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Non-Neuronal Acetylcholine Secretion by Neonatal Murine Cardiomyocytes

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Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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NON-NEURONAL ACETYLCHOLINE SECRETION BY NEONATAL MURINE CARDIOMYOCYTES

(Thesis format: Monograph)

by

William C. Fields

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Recent studies have shown that murine cardiomyocytes possess a functional intrinsic cholinergic system that can synthesize and release acetylcholine (ACh). The way by which ACh release is regulated however, is not fully understood. Classically, ACh released from neuronal sources is regulated by the vesicular ACh transporter (VAChT). We tested the hypothesis that ACh released from neonatal murine cardiomyocytes is regulated via the VAChT by pharmacologically inhibiting or genetically removing this transporter selectively from cardiomyocytes. Pharmacological inhibition of the VAChT using vesamicol (VES) revealed a significant reduction in ACh release from cultured cardiomyocytes. Cardiomyocytes from genetically-modified mice where the VAChT was selectively removed revealed similar reductions in ACh release. Similar to its role within nerve terminals, these data suggest that the VAChT serves an essential role in regulating ACh release from cardiomyocytes. These experiments suggest that non-neuronal ACh released from cardiomyocytes may act to amplify the effects of neuronally released ACh.

Keywords

Non-neuronal cholinergic system, Acetylcholine (ACh) release, Cardiomyocytes, Regulated, Vesicular acetylcholine transporter (VAChT), High affinity choline transporter (CHT1), Choline acetyltransferase (ChAT), Vesamicol (VES), Hemicholinium-3 (HEM), Pyridostigmine (PYR), Atropine (ATR), Confocal microscopy, DAF-FM, Choline/ACh fluorometric assay, HPLC-ED.
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“Nothing in the world can take the place of Persistence. Talent will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent. The slogan 'Press On' has solved and always will solve the problems of the human race.”- Calvin Coolidge

And lastly to my mother and my father; without the life lessons you taught me along the way and the constant drive for perfection that you instilled in me, my dreams would only be a fantasy of my imagination rather than a pursuit of my reality. For that I thank and love you more than you will ever know.
# Table of Contents

Abstract .................................................................................................................................................. ii  
Acknowledgments ........................................................................................................................iii  
Table of Contents ..........................................................................................................................iv  
List of Figures ........................................................................................................................................vi  
Abbreviations ....................................................................................................................................... vii  
Chapter 1 .............................................................................................................................................. 1  
1 Introduction ......................................................................................................................................... 1  
1.1 Cholinergic Neurotransmission ................................................................................................. 1  
1.2 Nicotinic and Muscarinic ACh Receptors ............................................................................... 4  
1.3 The Role of Acetylcholinesterase in Cholinergic Neurotransmission ................................ 6  
1.4 An Overview of Cholinergic Neurotransmission ....................................................................... 7  
1.5 Neural and Humoral Control of the Heart ............................................................................... 8  
1.6 The Importance of ACh in Maintaining Cardiovascular Homeostasis ..................................... 11  
1.7 The Emergence of Non-Neuronal Cholinergic Systems ............................................................ 14  
1.8 Direction, Aims, and Hypothesis .............................................................................................. 16  
Chapter 2 .............................................................................................................................................. 18  
2 Methods ............................................................................................................................................ 18  
2.1 Animals .......................................................................................................................................... 18  
2.2 Neonatal Cardiomyocyte Cultures .......................................................................................... 18  
2.3 Genotyping ....................................................................................................................................... 20  
2.4 Cell Fixation and Immunofluorescence ................................................................................... 20  
2.5 DAF-FM Fluorescence ................................................................................................................. 21  
2.6 Choline/ACh Fluorometric Assay ............................................................................................... 21
2.7 HPLC-Electrochemical Detection (HPLC-ED) .......................................................... 22
2.8 Statistical Analysis ................................................................................................. 23

Chapter 3 ............................................................................................................................ 24

3 Results ........................................................................................................................... 24
3.1 Neonatal Cardiomyocytes Secrete ACh in an Autocrine or Paracrine Fashion .... 24
3.2 Pharmacological Inhibition of CHT1 by Hemicholinium-3 Compromises ACh Release from Neonatal Cardiomyocytes .......................................................... 26
3.3 Pharmacological Inhibition of the Vesicular Acetylcholine Transporter by Vesamicol Compromises ACh Release from Neonatal Cardiomyocytes .............. 28
3.4 The Conditional Removal of the Vesicular ACh Transporter from Neonatal Cardiomyocytes ................................................................................................. 30
3.5 The Conditional Removal of the Vesicular ACh Transporter from Neonatal Cardiomyocytes Severely Compromises ACh Release ........................................ 33

Chapter 4 ............................................................................................................................ 39

4 Discussion ..................................................................................................................... 39
4.1 Overview .................................................................................................................. 39
4.2 ACh Release from Cardiomyocytes can Act in an Autocrine or Paracrine Fashion to Further Stimulate the Production of ACh .................................................. 40
4.3 ACh Release from Cardiomyocytes is Compromised Following Inhibition of CHT1 by Hemicholinium-3 ................................................................. 42
4.4 ACh Release from Cardiomyocytes is Regulated by the Vesicular ACh Transporter ............................................................................................................. 43
4.5 Conclusions and Future Directions ......................................................................... 45

References ......................................................................................................................... 47

Publication/Contributions ............................................................................................... 55

Curriculum Vitae ............................................................................................................. 67
List of Figures

Figure 1. Acetylcholine release from cultured neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments. .............................. 25

Figure 2. Acetylcholine release from cultured neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments. ............................ 27

Figure 3. Acetylcholine release from cultured neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments. .............................. 29

Figure 4. Genotyping analysis of a cardiomyocyte specific VACH T KO mouse strain. ....... 31

Figure 5. Conditional removal of the VACH T from cardiomyocytes in VACH T^{Myh-6-Cre-}\textit{flox/flox} mice .................................................................................................................................................. 32

Figure 6. Acetylcholine release from cultured VACH T^{flox/flox} and VACH T^{Myh-6-Cre-flox/flox} neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments. .................................................................................................................................... 35

Figure 7. Acetylcholine secreted by VACH T^{flox/flox} and VACH T^{Myh-6-Cre-flox/flox} neonatal cardiomyocytes was measured using a choline/ACH Fluorometric Assay. ................................. 36

Figure 8. Acetylcholine secreted by VACH T^{flox/flox} and VACH T^{Myh-6-Cre-flox/flox} neonatal cardiomyocytes was measured using HPLC with electrochemical detection (ED)............... 37
Abbreviations

ACh-Acetylcholine
AChE-Acetylcholinesterase
AChEI-Acetylcholinesterase Inhibitor
AP-Action Potential
ATR-Atropine
ANS-Autonomic Nervous System
AV-Atrioventricular
cAMP-Cyclic Adenosine Monophosphate
Carb-Carbachol
ChAT-Choline Acetyltransferase
CHF-Chronic Heart Failure
CHT1-Choline Transporter 1
CNS-Central Nervous System
DAG-Diacyl Glycerol
ED-Electrochemical Detection
GPCR-G-Protein Coupled Receptor
HEM-Hemicholinium-3
HR-Heart Rate
IP3-Inositol Triphosphate
LVEF-Left Ventricle Ejection Fraction
LVESV-Left Ventricle End Systolic Volume
mAChR-Muscarinic Acetylcholine Receptor
mAChR-2-Muscarinic Type 2 Acetylcholine Receptor
MI-Myocardial Infarction
NEO-Neostigmine
nAChR-Nicotinic Acetylcholine Receptor
OTC-On the Column
PKA-Protein Kinase A
PLC-Phospholipase C
PNS-Peripheral Nervous System
PIP2- Phosphatidylinositol 4,5-bisphosphate
PSNS-Parasympathetic Nervous System
PYR-Pyridostigmine
SA-Sinoatrial
SNP-Sodium Nitroprusside
SNS-Sympathetic Nervous System
VACHT-Vesicular Acetylcholine Transporter
VES-Vesamicol
Chapter 1

1 Introduction

1.1 Cholinergic Neurotransmission

Neurotransmitters are those chemicals endogenous to an organism which act by transmitting impulses across synaptic clefts located either between pre- and post-synaptic neurons or pre-synaptic neurons and target cells (Purves D, 2001). Acetylcholine (ACh), one of the most widely studied neurotransmitters to date, was discovered in 1926 by the German physiologist, Otto Loewi. The discovery came following the dissection of two beating frog hearts; one with an intact vagus nerve and the other lacking it. Loewi then placed both hearts into a saline solution, stimulated the vagus nerve of the intact heart, and the result was a decrease in heart rate (HR). Loewi then took a portion of the fluid from the stimulated heart and applied it to the second heart where he found a similar reduction in HR. This led Loewi to conclude that HR was being regulated by a chemical released from the vagus nerve (Purves D, 2001). The molecule that would eventually be given the name ACh, serves as a neurotransmitter for both the central (CNS) and peripheral nervous systems (PNS).

In order for ACh to function as a neurotransmitter, it must be synthesized, packaged into vesicles, released into the synaptic cleft, and finally degraded by an acetylcholinesterase enzyme (AChE). These processes are tightly regulated through three main cholinergic proteins including the high affinity choline transporter (CHT1), the choline acetyltransferase (ChAT) enzyme, and the vesicular acetylcholine transporter (VAChT).

Choline, a water soluble essential nutrient required for sustaining life, serves a role in brain development, cellular membrane structure, and the modulation of several cell signaling processes (Zeisel & Blusztajn, 1994). The uptake of choline from extracellular sources, for the purposes of cholinergic signaling, is under the regulation of the high affinity choline transporter, CHT1 (Collier & Katz, 1974; R. Birks, 1961). This protein is a 63 kDa protein which belongs to the Na⁺-dependent glucose transporter family, SLC5 (SLC5A7) (Apparsundaram, Ferguson, George, & Blakely, 2000; Okuda et al., 2000). As
the name implies, CHT1 is a membrane embedded protein that exhibits high affinity for choline molecules present within extracellular spaces (Brandon et al., 2004; Collier & Katz, 1974; R. Birks, 1961). The high affinity choline transporter localizes mainly at the membrane of synaptic vesicles, but has also been found, albeit at lower levels, at the membrane of pre-synaptic nerve endings (Ferguson et al., 2003; Ribeiro et al., 2003). The high affinity choline transporter has often been described as the rate-limiting step in the synthesis of ACh considering that the specific inhibition of CHT1 by hemicholinium-3 (HEM) results in severe deficits in cholinergic function (R. Birks, 1961; Ribeiro et al., 2003). However, under circumstances in which there is a period of prolonged neuronal stimulation, the synthesis and release of ACh is sustained as long as CHT1 is functioning properly and as long as there is sufficient choline available for uptake (Ribeiro et al., 2006). Considering CHT1 localizes predominately at the membrane of synaptic vesicles, prolonged neuronal stimulation not only increases synaptic vesicle exocytosis, but it increases the amount of CHT1 present at the plasma membrane following exocytosis. These conditions facilitate an increase in choline re-uptake by the pre-synaptic neuron which mediates increased ACh synthesis and provides the foundation required to sustain prolonged neuronal stimulation (Ribeiro et al., 2006).

