et al., 2004). Electrophysiologic studies conducted at sternomastoid NMJ show that while CHT-/- pups are born with normal stores of ACh, they are unable to sustain ACh availability during increased demands, resulting in motor and respiratory paralysis (Ferguson et al., 2004). Similarly, immunofluorescence studies show that CHT-/- mice have disrupted NMJ formation with more diffuse nAChRs patterning compared to WT mice (Ferguson et al., 2004). The post-natal lethality exhibited in CHT-/- mice limited further physiologic studies on the role of CHT loss on neuromuscular and autonomic regulation. Although limited, studied conducted in CHT-/- mice demonstrated the requirement for CHT and its maintenance of ACh stores for viability.

Conversely, the generation of CHT+/- mice provided a viable murine model for expanded physiologic studies. Early studies conducted in CHT+/- mice show reduced CHT expression, but normal levels of [^{3}H]choline uptake in whole brain extracts. The findings of normal choline uptake in the presence of reduced CHT expression may reflect the increased shift of the vesicular pool of CHT to the plasma membrane to compensate for the reduced expression. Subsequent studies however have shown that CHT+/- mice

show reduced choline uptake in synaptosomal preparations of the striatum and hippocampus, areas with significant cholinergic innervation (David Lund, unpublished observations).

CHT+/- mice are born at normal Mendelian rates and appear and develop normal when compared to WT littermates. Though CHT+/- mice do not display an overt phenotype, solicitation of behaviors or pharmacologic challenges dependent upon sustainable cholinergic tone result in phenotypic differences compared to CHT+/+ mice. For instance, CHT+/- mice show an increased sensitivity to the lethal effects of HC-3 when compared to CHT+/+ mice demonstrated a reduced reserve capacity of CHT stores (Ferguson et al., 2004). CHT+/- mice also display normal spontaneous behavioral characteristics, failing to show differences in sensory-motor, motor coordination, overall locomotor activity, anxiety and spatial learning and memory tests when compared to CHT+/+ mice (Bazalakova et al., 2007). However, when CHT+/- mice are placed on a treadmill, they were incapable of sustaining exercise endurance or intensity compared to CHT+/+ mice. Given that motor function requires continuous cholinergic transmission at the NMJ, deficits in the maintenance of ACh stores due to loss of CHT reserve is predicted to produce deficits in motor function. Conversely, studies conducted in CHT mice displaying overexpression of CHT driven by a neuromuscular junction specific promoter (Hb9:CHT) show enhanced treadmill performance (David Lund, unpublished observations) demonstrating the role of CHT in maintaining cholinergic tone at the NMJ.

CHT Heterozygous Mice as Genetic Susceptibility Models of Cardiovascular Disease

Several murine models of cholinergic dysfunction have identified changes in CHT function and expression demonstrating compensatory mechanisms for CHT regulation in the maintenance of ACh transmission (Bazalakova and Blakely, 2006). The observations of reduced sensitivity to pharmacologic challenge to HC-3 and diminished exercise capacity in CHT+/- mice (Bazalakova et al., 2007) led us to search for additional physiologic functions dependent upon sustained cholinergic activation. Homeostatic regulation of HR and BP is mediated by the sympathetic and parasympathetic branches of the autonomic nervous system. Parasympathetic regulation of the heart produces direct negative inotropic and chronotropic effects, while parasympathetic-mediated regulation of endothelial nitric oxide, produce vasodilatory effects, both mediated by sustainable ACh transmission. Therefore, we hypothesized that CHT+/- mice would exhibit deficits in parasympathetic (vagal) regulation of HR and BP.

CHT+/- Mice Exhibit Tachycardia and Hypertension

CHT+/- mice develop normally into adulthood exhibiting similar weight, motor and behavioral characteristics to CHT+/+ mice under normal conditions. The lack of distinguishable phenotypes may reflect compensatory changes in CHT regulation as demonstrated by biochemical studies in the CNS (Bazalakova et al., 2007). Similar compensatory changes in CHT function and regulation have been seen in other models of cholinergic dysfunction (Bazalakova and Blakely, 2006; Xie et al., 2000), providing

evidence of the dynamic regulation of cholinergic synthesis pathways in maintaining ACh transmission.