Choline acetyltransferase, an enzyme often attributed as being the phenotypical marker of cholinergic neurons, is a ~69 KD (Dobransky & Rylett, 2003), monomeric, globular protein (Oda, 1999) responsible for synthesizing ACh from the precursors, choline and acetyl-CoA (Dobransky & Rylett, 2003). Acetyl-CoA, a biologically important molecule used in several biochemical reactions, is obtained through three main endogenous reactions. These reactions include glycolysis, the degradation of amino acids, and the oxidation of fatty acids (Oda, 1999). Although ChAT is located all throughout cholinergic neurons, its highest concentration exists within the nerve terminals (Oda, 1999). Choline acetyltransferase is initially produced within the cell body of cholinergic neurons before being transported to the nerve terminal through axoplasmic transport (Dziegielewska et al., 1976; Frizell, Hasselgren, & Sjostrand, 1970). Synthesis of ACh by ChAT commences in the cytoplasm of nerve terminals (Govindasamy et al., 2004; Oda, 1999; Parsons, 2000; M. A. Prado et al., 2002; V. F. Prado, Roy, Kolisnyk, Gros, & Prado, 2013). Synthesis occurs through a single step reaction in which an acetyl group
from acetyl-CoA is transferred to a single molecule of choline (Govindasamy et al., 2004; Oda, 1999; Yamada, Imajoh-Ohmi, & Haga, 2012). It has also been shown that the open reading frame of the VAChT, another important protein used in cholinergic signaling, lies within the first intron of the ChAT gene (Alfonso, Grundahl, McManus, Asbury, & Rand, 1994; Bejanin, Cervini, Mallet, & Berrard, 1994). This may be of particular importance as it could suggest that the expression of ChAT and the VAChT are coregulated within cholinergic neurons.

The VAChT is a ~75 kDA protein (Alfonso, Grundahl, Duerr, Han, & Rand, 1993; Roghani et al., 1994; Varoqui & Erickson, 1996) responsible for loading ACh synthesized by ChAT into acidic secretory vesicles (Parsons, Prior, & Marshall, 1993; Usdin, Eiden, Bonner, & Erickson, 1995). The VAChT, a member of the solute carrier family, SLC18, is a protein found in both neuronal cell bodies, as well as the nerve terminals of cells containing the ChAT protein (Roghani, Shirzadi, Butcher, & Edwards, 1998; Schafer, Eiden, & Weihe, 1998). The VAChT consists of 12 transmembrane spanning domains which orient themselves into two main bundles such that the amino-terminus and carboxyl-terminus are both directed towards the cytosolic side of the cell (V. F. Prado et al., 2013). The two main bundles undergo a back and forth-like motion such that the substrate-binding site is exposed to either the cytoplasm of the cell or the interior of the synaptic vesicle (Nguyen, Cox, & Parsons, 1998). This motion allows ACh to be packaged into acidic vesicles through the exchange of two luminal protons per cytoplasmic molecule of ACh (Nguyen et al., 1998).

The VAChT mediated storage of ACh can be pharmacologically inhibited through the use of vesamicol [(-)-trans-2-(4-phenylpiperidino) cyclohexanol] (VES) (V. F. Prado et al., 2013; Prior, Marshall, & Parsons, 1992). Vesamicol is a tertiary amine that can traverse the cell membrane and bind to the VAChT such that it inhibits the transport of ACh (Nguyen et al., 1998; Parsons et al., 1993). Inhibition by VES has been shown to be non-competitive in nature indicating that its inhibitory actions take place at a site external to the ACh binding site (Bahr, Clarkson, Rogers, Noremberg, & Parsons, 1992).
1.2 Nicotinic and Muscarinic ACh Receptors

Nicotinic ACh receptors (nAChRs), a class of integral membrane proteins, are receptors which serve as ligand gated ion channels within cholinergic neurotransmission (Chesher & James, 1966; Kalamida et al., 2007). Nicotinic ACh receptors are composed of 5 transmembrane subunits organized around a central pore and can vary in function depending on the polypeptide subunits that are present (α1-10, β1-4, γ, δ, and ε) (Lindstrom, 1997). Although all nAChRs are composed of 5 transmembrane subunits organized around a central pore, these receptors can be further divided into neuronal and muscular subtypes (Marshall, 1981). Neuronal subtypes are those receptors that associate with the CNS, the PNS, and non-neuronal ACh sources (Kalamida et al., 2007). In contrast, muscular subtypes, as the name implies, are those receptors that associate with the neuromuscular junction and act to mediate muscular contraction (Kalamida et al., 2007). Although nAChRs serve an important role within cholinergic neurotransmission, particular focus will be placed on the role muscarinic acetylcholine receptors (mAChRs) serve in such transmission.

Muscarinic acetylcholine receptors, a class of G-protein coupled receptors (GPCRs), are membrane embedded proteins that are present within various cell types, including neurons, and are responsible for mediating several diverse functions within an organism (Ashkenazi, Peralta, Winslow, Ramachandran, & Capon, 1988). Named as such because of their increased sensitivity to muscarine, five different mAChR subtypes have been identified (M1-M5) (Bonner, Young, Brann, & Buckley, 1988; Hulme, Birdsall, & Buckley, 1990; Kubo et al., 1986; Liao et al., 1989). All muscarinic receptor subtypes are composed of seven transmembrane spanning domains with a carboxyl-terminus directed intracellularly and an amino-terminus directed extracellularly (Dhein, van Koppen, & Brodde, 2001). The ligand binding site of these receptors are situated within the outer half of the membrane embedded portion of the receptor protein (Dhein et al., 2001). Ligand binding and subsequent activation of the various receptor subtypes can produce differing responses depending on the G-protein the receptor subtype is coupled to (Dhein et al., 2001). Odd numbered muscarinic receptor subtypes (M1, M3, and M5) for instance, are coupled to Gq/11 G-proteins, which when activated, results in phospholipase
C (PLC) stimulation (Dhein et al., 2001; Ishii & Kurachi, 2006). Activation of PLC initiates a cascade of events beginning with the hydrolysis of Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to its constituents, diacyl glycerol (DAG) and inositol triphosphate (IP$_3$) (Dhein et al., 2001; Ishii & Kurachi, 2006). Following PIP$_2$ hydrolysis, DAG acts as a second messenger to activate Protein Kinase C, while IP$_3$ acts to increase intracellular calcium levels (Dhein et al., 2001; Ishii & Kurachi, 2006). In contrast, even numbered receptor subtypes (M2 and M4) are coupled to G$_{i/o}$ G-proteins, which when activated, serve to inhibit adenylyl cyclase activity (Nathanson, 1987). Consequently there is decreased production of cyclic adenosine monophosphate (cAMP) and a subsequent decrease in Protein Kinase A (PKA) activity (Dhein et al., 2001; Ishii & Kurachi, 2006). G$_{i/o}$ G-proteins also mediate the activation of G protein-gated potassium channels resulting in the hyperpolarization of excitable cells (Kim et al., 1989; Logothetis, Kurachi, Galper, Neer, & Clapham, 1987; Yatani et al., 1988).

There are five different mAChR subtypes which exist and a particular tissue is capable of expressing multiple receptor subtypes at any given time (Brodde & Michel, 1999). For instance, the heart expresses, albeit at different levels, both M2 and M3 receptor subtypes (Sharma, Colecraft, Rubin, & Sheu, 1997; Z. Wang, Shi, & Wang, 2004; Yang, Yeh, Sung, Chen, & Wang, 1992). The M2 receptor subtype, denoted as the muscarinic type-2 ACh receptor (mAChR-2), is the predominant receptor subtype present within the heart. The M3 receptor subtype seems to serve only a minor role as it has been described as carrying-out functions associated mainly with the coronary circulation (Dhein et al., 2001). For this reason, the mAChR-2 and the effects it exerts on the heart are of particular importance.

Muscarinic type-2 ACh receptors can be activated by a plethora of different agonists including ACh and carbachol (Carb) (Brodde & Michel, 1999). When these receptors are activated at the heart, a reduction in heart rate (chronotropy), force of contraction (inotropy), and conduction velocity (dromotropy) ensues (Brodde & Michel, 1999). More specifically, activation of these receptors at the sinoatrial node (SA) elicits an increase in outward potassium conductance, while decreasing the inward conductance of calcium and sodium ions. Consequently, SA nodal cells hyperpolarize and reduce the rate at
which these cells spontaneously depolarize. The net result is a reduction in the rate of action potential (AP) firing from these cells (Giessler, Dhein, Ponicke, & Brodde, 1999). Furthermore, activation of mACHRs-2 produces a shortened AP within the atria, as well as a decrease in the velocity at which the AP propagates through the atrioventricular (AV) node (Giessler et al., 1999). Lastly, activation of these receptors can elicit a slight reduction in the force at which the ventricles contract, as long as the cAMP pathway has been stimulated beforehand in a phenomenon termed accentuated antagonism (Brodde & Michel, 1999; Giessler et al., 1999; Levy, 1984). The effects produced by mAChR-2 activation can be competitively inhibited through the use of atropine (ATR), a non-specific muscarinic receptor antagonist (Bhattacharjee, Pomponio, Evans, Pervitsky, & Gordon, 2013).

1.3 The Role of Acetylcholinesterase in Cholinergic Neurotransmission

Acetylcholinesterase, a hydrolase enzyme located within the synaptic cleft of cholinergic neurons, serves to hydrolyze ACh into acetic acid and choline (Sussman et al., 1991; Pohanka, 2011). The enzymatic breakdown of ACh is a crucial process required by organisms to maintain cholinergic neurotransmission. The active site of these enzymes is composed of two subsites including the anionic subsite, as well as the esteratic subsite (Sussman et al., 1991). The anionic subsite is responsible for interacting with the quartenary amine of the ACh molecule to ensure the molecule is in the correct orientation for subsequent hydrolysis (Sussman et al., 1991). The esteratic subsite however, is the portion of the active site where the ester bond joining acetic acid and choline together is broken through hydrolysis. This site contains three essential amino acids including serine, histidine, and glutamate and is often referred to as the catalytic triad (Silman & Sussman, 2008; Sussman et al., 1991). The hydrolysis of ACh by AChEs to acetic acid and choline can be slowed however, through the use of acetylcholinesterase inhibitors (AChEIs). The method by which AChEIs exerts their inhibitory effects depends on the type of AChEI present. The two AChEIs of particular importance, pyridostigmine (PYR) and neostigmine (NEO), act as reversible inhibitors.
to the AChE enzyme (Androne, Hryniewicz, Goldsmith, Arwady, & Katz, 2003; Miller, Van Nyhuis, Eger, Vitez, & Way, 1974). Although both inhibitors act in a similar fashion, PYR, compared to NEO, requires more time to reach peak onset, but its actions as a cholinesterase inhibitor are of a longer duration (Miller et al., 1974; Smith, Mead, & Unna, 1957).

### 1.4 An Overview of Cholinergic Neurotransmission

After discussing the roles of CHT1, ChAT, the VACHT, nAChRs, mAChRs, AChEs and AChEIs in cholinergic neurotransmission, a broader picture depicting how cholinergic neurotransmission proceeds can be put forth. The initial stage of cholinergic neurotransmission begins in the cytoplasm of the nerve terminal where ACh is synthesized through the enzymatic actions of ChAT (Dobransky & Rylett, 2003). Following its synthesis, ACh is then packaged into acidic vesicles through the actions of the VACHT (V. F. Prado et al., 2013). Once ACh is packaged into acidic vesicles, ACh is ready for release into the synaptic cleft where it can bind to nAChRs or mAChRs to elicit a given response. In order for the vesicular contents to be released into the synaptic cleft in a synchronous fashion however, an AP must first be generated at the cell body of the neuron. Following its generation, the AP then propagates to the nerve terminal (Hall, 2010; Katz, 1969). Depolarization of the nerve terminal results in the opening of voltage-gated calcium channels causing an influx of extracellular calcium into the pre-synaptic neuron (Katz, 1969). Calcium influx in turn causes ACh-containing vesicles to fuse with the membrane of the pre-synaptic neuron at which point ACh is released into the synaptic cleft through a process known as exocytosis (Hall, 2010; Katz, 1969). Depending on the location of the synapse, ACh released into the synaptic cleft is free to act on either nAChRs (Guo, Liu, Sorenson, & Chiappinelli, 2005) or mAChRs (Abramochkin et al., 2010). These effects are short lived however, because of the actions of the AChE enzyme. These enzymes work at an extremely rapid rate to hydrolyze ACh to acetic acid and choline (Minic, Molgo, Karlsson, & Krejci, 2002; Soreq & Seidman, 2001). Following the enzymatic breakdown of ACh, choline can be recycled back into the nerve
terminal through the actions of CHT1 (Collier & Katz, 1974; R. Birks, 1961) where it can then be utilized for the synthesis of additional molecules of ACh.