To determine the role of CHT hemizygosity on vagal regulation of HR and BP, we utilized implantable cardiac telemetric devices in conscious mice. Our initial findings demonstrated that CHT+/- mice exhibited higher resting HRs and BP compared to CHT+/+ mice. Since both parasympathetic and sympathetic branches utilize ACh as their neurotransmitter at the preganglionic junction, we hypothesized that since the parasympathetic branch relied on ACh at both pre and post-ganglionic junctions that autonomic deficits would reflect deficits in PNS tone to the heart. Preliminary data in older CHT mice (1.5 years) showed that CHT+/- mice displayed hypersensitivity to ganglionic blockade (Mihaela Bazalakova; unpublished data) by demonstrating significant reductions in HR. These results demonstrate that CHT+/- mice exhibit nAChR hypersensitivity with the sympathetic branch as the predominant regulator of HR in CHT+/- mice. Similar effects have been seen in patients suffering from multiple system atrophy (MSA) whereby lesions within the rostral ventrolateral medulla affect autonomic regulation of HR and BP (Diedrich et al., 2002; Diedrich et al., 2003; Parikh et al., 2002). Patients suffering from MSA have been shown to have greater residual sympathetic tone as evidenced by dramatic changes in BP and HRV with the addition of the ganglionic blocker trimethephan (Diedrich et al., 2003).

The role of cholinergic neurotransmission in BP regulation is less clear. The modulation of BP control via cholinergic mechanisms seems to involve both central and peripheral mechanisms. For instance, direct microinjection of ACh into the NTS involves the activation of NO from nNOS and facilitates sympathoinhibitory responses

resulting in reduction of HR and BP (da Silva et al., 2008). Within the periphery, cholingeric blockade via atropine markedly potentiated the NOS inhibitor L-NMMA increases in MAP (Lepori et al., 2001). These studies point to the possibility of both central and peripheral effects of cholinergic regulation of BP mediated by the effects of NO and activation of the cGMP pathways resulting in vasodilation. Thus, in addition to tachycardia, the hypertensive phenotype observed in CHT+/- mice may be the result of loss of cholinergic tone mediated via NO pathways. Though CHT+/- mice displayed reduced sensitivity to PE, there were no observable differences between genotypes when examining the impact of the nitric oxide donor, SNP during the BRS experiments.

Given the findings of elevated resting HR and BP in the CHT+/- mice, it would be interesting to examine further the specific contributions of either the sympathetic and parasympathetic branches to modulating these cardiovascular parameters. Determination of baseline and pharmacologic manipulation of heart rate variability would provide useful information on the relative contributions of both branches of the ANS on HR and BP. Unlike in humans, where the HF component represents the PNS contribution to HRV, PNS modulation of HRV is seen within the LF component in mice (Gehrmann et al., 2000). Decreased HRV as reflected by reductions in the LF and HF/LF ratio has been seen in β_1-adrenergic receptor overexpressing mice and mice with reduced functional $G\alpha_i$-I_{KACh} coupling (Gehrmann et al., 2002; Mansier et al., 1996). Therefore, I would predict that the CHT+/- mice would have reduced HRV as reflected by reductions in the LF component, indicative of reduced parasympathetic tone.

Alterations in Cholinergic Synthesis and Transmission Pathways Underlie CHT+/- Cardiovascular Dysfunction

Elevations in HR and BP observed in CHT+/- mice may be explained by dysregulation of parasympathetic tone to the heart at either the presynaptic or postsynaptic level. Indeed, we observed that CHT+/- mice had reduced atrial HACU and CHT protein expression compared to CHT+/+ mice. Similarly we observed reductions in cardiac tissue levels of ACh and choline. These results lead to the hypothesis that reduced presynaptic synthesis of ACh results in altered parasympathetic tone to the heart resulting in tachycardia. At the postsynaptic level, we observed increased M_2AChR protein expression in CHT+/- mice compared to CHT+/+ mice. This may reflect a compensatory mechanism by which to maintain chronotropic and inotropic homeostasis of the heart, as these effects are similarly seen in patients and animal models of MI and HF (Brodde and Leineweber, 2004; Dunlap et al., 2003).