1.5 Neural and Humoral Control of the Heart

The pumping and subsequent delivery of blood through the use of a connected circulatory system is critical for the delivery of oxygen and essential nutrients to each and every cell of the body (Vadakkumpadan et al., 2010). The delivery of blood can be altered however, in order to meet the metabolic demands of the body by altering vascular resistance or by altering the heart’s inotropic and chronotropic responses. The frequency and strength at which these contractions occur is regulated by both a system intrinsic to the heart, as well as system extrinsic to the heart (Randall, Evans, Billman, Ordway, & Knapp, 1981).

Intrinsic control of the heart refers to the hearts’ ability to regulate its inotropic and chronotropic responses in the absence of external neural control. Intrinsic control of the heart is regulated by a specialized group of cells often referred to as pacemaker cells (Tessadori et al., 2012). Pacemaker cells are responsible for initiating and distributing electrical impulses in a coordinated fashion throughout the heart (Tessadori et al., 2012). In other words, the heart can be described as having automaticity or self-excitability, since cardiomyocytes are capable of initiating spontaneous electrical impulses in the absence of external neural control. The spontaneous depolarization of pacemaker cells is the result of changes in membrane conductance to sodium, potassium, and calcium ions (Jones Bernardes Graceli, 2012). Once pacemaker cells reach threshold, an impulse is generated where it then propagates through internodal pathways (Kafer, 1991). Propagation through these pathways allows for atrial depolarization followed by atrial contraction (Kafer, 1991). During atrial contraction, the impulse propagates to a specialized group of cells located within the atrial septum known as the AV node (Chi et al., 2008). While the AV node depolarizes, repolarization of both the SA node and the internodal pathways commences (Nada et al., 2013). Following a slight delay at the AV node to allow for ventricular filling (Chi et al., 2008; Marger et al., 2011), the impulse then propagates through the bundle of His, as well as the left and right bundle branches,
before finally reaching the Purkinje fibers (Chi et al., 2008). Depolarization of the
Purkinje fibers causes ventricular contraction from the apex of the heart to the base of the
heart resulting in the expulsion of blood from the ventricles (Chi et al., 2008). During
ventricular contraction, the atria rest and undergo repolarization (Nada et al., 2013).
Following ventricular contraction, the AV node, the bundle of His, and the Purkinje
fibers repolarize while the ventricles relax (Nada et al., 2013). In some cases however,
the pacemaker cells of the SA node lose their automaticity. Considering that other cells in
the heart are self-excitable, the role of the pacemaker can be assumed by these cells,
albeit at a slower rate. The first area of the heart to embrace this role once pacemaker
cells lose their automaticity, are the cells of the AV node (Marger et al., 2011). If for
some reason the cells of the AV node also lose their automaticity, the role can be
assumed by the bundle of His and the Purkinje fibers (Chay & Lee, 1984). Under normal
circumstances, the self-excitability of the AV node, the bundle of His, and the Purkinje
fibers are much slower than the pacemaker cells of the SA node (Tessadori et al., 2012).
Consequently, APs generated by the SA node are able to propagate through the
conduction system and excite the AV node, the bundle branches, and the Purkinje fibers
before these areas have a chance to generate their own spontaneous AP (Tessadori et al.,
2012). For this reason, the spontaneous generation of APs by the SA node tends to be
approximately 100 times per minute but because of extrinsic regulation, this rate is
modified to approximately 70 times per minute.

Extrinsic control of the heart refers to the organism’s ability to externally regulate heart
function through the release of neurotransmitters [ie. Norepinephrine (NE) and ACh]
from the autonomic nervous system (ANS) (Vaseghi & Shivkumar, 2008). The ANS, a
division of the PNS, is responsible for controlling involuntary and visceral functions
(Chen, Wasmund, & Hamdan, 2006). At the heart specifically, the ANS is responsible for
regulating the hearts’ chronotropic, inotropic, and dromotropic effects through the
coordinated interplay of its two subdivisions (Vaseghi & Shivkumar, 2008) which
include the sympathetic (SNS) and parasympathetic nervous systems (PSNS).

The SNS is a division of the PNS often referred to as the “fight-or-flight” system and its
actions predominate over the PSNS during time of physiological stress (Floras, 2009).
With reference to the heart, adrenergic nerves of the SNS originate in the thoracic and lumbar regions of the spine before eventually innervating the atria, the ventricles, and the ventricular conducting system (Camici, 2002; Chen et al., 2006; Ieda & Fukuda, 2009; Mitchell, 1953). During times of physiological stress, an increase in SNS activity causes an increase in NE secretion from adrenergic fibers. Consequently, there is an increased concentration of circulating NE which can be utilized for the activation of $\beta$-adrenergic receptors (Camici, 2002; Parati & Esler, 2012). $\beta$-adrenergic receptors are a class of GPCRs that are activated by catecholamines including epinephrine and NE (Ablad et al., 1974). Activation of these receptors increases the level of activity in the c-AMP-dependent pathway (Bristow, Hershberger, Port, Minobe, & Rasmussen, 1989). In short, activation of adenylyl cyclase by the $G_\text{s}$ alpha subunit of the G-protein complex increases cAMP production and ultimately causes an increase in PKA activity (Bristow et al., 1989). Activation of PKA leads to several downstream effects at the heart including those effects aforementioned (Vaseghi & Shivkumar, 2008).

The PSNS however, is a division of the PNS often referred to as the “rest-and-digest” system and its actions predominate over the SNS during times of rest and relaxation (Stewart, Huang, & Fisher, 2012). In contrast to the SNS, the vagus nerve of the PSNS originates in the brainstem and innervates the SA/AV nodes, the atria, the ventricular conducting system, but only sparsely innervates the ventricles (Blotnick, Hamra-Amitai, Wald, Brenner, & Anglister, 2012; Chen et al., 2006; Hildreth, Anderson, & Henderson, 2009; Mitchell, 1953). Activation of the PSNS elicits an increase in ACh release from cholinergic neurons. Consequently, there is an increase in mAChR-2 activation resulting in an increased cholinergic tone (Abramochkin et al., 2010; Minic et al., 2002; Wessler & Kirkpatrick, 2008). Like $\beta$-adrenergic receptors, mAChRs-2, as previously described, are a class of GPCRs that regulate chronotropy, inotropy, and dromotropy (Brodde & Michel, 1999; Giessler et al., 1999). In contrast to $\beta$-adrenergic receptors however, mAChRs-2 work to decrease those effects previously mentioned (Brodde & Michel, 1999; Giessler et al., 1999) It should also be noted that chemical stimuli present within the circulation (ie. tissue metabolites, $\text{CO}_2$, $\text{O}_2$, and adenosine) can effect cardiac function by altering the degree of neural outflow from the SNS and the PSNS (Thomas, 2011).
1.6 The importance of ACh in Maintaining Cardiovascular Homeostasis

Cholinergic neurotransmission plays an important role in maintaining cardiovascular health considering that alterations in cholinergic protein expression and/or a withdrawal of PSNS activity elicits several cardiovascular deficits. Although decreased cholinergic tone results in deficits including left ventricular dysfunction (Lara et al., 2010), decreased HR variability (Serra, Costa, Teixeira De Castro, Xavier, & Nobrega, 2009), and cardiac remodeling (Li et al., 2004), normal cardiovascular function can be restored, or at least improved, following AChE inhibition or treatment with chronic vagal stimulation (Lara et al., 2010; Li et al., 2004).

Both animal studies, as well as human studies, have shown that AChE inhibition can be beneficial in restoring normal cardiovascular function in models with cardiac dysfunction. For instance, those mice experiencing a reduction in the level of functioning VACHT protein (VACHT KD\textsuperscript{HOM}) to approximately 30% of normal levels, presented with decreased levels of peak calcium, as well as a decrease in the rate of calcium decay (Lara et al., 2010). Inefficient calcium handling by cardiomyocytes has been shown to have a significant influence on the normal physiology of these cells (Bers, 2002, 2006). Furthermore, altered calcium handling is considered to be a key biological marker for chronic heart failure (CHF) and several cardiac pathologies (Vahl, Bonz, Timek, & Hagl, 1994). In addition to altered calcium handling, VACHT KD\textsuperscript{HOM} mice exhibited significant reductions in left ventricular fractional shortening, indicative of left ventricular dysfunction (Lara et al., 2010). Interestingly, peak calcium and calcium decay, as well as left ventricular fractional shortening, were all restored to WT levels following AChE inhibition by PYR (Lara et al., 2010). These results suggest that cholinergic neurotransmission is critical for maintaining normal cardiovascular function, since a decrease in the level of functioning VACHT protein produced a phenotype consistent with heart dysfunction which was restored following AChE inhibition. The beneficial effects of AChE inhibition on cardiovascular function were also shown in a study by Okazaki et al., 2010 in which rats with CHF were treated with the AChE inhibitor donepezil. When these rats were subjected to AChE inhibition using donepezil, their left ventricular end diastolic
pressure (LVEDP) and heart weights were significantly reduced compared to controls. Similarly, neurohumoral factors associated with the SNS, including NE and epinephrine, were significantly reduced following treatment with donepezil. (Okazaki, Zheng, Li, & Sugimachi, 2010). Interestingly, the beneficial effects of AChE inhibition have been described in clinical trials as well (Serra et al., 2009). One such trial showed that AChE inhibition by PYR in patients with CHF elicited an increase resting HR variability and decreased the incidence of ventricular arrhythmias, while also improving HR recovery following exercise (Serra et al., 2009). Furthermore, a recent study examining the correlation between AChE inhibition and myocardial infarctions (MI) revealed that the use of AChEI was correlated to a reduction in the risk of MI and mortality rate (Nordstrom, Religa, Wimo, Winblad, & Eriksdotter, 2013). Interestingly, Nordstrom et al., 2013 reported that this correlation was even stronger when increasing doses of the AChEI were utilized.

Similar to the beneficial effects of AChE inhibition on cardiovascular function, studies involving chronic vagal stimulation have also shown to be quite beneficial. A study performed by Li et al., 2004 showed that when rats with CHF underwent chronic vagal stimulation, there was a significant decrease in both hemodynamic changes, as well as heart weights, compared to control rats. Li et al., 2004 also reported that when rats with CHF underwent chronic vagal stimulation, there was a marked improvement in long-term survival rate by preventing the progression of pumping failure and cardiac remodeling. A study performed by Sabbah and Cohen in 2005 in which canines were induced with a MI through the use of an intracoronary embolism, revealed that there was a significant improvement in left ventricular hemodynamics following chronic vagal stimulation compared to control canines. Sabbah and Cohen (2005) also showed that concomitant use of a β-blocker, along with chronic vagal stimulation, resulted in a significant increase in left ventricular ejection fraction (LVEF), as well as a significant decrease in left ventricular end systolic volume (LVESV) compared to canines treated simply with a β-blocker (Sabbah HN & Cohen U, 2005). It has also been reported that there was a significant increase in proinflammatory cytokines in models of CHF, including tumor necrosis factor alpha and interleukin 6, both of which are highly correlated with morbidity and mortality (H. Wang et al., 2003). Interestingly, there is a reduction in those
proinflammatory cytokines aforementioned following chronic vagal stimulation (H. Wang et al., 2003). Clinical trials performed on individuals whose LVEF was less than 35% also reaped the beneficial effects of chronic vagal stimulation. In these patients, electrical pulser were implanted in such a fashion to ensure that vagal stimulation occurred at specific intervals based on the patient’s prior health. Data acquired 3 months following implantation showed that chronic vagal stimulation significantly reduced basal HR and significantly improved the distance in which these patients could travel during a six minute walk test (De Ferrari, Sanzo, & Schwartz, 2009). Furthermore, data acquired one year post implantation revealed a significant increase in the patient’s LVEF (De Ferrari et al., 2009). Not only did these data show the beneficial effect of chronic vagal stimulation on cardiovascular function, but patient interviews following this treatment also revealed that there was a general improvement in their quality of life (De Ferrari et al., 2009).

Although those studies mentioned above show how AChE inhibition and chronic vagal stimulation are beneficial in restoring or maintaining normal cardiovascular function, other studies have shown more generally just how important the PSNS and its associated proteins are in maintaining cardiovascular homeostasis. For instance, it has been shown that mAChR-2 knockout mice display impaired ventricular function, as well as an increased susceptibility to cardiac stress and cardiomyocyte remodeling (LaCroix, Freeling, Giles, Wess, & Li, 2008; Lara et al., 2010). Furthermore, a study performed by Nolan et al., 1992 revealed a significant correlation between the degree of parasympathetic impairment and the severity of left ventricular dysfunction. Finally, it has been shown in both animal models and humans with CHF, that there is cholinergic transdifferentiation of the cardiac SNS. Transdifferentiation of the cardiac SNS is the result of several factors being secreted from failing cardiomyocytes which switch neurons normally sympathetic in nature, to cholinergic neurons by altering the neurotransmitter that is released (Kanazawa et al., 2010). Inhibiting this transdifferentiation resulted in increased mortality within these mice.
1.7 The Emergence of Non-Neuronal Cholinergic Systems

If one considers that essentially all living cells, including epithelial cells, endothelial cells, immune cells, etc., have the ability to synthesize ACh, then the idea of ACh acting solely as a neurotransmitter must be revised (Wessler & Kirkpatrick, 2008). Evidence supporting the synthesis of ACh by multiple non-neuronal cell types has been confirmed through several experiments. Such experiments have revealed ChAT immunoreactivity and ChAT enzymatic activity, as well as the intracellular presence of ACh in non-neuronal cells (Wessler & Kirkpatrick, 2008). Although ACh has been found in several non-neuronal sources, an argument suggesting that its presence in these sources is due to neuronal contamination could be made. However, based on several findings from the last few decades, the possibility of non-neuronal contamination by neuronal sources can be ruled out. Two such studies were performed on isolated epithelial cells, as well as immune cells, where the presence of both ChAT mRNA and ChAT protein were found intracellularly (Grando et al., 1993; Klapproth et al., 1997). Several other studies have provided evidence supporting the work completed by Grando et al., 1993 and Klapproth et al., 1997, where they were able to detect ChAT within non-neuronal cells by using antibodies targeted against it (Wessler & Kirkpatrick, 2008). Perhaps the most convincing finding comes from the human placenta and its ability to synthesize, store, and release ACh in the absence of cholinergic innervation (Wessler et al., 2001). Finally, studies performed by Kao et al., 1976 and Schlereth et al., 2006 have provided evidence refuting the argument that ACh present within non-neuronal sources was due to neuronal contamination. In these two studies, botulism toxin was used to inhibit the exocytotic release of ACh. When dermal microdialysis was utilized following this treatment, it was determined in vivo, that human skin cells could secrete non-neuronal ACh (Kao, Drachman, & Price, 1976; Schlereth et al., 2006). Considering the studies aforementioned, it becomes apparent that non-neuronal cells possess the ability to synthesize, store, and release ACh.

Although several functions have been attributed to the non-neuronal release of ACh, perhaps its role in signal transduction is most important (Wessler & Kirkpatrick, 2008). As a signal transducing molecule, ACh can act through autocrine and paracrine
mechanisms to regulate basic cellular functions including cell proliferation, cell differentiation, and cell-cell contact (Wessler, Herschel, Bittinger, & Kirkpatrick, 2007). Its role in signal transduction has been revealed in several physiological systems, as well as pathological conditions, including the placenta (Wessler et al., 2007), the pancreas (Rodriguez-Diaz et al., 2011), the colon (Yajima, Inoue, Matsumoto, & Yajima, 2011), lymphocytes (Kawashima & Fujii, 2004), and a variety of different cancers (Wessler & Kirkpatrick, 2008; Yajima et al., 2011). In the placenta for example, non-neuronal ACh acts through nAChRs to regulate blood flow and fluid volume in placental vessels, the uptake of nutrients, and vascularization during placental development (Wessler et al., 2007). In the pancreas, non-neuronal ACh released by the alpha cells serves as a feed-forwarding signal to sensitize the beta cell response to increasing glucose concentrations, thereby limiting glucose fluctuations within the plasma (Rodriguez-Diaz et al., 2011). Furthermore, it has been suggested that the intracellular signaling pathways activated by ACh may promote long-term survival of beta cells through the use of tropic factors (Rodriguez-Diaz et al., 2011). In the colon, non-neuronal ACh released from colonocytes, in conjunction with propionate stimulation, serves as a signaling molecule which can act in a paracrine fashion through muscarinic receptors to regulate chloride secretion (Yajima et al., 2011). Chloride secretion in the colon is critical to maintaining normal physiological function as it plays an essential role in regulating mucosal hydration (Barrett & Keely, 2000). Finally, studies have revealed the synthesis and release of non-neuronal ACh from tumor cells (Song & Spindel, 2008) and lymphocytes (Kawashima & Fujii, 2004). Tumor cells, such as those found in lung cancers, have revealed that non-neuronal ACh can act in an autocrine or paracrine fashion to stimulate an increase in tumor cell growth (Song & Spindel, 2008). Furthermore, it has been shown that lymphocytes possess an intrinsic non-neuronal cholinergic system as well (Kawashima & Fujii, 2004). This system has been shown to serve a role in the cholinergic anti-inflammatory pathway by regulating immune function as well as local circulation (Kawashima & Fujii, 2004; Rosas-Ballina et al., 2011).

Interestingly, recent evidence has revealed that the machinery required for the synthesis and release of ACh is present within cardiomyocytes themselves (Kakinuma, Akiyama, & Sato, 2009; Rana et al., 2010; Rocha-Resende et al., 2012). Results from these studies
have shown that the VAChT localizes in the perinuclear region of cardiomyocytes (Kakinuma et al., 2009; Rana et al., 2010; Rocha-Resende et al., 2012). Similarly, these studies have shown that ChAT localizes in and around the nucleus, while CHT1 localizes at the plasma membrane (Kakinuma et al., 2009; Rana et al., 2010; Rocha-Resende et al., 2012). The presence of the VAChT, ChAT, and CHT1 and their locations throughout cardiomyocytes puts them in an ideal position to synthesize and secrete ACh. Since it has been shown previously that cardiomyocytes can in fact secrete ACh (Kakinuma et al., 2009; Rocha-Resende et al., 2012), it would be interesting to determine the mechanism by which cardiomyocytes are regulating this release.

1.8 Direction, Aims, and Hypothesis

As mentioned previously, ACh release at the heart is essential in maintaining cardiovascular function. More specifically, AChE inhibition, as well as chronic vagal stimulation, has been shown to improve left ventricular function in models of CHF. These findings are perplexing as parasympathetic innervation is found mainly at the SA/AV node and only sparsely at the ventricles. Based on the recent emergence of a non-neuronal cholinergic system within cardiomyocytes, (Kakinuma et al., 2009; Rana et al., 2010; Rocha-Resende et al., 2012) along with their ability to secrete ACh (Kakinuma et al., 2009; Rocha-Resende et al., 2012; A. Roy, Fields, W.C., Rocha-Resende, C., Resende, R.R., Guatimosim, S.,Prado, V.F.,Gros, R., and Prado, M.A. , 2013), these results taken together could suggest that this system serves a physiologically important role in the heart. To better understand how a non-neuronal cholinergic system functions in the heart, the aim of this project was to determine the mechanism regulating ACh release from cardiomyocytes. Considering ACh released from cholinergic neurons is regulated by the VAChT (de Castro et al., 2009; Lara et al., 2010), it is thought that ACh released from cardiomyocytes may be regulated in a similar fashion. Therefore, the hypothesis of this study is that ACh secreted from neonatal murine cardiomyocytes is regulated by the actions of the VAChT. To test this hypothesis, we will pharmacologically inhibit the VAChT in control mice using VES and we will also
analyze a genetically modified mouse line in which this transporter has been conditionally removed from cardiomyocytes.
Chapter 2

2 Methods

2.1 Animals

Transgenic mice used for the present study were acquired from JAX Laboratories (B6.FVB-Tg(Myh6-cre)2182Mds/J, stock #011038). Expression of Cre in these mice was under the control of the cardiomyocyte-specific murine alpha myosin-heavy chain promoter (Myh-6-cre). Once acquired, transgenic mice were then bred to VACHT-floxed mice (VACHT\textsuperscript{flox/flox}) (Guzman et al., 2011; Martins-Silva et al., 2011). All procedures and animals used in this thesis were approved by the local animal users committee (Protocol# 2008-127). As described previously, VACHT\textsuperscript{flox/flox} mice were backcrossed 5 times to C57BL6/j mice (Guzman et al., 2011). Furthermore, it should be noted that VACHT\textsuperscript{flox/flox} mice were no different from wild-type mice (Martins-Silva et al., 2011). In order to generate VACHT\textsuperscript{flox/flox,Myh6-Cre\textsuperscript{+}} and VACHT\textsuperscript{flox/flox,Myh6-Cre\textsuperscript{-}} mice, VACHT\textsuperscript{WT/flox,Myh6-Cre\textsuperscript{+}} littermates were crossed with VACHT\textsuperscript{WT/flox,Myh6-Cre\textsuperscript{-}} littermates. Mice used for the present study were generated by breeding F2 littermates together. For the present study, VACHT\textsuperscript{flox/flox,Myh6-Cre\textsuperscript{-}} littermates were considered as controls.

2.2 Neonatal Cardiomyocyte Cultures

Neonatal cardiomyocytes were cultured as previously described (Guatimosim et al., 2008) and used for all subsequent experiments. Neonatal cardiomyocytes were used over their adult counterparts as adult cardiomyocytes are only viable in culture for a short period of time consequently making them difficult to work with and obtain accurate results. In short, neonatal mice were sterilized, decapitated, and their hearts were extracted with any excess blood being removed by gently shaking the hearts in Hanks’ Balanced Salt Solution [(HBSS; Sigma Aldrich; 55021C) (5.37 mmol/L potassium chloride, 0.44 mmol/L potassium phosphate monobasic anhydrous, 4.17 mmol/L sodium bicarbonate, 136.89 mmol/L sodium chloride, 0.336 mmol/L sodium phosphate dibasic]
heptahydrate, 5.55 mmol/L dextrose anhydrous, and 0.028 mmol/L phenol red sodium salt). Hearts were then cut, washed, and supplemented with 2 mL of Liberase solution [Liberase TH enzyme (Roche; 05401135001) stock solution reconstituted in 10 mL of HBSS] before being left in a water bath at 37°C with agitation for 10 minutes. The tissue was replaced with 4 mL of fresh Liberase solution and incubated at 37°C for 15 minutes. Cardiomyocytes were suspended in the Liberase solution and centrifuged at 200 x g for 5 minutes at 23°C. The supernatant was discarded and the pellet was re-suspended in 3 mL of complete M199 medium (Sigma M4530) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 10% Fetal Bovine Serum and 2 mmol/L L-glutamine. The process was repeated with the remaining undigested tissue before the cardiomyocytes were pre-plated in a petri dish (diameter x height: 100 mm x 25 mm) and incubated in a 37°C incubator with 5% CO₂ for 90 minutes. Following incubation, any tissue residue present at the surface of the medium was removed before the cardiomyocytes were re-suspended. The resulting medium was centrifuged using the same parameters as previously described and the supernatant was discarded with the pellet being re-suspended in complete M199 medium. In the case of assay-based studies, cardiomyocytes were re-suspended in enough medium such that each well of a 4 well plate contained 3 x 10⁵ cells. In the case of microscopy-based studies, cardiomyocytes were re-suspended in enough medium such that each plate contained 1.5 x 10⁵ cells. Cardiomyocytes used for assay-based studies were left to incubate overnight while cardiomyocytes used for microscopy-based studies were left in the incubator for 30 minutes before additional medium was added. These plates were then placed in the incubator for overnight incubation. The following morning, cardiomyocytes were examined and the medium was changed to complete M199 medium supplemented with 20 μg/mL of cytosine-D-arabinofuranoside (ARA-c) (Sigma Aldrich; C1768). Following incubation with ARA-c for 48 hours, the medium was changed to warmed M199 medium. Cardiomyocytes were then used for their respective experiments (ie. Immunofluorescence/confocal microscopy, DAF-FM Diacetate Imaging, HPLC-ED, or the Choline/ACh Fluorometric Assay). Cardiomyocytes to be used for these experiments could be left in a 37°C incubator with 5% CO₂ for up to one week from the initial culture day before they were no longer viable.
2.3 Genotyping

Tail snips were taken from 1-2 day old neonatal mice, placed in Eppendorf tubes, and supplemented with 25 μL of extraction solution (Sigma Aldrich; E7526) and 7 μL of tissue preparation solution (Sigma Aldrich; T3073). Eppendorf tubes were placed in a water bath at 37°C for 10 minutes with agitation and then placed onto a hot plate at 95°C for 5 minutes. Eppendorf tubes were supplemented with 25 μL of neutralization buffer (Sigma Aldrich; N9310) and vortexed before each neutralized solution was added to a PCR tube containing REDExtract-N-AMP PCR Reaction Mix (Sigma Aldrich; R4775) and primers (Flox GF, Flox GR, Cre-1, Cre-2, and VAT2DET2S). The positive control was supplemented with 1.5 μL of solution from a tube containing a tail snip of a Myh-6-Cre Fx2 mouse. PCR tubes were then vortexed and spun down before being run on a PCR machine using a pre-made program. Upon completion of the program, each well of a 2% agarose gel was loaded with 7 μL of the resulting solution from its corresponding PCR tube. A 1 kb DNA ladder was also loaded for reference. The agarose gel was then run on a GE Gel Electrophoresis machine at 120 mv for 40 minutes and imaged.