Though we observed a decrease atrial HACU and tissue ACh levels, the impact of partial loss of CHT on vagal release of ACh needs to be explored further *in vivo*. Dialysate ACh has been measured during vagal nerve stimulation in the cat as a measure of postsynaptic ganglionic vagal regulation of the myocardium (Akiyama and Yamazaki, 2001; Kawada et al., 2001). Similar studies conducted in CHT+/- mice would provide information on the impact of genetic loss of CHT on ACh release and disposition at the heart. Additionally, though CHT+/- mice exhibited similar M_2AChR protein expression levels compared to wildtypes, in light of hypersensitivity of bradycardic response to VNS, these results should be examined functionally via carbachol induced GTPγS stimulation of atrial preparations to reveal detailed information on the receptor-coupling

modulation of HR regulation. As previously mentioned, changes in cardiac postsynaptic M_2AChR expression and coupling have been identified in patients with HF (Dunlap et al., 2003), a condition often associated with tachycardia and elevated sympathetic tone and may represent a protective compensatory mechanism as deficits in M2AChRs have been shown to increase ventricular function to adrenergic stress (Kawada et al., 2001; LaCroix et al., 2008).

In vivo dialysis experiments examining ACh release during vagal nerve stimulation would provide detailed information on the disposition of ACh in CHT+/- and may explain why CHT+/- mice have elevated resting HRs whereas vagal tone is expected to increase. Additionally, examination of M_2AChR coupling in CHT+/- mice would reveal whether CHT+/- mice show increased coupling in addition to M_2AChR protein expression. Increased M_2AChR protein expression and coupling would be expected to result in a increased parasympathetic effect at the heart, producing bradycardia rather than tachycardia, however it would be expected that CHT+/- mice though eliciting a hyperresponsive effect during acute ACh release, would not be able to sustain this bradycardic effect given to their inability to sustain ACh synthesis with increased cholinergic demand.

The findings of differential effects of vagal nerve stimulation on HR regulation in CHT+/- mice provided evidence for the acute vs. chronic vagal activation on HR. During our acute VNS trials, CHT+/- mice exhibited an increased bradycardic effect compared to CHT+/+ mice, leading to the possibility of increased postsynaptic hypersensitivity of M_2AChRs. However, during our chronic VNS experiments, CHT+/- mice that exhibit a significant bradycardic effect were unable to sustain this bradycardic effect and showed

greater HR recovery of their baseline HRs compared to CHT+/+ mice. These finding point to possible compensatory alterations at the postsynaptic level in the heart due to changes in presynaptic CHT expression and function. Similar findings have been observed within the CNS of CHT+/- mice (Bazalakova et al., 2007). It will be of interest to conduct *in vivo* dialysis and experiments to determine the impact of CHT heterzygosity on ACh release during acute and chronic vagal nerve stimulation. Similarly, direct GTPγS stimulation experiments will determine changes in receptor-effector coupling mediating these postsynaptic effects on HR regulation.

In our *in vivo* studies examining baroreceptor reflex and heart rate recovery post-exercise challenge, CHT+/- mice demonstrated deficits blunted BRS sensitivity and reduced HRR after-exercise, both functional tests which have been shown to be independent predictors of increased mortality (Cole et al., 2000; La Rovere et al., 2008). Functionally, these studies are an index of parasympathetic tone of HR and BP regulation and in CHT+/- mice demonstrate deficits in parasympathetic tone producing increased resting HR and elevated BP. These studies, though complimenting the VNS and telemetric studies, do not rule out the possibility of elevated sympathetic tone contributing to the blunted BRS and HRR. We demonstrated that CHT+/- mice have both increased urinary NE levels and blunted HR response to the beta-adrenergic antagonist, metoprolol. These findings point to elevated sympathetic tone in CHT+/- mice, but whether this is due to a loss of direct actions of parasympathetic tone to the heart or loss of vagal feedback on sympathetic tone at the level of the brainstem remains to be elucidated. It would be interesting to see if CHT+/- mice display altered sensitivity

to the α_2-adrenergic agonist clonidine, which exerts its BP reducing properties by centrally acting mechanisms.