2.4 Cell Fixation and Immunofluorescence

1-2 day old neonatal cardiomyocytes were cultured as previously described. Following a 48 hour treatment with 20 μg/mL of ARA-c, medium present on cell culture plates was aspirated and cardiomyocytes were washed 3x using 1x Phosphate Buffer Solution (PBS). Cardiomyocytes were then covered with 4% paraformaldehyde (PFA), protected from light, and allowed to fix before being blocked with blocking solution (1x PBS + 1%BSA + 0.5% Triton X-100). Cardiomyocytes were then incubated with anti-VACHT (Synaptic Systems) and α-Actinin (Sigma Aldrich) antibodies diluted 1:50 in blocking solution. Plates containing the anti-VACHT and α-Actinin antibodies were incubated overnight at 4°C. The following morning, cardiomyocytes were washed as previously described and incubated with secondary antibodies (Goat-α-Rabbit 488 and α-mouse 546) diluted 1:1000 in blocking solution. Each plate was left covered at room temperature for
1 hour before cardiomyocytes were subjected to nuclear staining using Hoescht Fluorescent Stain. Finally, cardiomyocytes were left in 2 mL of PBS, placed in a wet chamber, and left at 4°C until they were to be imaged. Imaging was completed using the 63x objective lens of a Zeiss LSM 510 Meta confocal system.

2.5 DAF-FM Fluorescence

1-2 day old neonatal cardiomyocytes were cultured as previously described. Following a 48 hour treatment with 20 μg/mL of ARA-c, the medium was removed, and cardiomyocytes were washed using 1mL of tyrode (140 mmol/L NaCl, 4 mmol/L KCL, 1 mmol/L MgCl2, 1.8 mmol/L CaCl2, 10 mmol/L Glucose, and 5 mmol/L HEPES). Tyrode was then removed and replaced with 250 μL of DAF-FM (Invitrogen; D-23842) diluted in tyrode at a final concentration of 5 μM for 30 minutes. DAF-FM in tyrode was removed and cardiomyocytes were washed with 250 μL of fresh tyrode for 30 minutes. Cardiomyocytes were then either imaged (controls) or treated with various drugs including Pyridostigmine (100 μM) (Sigma Aldrich; P9797), Carbachol (10 μM) (Sigma Aldrich; C2409), Atropine (10 μM) (Sigma Aldrich; A0132), Hemicholinium-3 (10 μM) (Sigma Aldrich; H108) or Vesamicol (10 μM) (Sigma Aldrich; V100) for 30 minutes and then imaged. For the positive control, 1 nM sodium nitroprusside (SNP), a known nitric oxide (NO) donor, was added to a plate for approximately 1 minute and then imaged to determine if the assay was functioning properly. Images from each treatment were acquired using the 63x objective lens of a Leica SP5 II confocal system. Images were then analyzed using ImageJ software.

2.6 Choline/ACh Fluorometric Assay

Cardiomyocytes were cultured from VACHT\textsuperscript{flox/flox} and VACHT\textsuperscript{Myh6-Cre-flox/flox} neonatal mice as previously described. Following a 48 hour treatment with 20 μg/mL of ARA-c, VACHT\textsuperscript{flox/flox} and VACHT\textsuperscript{Myh6-Cre-flox/flox} cardiomyocytes were treated with either
pyridostigmine (100 µM) or pyridostigmine and vesamicol (10 µM). The cultured medium was then collected, centrifuged at 13,200 RPM for 5 minutes at 4°C, and filtered using a 0.2 µM Acrodisc Syringe Filter (PALL Life Sciences; PN=4602). Known concentrations of ACh (0, 10, 20, 30, 40, and 50 pmol/well), as well as filtered cultured medium samples, were added to a 96-well plate and the volumes were adjusted to 50 µL/well using choline assay buffer (Biovision; K615-100-1). Next, two separate reaction mixes were created. The first reaction mix was created such that 50 µL of mix could be added to each well in the following proportions: 44 µL choline assay buffer, 2 µL choline probe (Biovision; K615-100-2A), 2 µL AChE (Biovision; K615-100-2), and 2 µL enzyme mix (Biovision; K615-100-4). The second reaction mix was created in a similar fashion with the exception of AChE. Instead, the second mix was created such that the AChE was replaced with an equivalent volume of choline assay buffer. Once the reaction mixes were added to their respective wells, the 96-well plate was removed from ice, covered with aluminum foil, and left at room temperature for 30 minutes. The 96-well plate was then placed into a micro-plate reader with an Ex/Em=535/590 and a readout with given values was produced. The results were then normalized by subtracting the blank (ie. the 0 pmol/well standard) value from those values obtained for each well containing either a known standard or cultured medium. To determine the amount of ACh present within the cultured medium, the normalized values from wells lacking AChE (ie. free choline) were subtracted from the normalized values of wells containing AChE (ie. total choline). These values were then applied to a standard curve generated using known ACh standards.

2.7 HPLC-Electrochemical Detection (HPLC-ED)

Cardiomyocytes from VACHT-flox/flox and VACHT-Myh6-Cre-flox/flox neonatal mice were cultured as previously described. Following a 48 hour treatment with 20 µg/mL of ARA-c, cardiomyocytes were treated with either pyridostigmine (100 µM) or pyridostigmine and vesamicol (10 µM) and left to incubate for 4 hours in a 37°C incubator with 5% CO2. Following incubation, the cultured medium was collected, centrifuged at 13,200 RPM for
5 minutes at 4°C, and filtered using a 0.2 µM Acrodisc Syringe Filter. The filtered cultured medium was loaded onto the autosampler of an UltiMATE 3000 HPLC system with a Coulometic electrochemical detector (ED). The cultured medium was then injected onto the UltiMATE 3000 system using the following components and parameters: Flow rate: 0.300 mL/min; Injection volume: 20 µL; Cell: Model 5040 amperometric cell with platinum target electrode (Thermo Scientific; 70-1074); Cell potential: 275 mV; Column: MGII CAPCELL PAK C18 column (Shiseido; Cat. No.= 92461); Column temperature: 40°C; ACh post-column solid phase reactor (Thermo Scientific; 70-0640A)

### 2.8 Statistical Analysis

GraphPad Prism 6 was used for all statistical analysis in this study. The results for each of the experiments are provided as mean ± SEM. A student’s t-test or a one-way ANOVA with a Tukey’s post-hoc test were used to determine the statistical significance between experimental groups. p<0.05 was considered to be statistically significant for all analyses.
Chapter 3

3 Results

3.1 Neonatal Cardiomyocytes Secrete ACh in an Autocrine or Paracrine Fashion

To determine if neonatal cardiomyocytes could secrete ACh, initially a DAF-FM bioassay was utilized. In this assay, secreted ACh elicits an increase in NO production and this production can be measured by DAF fluorescence. If neonatal cardiomyocytes could secrete ACh, it would be interesting to determine whether or not neonatal cardiomyocytes do so in an autocrine/paracrine fashion. Results obtained from the DAF-FM bioassay would provide a foundation from which we could expand upon to better understand the way in which the non-neuronal cholinergic system functions in neonatal cardiomyocytes (Figure 1).

Here we show that when VACHT^{flox/flox} neonatal cardiomyocytes were subjected to AChE inhibition by PYR (100 µM), those cells exhibited a significant increase in DAF fluorescence similar to that observed in cardiomyocytes treated with exogenous Carb (10 µM). The increase in DAF fluorescence following AChE inhibition signifies the preservation of ACh release by neonatal cardiomyocytes. The effects of PYR, as well as Carb, were blocked however, following pre-incubation with ATR (10 µM). To ensure the assay was functioning properly, VACHT^{flox/flox} neonatal cardiomyocytes were treated with sodium nitroprusside (SNP), a known NO donor. Treatment with SNP resulted in a significant increase in DAF fluorescence compared to cardiomyocytes treated with exogenous Carb or PYR.
Figure 1. Acetylcholine release from cultured neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments. 

(A) Representative images obtained using confocal microscopy depicting increased DAF fluorescence in VACHT<sup>flox/flox</sup> neonatal cardiomyocytes following acute treatment with 10 μM exogenous carbachol (Carb) or 100 μM pyridostigmine (PYR) and no change in fluorescence when treated with 10 μM atropine (ATR), 10 μM ATR + 10 μM Carb, or 10 μM ATR + 100 μM PYR. (B) Normalized DAF fluorescence in 1-2 day old VACHT<sup>flox/flox</sup> neonatal cardiomyocytes following acute treatment with exogenously added Carb, PYR, ATR or SNP. Number of cells subjected to each treatment are shown within their respective bars (n=). Cells were acquired from five separate neonatal cardiomyocyte cultures. Scale bar=20 μm. ***p<0.0001. Data are shown as mean ± SEM.
3.2 Pharmacological Inhibition of CHT1 by Hemicholinium-3 Compromises ACh Release from Neonatal Cardiomyocytes

The high affinity choline transporter, CHT1, has been shown to serve a critical role in regulating the re-uptake of choline into presynaptic cholinergic neurons (Ferguson et al., 2004). This is evident considering that a disruption in the CHT1 gene results in impaired cholinergic neurotransmission (Ferguson et al., 2004). Interestingly, work by Rocha-Resende et al., 2012 revealed the importance of CHT1 in the synthesis and subsequent release of ACh by adult cardiomyocytes, as pharmacological inhibition of this transporter by HEM severely compromised ACh release. Interestingly, Rana et al., 2010 were unable to locate CHT1, as well as other cholinergic proteins, within neonatal rat cardiomyocytes. Therefore, the purpose of this experiment was to determine, using DAF fluorescence, whether or not CHT1 regulates choline re-uptake and the subsequent release of ACh from neonatal murine cardiomyocytes (Figure 2). These data would not only provide evidence supporting ACh release by neonatal cardiomyocytes but they would inform us on whether or not a functional non-neuronal cholinergic system is present at birth and the possible physiological implications that this may have on cardiac development. Furthermore, they would provide greater insight into how a non-neuronal cholinergic system functions in cardiomyocytes.

Following experimentation, we once again show that ACh release from VACHT$^{flox/flox}$ neonatal cardiomyocytes is preserved following AChE inhibition. Interestingly however, the effects of AChE inhibition were abolished when VACHT$^{flox/flox}$ neonatal cardiomyocytes were pre-incubated with HEM (10 µM). Treatment with SNP resulted in a significant increase in DAF fluorescence compared to those cardiomyocytes treated with exogenous Carb, PYR, or HEM + Carb signifying that the assay was functioning properly.
Figure 2. Acetylcholine release from cultured neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments.