CHT plays a critical role in the maintenance of ACh biosynthesis and cholinergic transmission. *In vivo* physiologic studies in CHT+/- mice reveal cardiovascular phenotypes of resting tachycardia and elevated BP, both of which can result in adverse cardiovascular events. Though our studies point to reduced parasympathetic tone to the heart, genetic loss of CHT does not appear to produce overt heart failure, but may contribute to cardiovascular and neuropsychiatric pathology as a disease susceptibility gene (**Figure 24**). Our echocardiographic and histologic findings of reduced fractional shortening and cardiomyocyte hypertrophy in older CHT+/- mice are consistent with those phenotypic findings in animal models and human patients with HF (Medeiros et al., 2008; Olshansky et al., 2008), and point to the role of CHT as a potential risk factor or disease modifying gene in age-dependent cardiovascular disorders. These findings of elevated HR, blunted BRS and reduced HRR in CHT+/- mice provide insight into the modulation of cardiovascular phenotypes in patients with cardiovascular disorders. However to fully explore the reserve capacity of CHT on the autonomic regulation of cardiovascular physiology, further reduction of CHT function using either tissue specific targeted deletion of CHT in vagal efferents or virally mediated strategies to produce additional suppression of CHT activity resulting in increased sympathetically-mediated pathology. The finding of a hypomorphic allele in the human CHT gene (Ile89Val) (Okuda et al., 2002) lends us the opportunity to examine the role of this variant as a disease risk allele in patients with cardiovascular disease.

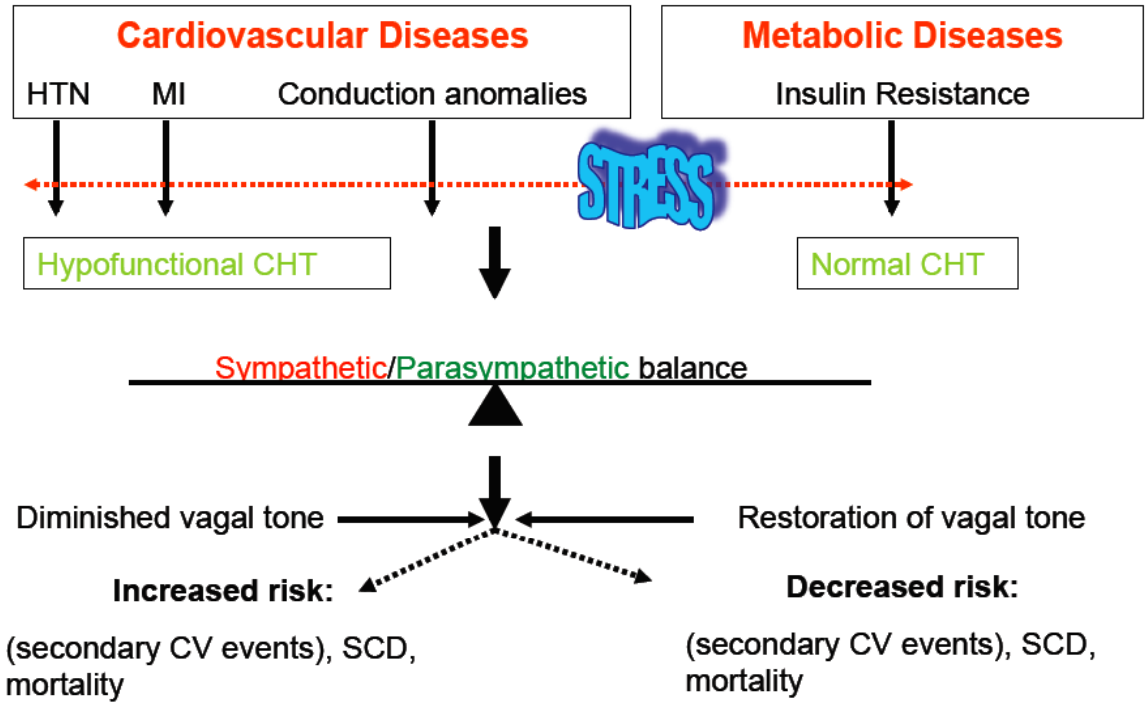


Fig. 24. Genetic loss of CHT as a disease modifying susceptibility gene. CHT mediated HACU contributes to the homeostatic functioning of parasympathetic (vagal) tone by maintenance of releasable pools of ACh. Genetic loss of CHT, reduces parasympathetic activity and increases the susceptibility to various cardiac, metabolic and neurologic insults by preventing the ability of the PNS to modulate these insults. Diminished vagal tone in addition to these physiologic and neurologic insults adds to increased mortality and progression of disease.