(A) Representative images obtained using confocal microscopy depicting increased DAF fluorescence in VAChT<sup>flox/flox</sup> neonatal cardiomyocytes following treatment with 10 μM exogenous carbachol (Carb), 100 μM pyridostigmine (PYR), or 10 μM hemicholinium-3 (HEM) + 10 μM Carb and no change in fluorescence when treated with 10 μM HEM or 10 μM HEM + 100 μM PYR. (B) Normalized DAF fluorescence in 1-2 day old VAChT<sup>flox/flox</sup> neonatal cardiomyocytes following acute treatment with exogenously added Carb, PYR, HEM, or SNP. Number of cells subjected to each treatment are shown within their respective bars (n=). Cells were acquired from six separate neonatal cardiomyocyte cultures. Scale bar=20 μm. **p<0.0001. Data are shown as mean ± SEM.
3.3 Pharmacological Inhibition of the Vesicular Acetylcholine Transporter by Vesamicol Compromises ACh Release from Neonatal Cardiomyocytes

Previously, it has been shown that the VACHT serves a critical role in mediating the release of ACh from cholinergic neurons (de Castro et al., 2009), and it has yet to be fully determined if this transporter serves a similar role within neonatal cardiomyocytes. To elucidate whether or not the VACHT serves a similar physiological role in regulating the release of ACh from neonatal cardiomyocytes, the transporter was pharmacologically inhibited using vesamicol (VES) (Figure 3).

Here we further confirm those observations previously reported (Rocha-Resende et al., 2012) that the release of ACh by VACHT$^{\text{flox/flox}}$ neonatal cardiomyocytes is preserved following AChE inhibition. When VACHT$^{\text{flox/flox}}$ neonatal cardiomyocytes were pre-incubated with VES however, the release of ACh was inhibited despite the cells being subjected to AChE inhibition. Treatment with SNP resulted in a significant increase in DAF fluorescence compared to those cardiomyocytes treated with exogenous Carb, PYR, or VES+Carb signifying that the assay was functioning properly.
Figure 3. Acetylcholine release from cultured neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments.

(A) Representative images obtained using confocal microscopy depicting increased DAF fluorescence in VACHT\textsuperscript{flox/flox} neonatal cardiomyocytes following acute treatment with 10 \( \mu \text{M} \) exogenous carbachol (Carb), 100 \( \mu \text{M} \) pyridostigmine (PYR), or 10 \( \mu \text{M} \) vesamicol (VES) + 10 \( \mu \text{M} \) Carb and no change in fluorescence when treated with 10 \( \mu \text{M} \) VES or 10 \( \mu \text{M} \) VES + 100 \( \mu \text{M} \) PYR. (B) Normalized DAF fluorescence in 1-2 day old VACHT\textsuperscript{flox/flox} neonatal cardiomyocytes following acute treatment with exogenously added Carb, PYR, VES, or SNP. Number of cells subjected to each treatment are shown within their respective bars (\( n= \)). Cells were acquired from six separate neonatal cardiomyocyte cultures. Scale bar=20 \( \mu \text{m} \). ***\( p<0.0001 \). Data are shown as mean ± SEM.
3.4 The Conditional Removal of the Vesicular ACh Transporter from Neonatal Cardiomyocytes

To further test for the role that the VAChT serves in mediating the release of ACh from neonatal cardiomyocytes, the transporter was conditionally removed from these cells. To differentiate VAChT<sup>flox/flox</sup> mice from VAChT<sup>Myh-6-Cre-flox/flox</sup> mice for subsequent experimentation, each mouse from a given litter was genotyped as previously described. Briefly, tail snips were taken from neonatal mice and subjected to PCR before the resulting DNA was run on a 2% agarose gel. For reference, a representative gel (Figure 4) is shown to illustrate VAChT<sup>flox/flox</sup> and VAChT<sup>Myh-6-Cre-flox/flox</sup> genotyping. Each numbered lane represents a lane corresponding to a given mouse. Lanes labeled “P” and “N” are positive and negative controls, respectively. “L” represents a 1kb DNA ladder. VAChT<sup>flox/flox</sup> and VAChT<sup>Myh-6-Cre-flox/flox</sup> genotypes were determined by the presence of the flox/flox gene and either the absence or presence of Cre, respectively. In Figure 4, lanes 7, 10-12, and 14 denote mice of the VAChT<sup>flox/flox</sup> genotype, whereas the remaining lanes (1-6, 8, 9, and 13) denote mice of the VAChT<sup>Myh-6-Cre-flox/flox</sup> genotype. To confirm that the VAChT had been conditionally removed from VAChT<sup>Myh-6-Cre-flox/flox</sup> cardiomyocytes, immunostaining followed by confocal microscopy were performed on both VAChT<sup>flox/flox</sup> (Figure 5a) and VAChT<sup>Myh-6-Cre-flox/flox</sup> (Figure 5b) neonatal cardiomyocytes. Following the analysis of VAChT<sup>flox/flox</sup> neonatal cardiomyocytes, immunostaining and confocal microscopy revealed the presence of the VAChT within the perinuclear region. However, immunostaining and confocal microscopy in VAChT<sup>Myh-6-Cre-flox/flox</sup> neonatal cardiomyocytes revealed no such VAChT staining within the perinuclear region. The lack of VAChT staining within these cardiomyocytes confirms the selective removal of the VAChT from neonatal cardiomyocytes.
Figure 4. Genotyping analysis of a cardiomyocyte specific VAChT KO mouse strain.

An agarose gel used to distinguish VAChT^{flox/flox} and VAChT^{Myh-6-Cre-flox/flox} genotypes has been shown. Bands denoting Cre have been boxed in red while bands denoting flox have been boxed in yellow. Lanes 1-6, 8, 9, & 13 denote floxed Cre+ mice (VAChT^{Myh-6-Cre-flox/flox}), while lanes 7, 10-12, & 14 denote floxed Cre- mice (VAChT^{flox/flox}). DNA obtained via tail snip from VAChT^{Myh-6-Cre-flox/flox} mice was used as a positive control (Lane P) to show the presence of Cre. DNA obtained via tail snip from VAChT^{flox/flox} mice was used as a negative control (Lane N) to show the absence of Cre. Lane L is a 1kb DNA ladder.
Figure 5. Conditional removal of the VACHT from cardiomyocytes in VACHT<sup>Myh-6-Cre-flox/flox</sup> mice

Representative images obtained by immunostaining and confocal microscopy showing the presence and absence of the VACHT within (A) VACHT<sup>flox/flox</sup> and (B) VACHT<sup>Myh-6-Cre-flox/flox</sup> neonatal cardiomyocytes, respectively. n=20 for both genotypes. Scale bar=20 µm.
3.5 The Conditional Removal of the Vesicular ACh Transporter from Neonatal Cardiomyocytes Severely Compromises ACh Release

Previous work has shown that the VACHT is essential in regulating the release of ACh from cholinergic neurons, as well as sustaining life, since deletion of this gene prevented the stimulated release of ACh from synaptosomes and resulted in death of the animals shortly after birth (de Castro et al., 2009). Furthermore, the essential role that the VACHT serves in mediating the release of ACh from cholinergic neurons has been shown in VACHT KD HOM mice. These mice exhibit alterations at the neuromuscular junction (de Castro et al., 2009) and display a myasthenic-like phenotype (V. F. Prado et al., 2006). To further determine if the VACHT serves a similar role within neonatal cardiomyocytes, we compared ACh release between VACHT flox/flox and VACHT Myh-6-Cre-flox/flox genotypes. Initially, VACHT flox/flox and VACHT Myh-6-Cre-flox/flox neonatal cardiomyocytes were subjected to analysis using the DAF-FM experimental approach as previously described (Figure 6). Here it was shown that the release of ACh by VACHT flox/flox neonatal cardiomyocytes was preserved following AChE inhibition, but no such preservation was observed in VACHT Myh-6-Cre-flox/flox neonatal cardiomyocytes subjected to the same treatment.

To further test these findings, cultured medium was collected from VACHT flox/flox and VACHT Myh-6-Cre-flox/flox neonatal cardiomyocytes treated with either PYR (100 µM) or PYR + VES (10 µM) and subjected to analysis by a choline/ACh fluorometric assay (Figure 7). Both VACHT flox/flox and VACHT Myh-6-Cre-flox/flox neonatal cardiomyocytes were maintained in medium containing PYR to preserve ACh from degradation by AChEs. To determine the amount of ACh released under each of these conditions, standard curves were created separately for each assay using known ACh standards. One such standard curve has been shown for representative purposes (Figure 7c). Analysis revealed that VACHT flox/flox neonatal cardiomyocytes treated with VES released significantly less ACh compared to control cardiomyocytes incubated with only PYR (PYR: 199.2 ± 17.65 pmols ACh/mg of protein, PYR+VES: 30.73 ± 3.89 pmols ACh/mg of protein, p<0.0001) (Figure 7a).
To further test these observations using genetic tools instead of pharmacology, VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes were treated with PYR (100 µM) before their cultured media were collected and subjected to fluorometric analysis (Figure 7b). Through the use of standard curves, it was determined that there was a significant reduction in ACh release from VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes compared to VACHT\textsuperscript{floxflox} neonatal cardiomyocytes (VACHT\textsuperscript{floxflox}: 203.7 ± 23.95 pmols ACh/mg of protein, VACHT\textsuperscript{Myh-6-Cre-floxflox}: 1.67 ± 1.67 pmols ACh/mg of protein, p<0.001).

To further confirm the lack of ACh release by VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes, cultured media were collected from VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes which were treated with either PYR (100 µM) or PYR + VES (10 µM) and then subjected to analysis by HPLC-ED (Figure 8). To determine the amount of ACh released under each of these conditions, standard curves were created separately for each run using known ACh standards. One of these curves has been shown for representative purposes (Figure 8e). Representative chromatograms for VACHT\textsuperscript{floxflox} neonatal cardiomyocytes treated with either PYR or PYR + VES, along with a 500 fmol on the column (OTC) standard, have been shown for comparison (Figure 8c). Following the analysis of all runs from four separate neonatal cultures, the data revealed a significant reduction in ACh release from VACHT\textsuperscript{floxflox} neonatal cardiomyocytes treated with PYR+VES compared to those cardiomyocytes treated with only PYR (PYR: 37.76 ± 4.51 pmols ACh/mg of protein, PYR+VES: 2.03 ± 1.17 pmols ACh/mg of protein, p<0.0001) (Figure 8a).

To further compare VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes, they were treated with PYR (100 µM) and subjected to the same analysis. Representative chromatograms for VACHT\textsuperscript{floxflox} or VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes treated with PYR, along with a 250 fmol OTC standard, have been shown for comparison (Figure 8d). Analysis revealed a significant reduction in ACh release from VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes compared to VACHT\textsuperscript{floxflox} neonatal cardiomyocytes (VACHT\textsuperscript{floxflox}: 47.89 ± 8.76 pmols ACh/mg of protein, VACHT\textsuperscript{Myh-6-Cre-floxflox}: 0.05 ± 0.03 pmols ACh/mg of protein, p<0.01) (Figure 8b).
Figure 6. Acetylcholine release from cultured VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes was determined using a DAF-FM bioassay following acute pharmacological treatments.

(A) Representative images obtained using confocal microscopy for VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes following acute treatment with either 10 μM carbachol (Carb) or 100 μM pyridostigmine (PYR). Acute treatment with 10 μM Carb revealed a significant increase in DAF fluorescence in both VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes, whereas treatment with 100 μM PYR produced a significant increase in DAF fluorescence only in VACHT\textsuperscript{floxflox} neonatal cardiomyocytes. B. Normalized fluorescence for 1-2 day old VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes following acute treatment with exogenously added Carb or PYR. Number of cells subjected to each treatment are shown within their respective bars (n=). Cells were acquired from six separate neonatal cardiomyocyte cultures. Scale bar=20 μm. \*p<0.05. Data are shown as mean ± SEM.
Figure 7. Acetylcholine secreted by VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes was measured using a choline/ACh Fluorometric Assay.

(A) Cultured VACHT\textsuperscript{floxflox} neonatal cardiomyocytes underwent acute treatment with either 100 μM pyridostigmine (PYR) or 100 μM PYR + 10 μM vesamicol (VES). Cultured media from a given treatment were separated from cells and the amount of secreted ACh was quantified. n=number of separate neonatal cardiomyocyte cultures for each treatment. ***p<0.0001 vs PYR. (B) Cultured VACHT\textsuperscript{floxflox} and VACHT\textsuperscript{Myh-6-Cre-floxflox} neonatal cardiomyocytes underwent acute treatment with 100 μM PYR. Cultured media from a given genotype were separated from cells and the amount of secreted ACh was quantified. n=number of separate neonatal cardiomyocyte cultures for each genotype. **p<0.001 vs CT. (C) Representative standard curve used for the measurement of ACh created using a Choline/ACh fluorometric assay.
Figure 8. Acetylcholine secreted by VACHT^{flox/flox} and VACHT^{Myh-6-Cre-flox/flox} neonatal cardiomyocytes was measured using HPLC with electrochemical detection (ED).

(A) Cultured VACHT^{flox/flox} neonatal cardiomyocytes underwent acute treatment with either 100 μM pyridostigmine (PYR) or 100 μM PYR + 10 μM vesamicol (VES). Cultured media from a given treatment were separated from cells and the amount of secreted ACh was quantified. n=number of separate neonatal cardiomyocyte cultures for each treatment. ***p<0.001 vs PYR (B) Cultured VACHT^{flox/flox} and VACHT^{Myh-6-Cre-flox/flox} neonatal cardiomyocytes underwent acute treatment with 100 μM PYR. Cultured media from a given genotype were separated from cells and the amount of secreted ACh was quantified. n=number of separate neonatal cardiomyocyte cultures for each genotype. **p<0.01 vs VACHT^{flox/flox}. (C) Representative chromatograms showing ACh release from VACHT^{flox/flox} neonatal cardiomyocytes following treatment with either 100 μM PYR or 10 μM VES + 100 μM PYR, along with a blank and 250 fmol OTC standard for comparison. Numbers present above each peak represent retention times. (D) Representative chromatograms showing ACh release from VACHT^{flox/flox} and VACHT^{Myh-6-Cre-flox/flox} neonatal cardiomyocytes following treatment with 100 μM PYR, along with a blank and 500 fmol OTC standard for comparison. Numbers present above each peak represent retention times. (E) A representative standard curve ranging from 125-1000 fmols OTC used to quantify ACh release.
Chapter 4

4 Discussion

4.1 Overview

The recent emergence of non-neuronal cholinergic systems within biological systems such as the pancreas, the placenta, and the colon has provided insight and understanding into both the physiology, as well as the pathophysiology, associated with these systems. Recently, it has been reported that the heart possess an intrinsic non-neuronal cholinergic system, as the machinery required to synthesize and release ACh is present within cardiomyocytes themselves (Kakinuma et al., 2009; Rana et al., 2010; Rocha-Resende et al., 2012). However, there are controversial results regarding whether or not neonatal cardiomyocytes also possess this machinery as a recent report was unable to show the presence of cholinergic machinery in neonatal cardiomyocytes (Rana et al., 2010). In order to investigate the mechanisms involved in regulating ACh release from neonatal cardiomyocytes, a mouse line in which the VAChT has been conditionally removed has been generated. By utilizing a DAF-FM bioassay, a choline/ACh fluorometric assay, and HPLC-ED, we have shown pharmacologically, as well as genetically, that neonatal cardiomyocytes secrete ACh in a VAChT-dependent manner. The results obtained from neonatal cardiomyocytes agree with previous observations by Rocha-Resende et al., 2012 that isolated adult cardiomyocytes can secrete ACh and can act in an autocrine/paracrine fashion through mAChRs-2. Furthermore, our results provide support for the role of CHT1 in the synthesis and subsequent release of ACh from adult cardiomyocytes (Rocha-Resende et al., 2012). These results also indicate that the non-neuronal cholinergic system is not only present in adult cardiomyocytes, but that it’s also present during the maturation of cardiomyocytes as well. The most important finding however, as previously mentioned, was determining how essential the role of the VAChT is in regulating ACh release from neonatal cardiomyocytes.
4.2 ACh Release from Cardiomyocytes can Act in an Autocrine or Paracrine Fashion to Further Stimulate the Production of ACh

The DAF-FM bioassay works based on the principle that secreted ACh elicits an increase in NO production. This production can then be measured through the use of DAF fluorescence. A study analyzing the non-neuronal cholinergic machinery within isolated adult cardiomyocytes utilized the DAF-FM bioassay to show the secretion of ACh by these cells (Rocha-Resende et al., 2012). It was also reported that non-neuronal ACh secreted from isolated adult cardiomyocytes could act as a local signaling molecule through the activation of mAChRs-2 (Rocha-Resende et al., 2012). The secretion of non-neuronal ACh by cardiomyocytes in an autocrine/paracrine fashion is of particular importance as it may serve to be cardioprotective in nature through the amplification of neuronally released ACh. By implementing a similar pharmacological approach on cultured VAChT\textsuperscript{flox/flox} neonatal cardiomyocytes, in conjunction with the DAF-FM bioassay, we have been able to corroborate the findings by Rocha-Resende et al., 2012.

Our findings reveal that the effects of preserved ACh release from VAChT\textsuperscript{flox/flox} neonatal cardiomyocytes are blocked following treatment with ATR, a muscarinic receptor antagonist, suggesting that non-neuronal ACh secreted by these cells acts in an autocrine/paracrine fashion through mAChRs-2. Similar to those observations presented by Rocha-Resende et al., 2012, our data also provide support to those findings presented by Kakinuma et al., 2009. In this particular study, rat cardiomyocytes underwent pharmacological treatment with 1 mM Pilocarpine, a non-selective muscarinic agonist, 1 μM of the cholinesterase inhibitor Donepezil, or 1 mM ATR. Following treatment with 1 mM Pilocarpine or 1 μM Donepezil, Kakinuma and colleagues observed a significant increase in intracellular ACh content (Kakinuma et al., 2009). Conversely, pre-incubation with 1 mM ATR prevented any significant increase in intracellular ACh and in fact, produced a significant reduction in intracellular ACh (Kakinuma et al., 2009). These observations suggest that ACh is synthesized and secreted constitutively by rat cardiomyocytes and acts through mAChRs-2 to actuate ACh production. The amount of ACh present within rat cardiomyocytes either increases or decreases from basal levels.
following mAChR-2 activation or inhibition, respectively. These findings suggest that ACh produced by rat cardiomyocytes for subsequent release is in some way mediated by the mAChR-2. Although not fully known, our findings could suggest a similar regulatory role for the mAChR-2 in murine cardiomyocytes as well.

The ability for non-neuronal ACh to act in an autocrine/paracrine fashion through muscarinic receptors is essential in maintaining normal physiology within several biological systems such as the pancreas (Rodriguez-Diaz et al., 2011). In the pancreas for instance, non-neuronal ACh secreted locally by the pancreatic alpha cells activates muscarinic type-3 ACh receptors (mAChR-3) to maintain glucose homeostasis, as well as proper insulin secretion. When the mAChR-3 is inhibited by J-104129, large fluctuations in plasma glucose concentrations ensue as a consequence of consistently reduced insulin responses (Rodriguez-Diaz et al., 2011).

In fact, the importance of non-neuronal ACh acting in an autocrine/paracrine fashion through muscarinic receptors has been shown recently in the cardiovascular system as well. It was reported that effectively abolishing the release of cardiomyocyte-derived ACh from murine cardiomyocytes through either pharmacological or genetic means, results in cardiomyocyte hypertrophy and cardiomyocyte remodeling (A. Roy et al., 2013). Moreover, when subjected to stress, these mice presented with altered cardiac contractility, as well as left ventricular function (A. Roy et al., 2013). These observations suggest that the release of cardiomyocyte-derived ACh and its ability to activate mAChRs-2 in an autocrine/paracrine fashion, is essential in maintaining cardiovascular homeostasis since inhibiting this release through either pharmacological or genetic means produces severe alterations in cardiomyocyte structure and function.

Taken together it becomes clear that several cells types, despite being non-neuronal in nature, can secrete ACh and do so in an autocrine/paracrine fashion through mAChR. More importantly, these data show that murine cardiomyocytes can secrete ACh in a similar fashion and can activate mAChRs-2 to ensure cardiovascular homeostasis, by maintaining cardiomyocyte structure and function.
4.3 ACh Release from Cardiomyocytes is Compromised Following Inhibition of CHT1 by Hemicholinium-3

As reported previously, the high affinity choline transporter, CHT1, is a protein essential to cholinergic neurotransmission, as it mediates the synthesis of ACh by providing cholinergic neurons with choline (Collier & Katz, 1974; R. Birks, 1961). This is evident considering that mutant mice possessing a disruption in the CHT1 gene produced alterations at the neuromuscular junction (Ferguson et al., 2004). Interestingly, these alterations paralleled those exhibited by ChAT mutant mice incapable of synthesizing ACh (Brandon et al., 2003; Misgeld et al., 2002) or VACHT mutants (de Castro et al., 2009), suggesting that when CHT1 activity is compromised, the production and release of ACh are also compromised. In the present study, CHT1 was pharmacologically inhibited in cultured VACHT\textsuperscript{flox/flox} neonatal cardiomyocytes using HEM. By pharmacologically inhibiting this transporter, we could determine if CHT1 regulates choline re-uptake in neonatal cardiomyocytes in a similar manner to neuronally-derived CHT1. Furthermore, by pharmacologically inhibiting CHT1 in neonatal cardiomyocytes, we could determine whether or not a non-neuronal cholinergic system is present and active during development.

These data show that ACh secreted by VACHT\textsuperscript{flox/flox} neonatal cardiomyocytes is abolished, despite AChE inhibition, following pre-incubation with HEM. These data provide support to those observations reported by Rocha-Resende et al., 2012 where they pharmacologically inhibited CHT1 in isolated adult cardiomyocytes. Similar to the observations we report, Rocha-Resende et al., 2012 reported that ACh secreted from isolated adult cardiomyocytes was abolished following pre-incubation with HEM. Considering both VACHT\textsuperscript{flox/flox} neonatal cardiomyocytes and isolated adult cardiomyocytes are able to preserve ACh release following AChE inhibition, but fail to do so following pre-incubation with HEM, suggests that the synthesis and subsequent release of ACh by murine cardiomyocytes is highly dependent upon CHT1 activity and its ability to regulate choline re-uptake. These data are important as they suggest that the role of CHT1 in cardiomyocytes is similar to its role in cholinergic neurons. Taken together, it becomes clearly evident that CHT1 is not only essential in regulating the re-
uptake of choline in cholinergic neurons, but it also serves a critical role in maintaining cholinergic tone within non-neuronal cell types as well.

The ability for CHT1 to regulate choline re-uptake not only in isolated adult cardiomyocytes, but in neonatal cardiomyocytes as well, suggests that an intrinsic non-neuronal cholinergic system is active in cardiomyocytes during development. It is likely that a non-neuronal cholinergic system is present in neonatal murine cardiomyocytes where it serves, like adult cardiomyocytes (A. Roy et al., 2013), to prevent cardiomyocyte remodeling and cardiomyocyte hypertrophy, as well as the functional implications associated with these molecular changes.

Not only do our data show the importance of CHT1 in regulating the re-uptake of choline by neonatal cardiomyocytes and the possible cardioprotective role it serves in the non-neuronal cholinergic system of cardiomyocytes, but they also suggest that these cells require extracellular sources of choline to properly synthesize ACh. This becomes important from a cholinergic signaling perspective as it has been previously reported that the de novo synthesis of ACh by cholinergic neurons is negligible and nearly all choline required for the synthesis of ACh must be acquired through dietary means (Fernstrom, 1981; Zeisel, 1981).