Though our human genotyping studies in cardiovascular populations were limited to predominantly healthy subjects, subjects suffering from MDD and ADHD show considerable co-morbidity with cardiovascular disorders such as HTN and MI. This suggests that patients with reduced functioning CHT may exhibit both brain and cardiovascular phenotypes. Our findings that the 89Val allele is preferentially overtransmitted to patients with the combined subtype (inattentive and hyperactive) of ADHD are consistent with cholinergic function in regulating both behavior and motor functions. Given the treatment refractoriness and aggressive treatment of this subtype with psychostimulants, these patients may require increased monitoring of their cardiovascular health given these associations.

In a study of healthy subjects undergoing moderate exercise testing, subjects with the 89Val allele had decreased QTc prolongation in the presence of ibutilide. These findings are intriguing in that calculation of QTc using Bazett's formula corrects for changes in QT interval due to HR, however the 89Val subjects fail to have a QT-prolonging effect with ibutilide after heart stress indicating the possibility of failure of the parasympathetic tone in increasing action potential duration (APD) in ventricular myocytes, potentially increasing the risk of fatal arrhythmias by promoting rapid repolarization of ventricular tissue post-exercise recovery (Magnano et al., 2002).

Additional genotyping studies in human subjects demonstrated an increased association of the 89Val allele with overall symptom severity in patients with major depressive disorder (Hahn et al., 2008). Although the cholinergic system receives less attention than the adrenergic system in mood, cholinergic mechanisms have been implicated in mood and mood disorders (Janowsky et al., 1983). Similarly, patients with

major depressive disorder have also been shown to exhibit reduced HRV (Brown et al., 2009). While our phenotypic data in our MDD subject group was limited to psychiatric phenotypes, it is possible that subjects with the 89Val variant exhibit reduced HRV. The reduction in vagal tone to the heart and subsequent reductions in HRV and HRR in patients with MDD, may explain why these patients are at increased risk for cardiovascular mortality (Hughes et al., 2008).

Further genetic studies in both healthy subjects and subjects with cardiovascular and neuropsychiatric disorders will reveal novel insights into the role of CHT as a disease modifying gene in a variety of cardiovascular and neuropsychiatric disorders. Interest in CHT as a disease-modifying gene affecting cognitive-related disorders is evident by several patents recently filed with the US Patent office for SNPs within the human CHT for use as genetic markers in identifying late-onset AD. Studies in rodent models showing changes in CHT activity and trafficking during sustained-attention tasks (Apparsundaram et al., 2005; Arnold et al., 2002), and the most recent finding of the CHT hypomorphic allele associated with combined subtype of ADHD implicate the role of CHT in sustaining cholinergic tone in a number of cognitive and movement disorders. Additionally the investigating the impact of CHT on HRV, BRS and HRR may serve as useful in providing a potential biomarker of cholinergic tone affecting both psychiatric and cardiovascular health. Thoroughly designed genetic studies involving the CHT Ile89Val allele in addition to other risk variants associated with cardiovascular and neuropsychiatric disorders, would provide great insights into potential gene-gene (GxG) and gene-environment (GxE) interactions as seen with the 5HTTLPR polymorphism in depression and posttraumatic stress disorder (PTSD) (Caspi et al., 2003; Xie et al., 2009).

In summary, the biochemical, physiologic and genetic association studies proposed above will provide further insight into the impact of CHT haploinsufficiency in sustaining ACh synthesis and cholinergic function in cardiovascular and neuropsychiatric disorders, and as a risk allele in modulating prognostic outcomes of these disorders.

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