### 4.4 ACh Release from Cardiomyocytes is Regulated by the Vesicular ACh Transporter

The role the VACHT serves within cholinergic neurotransmission has been well documented previously (Parsons et al., 1993; Usdin et al., 1995). The role it serves in packaging ACh into acidic vesicles is essential for sustaining life considering that VACHT KO\textsuperscript{HOM} mice are not viable shortly after birth (de Castro et al., 2009). In the present study, we both confirm and extend those data describing the role of the VACHT within cardiomyocytes by pharmacologically inhibiting the transporter, as well as conditionally removing it, from neonatal cardiomyocytes.
To more thoroughly understand the role this transporter serves in regulating ACh release from neonatal cardiomyocytes, multiple studies involving the pharmacological inhibition or conditional removal of the VACHT were performed. Experiments implementing the DAF-FM bioassay indicate that the release of ACh by VACHT\textsuperscript{flox/flox} neonatal cardiomyocytes is compromised following treatment with VES. These findings are consistent with DAF-FM data acquired from isolated ventricular cardiomyocytes of mice with significant reductions in functioning VACHT protein (VACHT KD\textsuperscript{HOM}), where it was reported that ACh release was severely compromised despite AChE inhibition (Rocha-Resende et al., 2012). Considering that AChEIs rely highly on preserved ACh release to properly exert their functions, the inability for PYR or NEO to mediate an increase in NO production (i.e. DAF fluorescence) when VACHT expression was reduced, or when the transporter was pharmacologically inhibited, indicates that the release of ACh was effectively abolished. This becomes even more evident considering that in both studies there was a significant increase in NO production when cardiomyocytes were treated with either exogenous Carb or ACh. Moreover, DAF-FM data acquired from VACHT\textsuperscript{Myh-6-Cre-flox/flox} neonatal cardiomyocytes revealed no significant increase in NO production following AChE inhibition indicating that the VACHT is absolutely essential for the release of ACh by neonatal cardiomyocytes. These observations indicate that ACh released by cardiomyocytes is completed in a VACHT-dependent manner similar to the manner in which ACh is released from cholinergic nerve terminals. This becomes evident considering that cardiomyocytes possess the recycling vesicles necessary for VACHT-dependent ACh release (Rocha-Resende et al., 2012). Interestingly, these vesicles have been shown to co-localize extensively with the VACHT in the perinuclear region of cardiomyocytes (Rocha-Resende et al., 2012).

To further exam whether the release of ACh from cardiomyocytes occurs in a VACHT-dependent manner, we utilized a choline/ACh fluorometric assay, as well as HPLC-ED, on both VACHT\textsuperscript{flox/flox} and VACHT\textsuperscript{Myh-6-Cre-flox/flox} neonatal cardiomyocytes. Both methods revealed results similar to those obtained following the pharmacological inhibition of the VACHT. These data not only support our previous findings, but they extend both our data, as well as the data of others, since until now no other study has shown through direct measurement, the secretion of ACh by cardiomyocytes. Even more convincing
however, is the fact that ACh release was almost completely undetectable by these methods when \( \text{VACHT}^{\text{Myh-6-Cre-flox/flox}} \) neonatal cardiomyocytes were used. Considering that ACh secreted from cholinergic neurons in both the central and peripheral nervous systems is strongly correlated with the level of VACHT expression (V. F. Prado et al., 2006), our data would seem to suggest that the level of VACHT expression within cardiomyocytes directly affects the amount of ACh released from these cells. Therefore, the release of ACh in a VACHT-dependent manner is not only a characteristic of cholinergic neurons, but also seems to be the manner in which ACh is secreted by cardiomyocytes. VACHT-dependent secretion of ACh by cardiomyocytes becomes even more plausible if one considers that other non-neuronal cholinergic systems have been shown to secrete ACh in this manner (Rodriguez-Diaz et al., 2011). In a study by Rodriguez-Diaz et al., 2011, AChE inhibition was ineffective in maintaining the pancreas’ insulin response when the VACHT was pharmacologically inhibited by VES.

Taken together, both the pharmacological inhibition and conditional removal of the VACHT severely compromised the release of ACh from cardiomyocytes. Moreover, there were similar reductions in ACh release following both approaches. As it has been well established that the VACHT is essential in mediating the release of ACh from cholinergic neurons, results obtained following the pharmacological inhibition and conditional removal of it would suggest a similar VACHT-dependent release by neonatal cardiomyocytes. Determining the mechanism by which ACh is secreted by neonatal cardiomyocytes allows for a better understanding of the intrinsic non-neuronal cholinergic system within cardiomyocytes.

### 4.5 Conclusions and Future Directions

These data indicate that neonatal murine cardiomyocytes possess an intrinsic non-neuronal cholinergic system that can synthesize and subsequently release ACh in an autocrine/paracrine fashion. Furthermore, pharmacologically inhibiting the VACHT or conditionally removing it from neonatal cardiomyocytes severely compromised ACh secretion suggesting that this process is VACHT-dependent. Although ACh release from
neonatal cardiomyocytes is VACHT-dependent, it is yet to be determined how this release changes under various physiological conditions such as acute and chronic stress. Determining how these conditions affect ACh release is not only critical to understanding cardiac physiology but it would also be critical to understanding the progression of several cardiac pathologies including CHF. Additionally, understanding how ACh release is altered under various physiological conditions may provide further insight into whether or not an intrinsic non-neuronal cholinergic system is cardioprotective in nature.
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Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart

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ABSTRACT Heart activity and long-term function are regulated by the sympathetic and parasympathetic branches of the nervous system. Parasympathetic neurons have received increased attention recently because acetylcholine (ACh) has been shown to play protective roles in heart disease. However, parasympathetic innervation is sparse in the heart, raising the question of how cholinergic signaling regulates cardiomyocytes. We hypothesized that non-neuronal secretion of ACh from cardiomyocytes plays a role in cholinergic regulation of cardiac activity. To test this possibility, we eliminated secretion of ACh exclusively from cardiomyocytes by targeting the vesicular acetylcholine transporter (VACHT). We find that lack of cardiomyocyte-secreted ACh disturbs the regulation of cardiac activity and causes cardiomyocyte remodeling. Mutant mice present normal hemodynamic parameters under nonstressed conditions; however, following exercise, their heart rate response is increased. Moreover, hearts from mutant mice present increased oxidative stress, altered calcium signaling, remodeling, and hypertrophy. Hence, without cardiomyocyte-derived ACh secretion, hearts from mutant mice show signs of imbalanced autonomic activity consistent with decreased cholinergic drive. These unexpected results suggest that cardiomyocyte-derived ACh is required for maintenance of cardiac homeostasis and regulates critical signaling pathways necessary to maintain normal heart activity. We propose that this non-neuronal source of ACh boosts parasympathetic cholinergic signaling to counterbalance sympathetic activity regulating multiple aspects of heart physiology.—Roy, A., Fields, W. C., Rocha Resende, Ė., Resende, R. R., Guaimimos, S., Prado, V. F., Gros, E., Prado, M. A. M. Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. FASEB J. 27, 000–000 (2013). www.fasebj.org

Key Words: cardiac remodeling • VACHT • choline acetyltransferase • parasympathetic activity • cardiac hypertrophy • autonomic function

Acetylcholine (ACh) released by parasympathetic nerves regulates the minute-to-minute changes in heart rate and contractility required for proper cardiovascular function via muscarinic receptors, opposing the activity of the sympathetic nervous system [1]. In addition to regulating atrial activity, ACh plays multiple roles in ventricular function [2, 3], notwithstanding limited parasympathetic innervation in ventricular regions [4, 5]. The exact mechanisms by which ACh can have such widespread effects, despite somewhat limited parasympathetic innervation in regions other than the atria, are not fully understood. Interestingly, in the pancreas [6] as well as the immune system [7, 8], non-neuronal sources of ACh secretion have recently been shown to play a role in regulating insulin secretion [9] and the cholinergic antiinflammatory system [9, 10], respectively. However, information is limited regarding the contribution of non-neuronal ACh for autonomic regulation of other bodily functions in vivo.

Recent experiments have indicated that cultured cardiomyocytes, similar to pancreatic alpha cells (6) and lymphocytes (7), can express the enzymatic machinery required for ACh synthesis (11, 12). Moreover, secretion of ACh from cultured cardiomyocytes depends on the activity of the vesicular acetylcholine transporter (VACHT; ref. 12). However, whether this non-neuronal source of ACh plays a physiological role in the regulation of heart activity

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ANP, atrial natriuretic peptide; α-MHC, α-myosin heavy chain (Myh6); β-MHC, β-myosin heavy chain; ChAT, choline acetyltransferase; CHT, high-affinity choline transporter; cVACHT, cardiomyocyte-specific vesicular acetylcholine transporter knockout (VACHT−/−); ECG, electrocardiography; floxed, flanked by loxP; Gαq, Gq-coupled receptor kinase; HIC, hemicolinium-8; ISO, isoprenaline; KO, knockout; LV, left ventricular; Myb6, myosin heavy chain (α-MHC); NO, nitric oxide; ROS, reactive oxygen species; S1, sinoatrial; VACHT, vesicular acetylcholine transporter; VES, vesamicol; WT, wild type

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or function is unknown. Here, we used the Cre-loxP system to eliminate VACHT exclusively in cardiomyocytes and test for potential roles for cardiomyocyte-secreted ACh in heart activity. We found that this novel form of cellular communication is required for physiological regulation of heart size and stress levels, likely by maintaining high levels of ACh at synaptic junctions. We also discovered that the recovery of heart rate following exercise or stress is disturbed in the absence of this non-neuronal source of ACh, which suggests the widespread effects of non-neuronal cardiomyocyte-derived ACh. Our study provides a novel mechanism for autocrine/paracrine regulation of cardiomyocytes by non-neuronal ACh secretion.

MATERIALS AND METHODS

Animals

Transgenic mice expressing Cre under the control of the cardiac-specific muscle enolase Cre/loxP uacrt transgene (MyleCat) were obtained from the Jackson Laboratory (Harbor, ME, USA). VACHT mice were bred to B6.Cg mice (C57BL/6J) and VACHT mice were bred to VACHT mice (C57BL/6J) and were backcrossed 5 times to C57BL/6J mice as described previously (13). VACHT/mice were not different from wild-type (WT) mice (14). Littermates (VACHT/mice × VACHT/mice) were crossed to generate VACHT/mice and VACHT/mice. F2 littermates were then bred to generate mice used for this study. VACHT/mice litters were used as controls.

To generate the cardiomyocyte-specific choline acetyltransferase (ChAT)-knockout (KO) mice, Myh6Cre mice were bred to ChAT-floxed mice (15) obtained from the Jackson Laboratory (B6.129-S6-Chen.Tg1.1 [J]; ChATfloxed) (13). Littermates (CHATfloxed × CHATfloxed) (13) were crossed to generate CHATfloxed mice and CHATfloxed mice.

The Rosa26FP strain (BD12SN-CAxROSA(26)Sortm1(FcR)) CovJ stock no. 0050648; Jackson Laboratory) used for a Cre reporter. Animals were maintained and cared for according to an approved animal protocol at the University of Western Ontario (2009-127) and following the Canadian Council on Animal Care. Only male mice were used for adult cardiomyocyte isolation and all in vivo experiments. Neonatal cardiomyocytes were isolated and cultured from mice of both genders.

Neonatal cardiomyocyte culture

Neonatal cardiomyocytes were isolated as described previously (16). Briefly, cardiac cells were plated in dishes containing 199 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% FBS, and 2 mM L-glutamine. Cytosine-β-D-arabinofuranoside (AraC-20 µg/ml) was used to prevent growth of fibroblasts. For hypertrophy studies, cardiomyocytes were cultured with 2 mM L-glutamine, vesicular (VES; 5 µM) or hemi-holinoxin (HC; 5 or 10 µM) for 48 h and then used for immunofluorescence or qPCR analyses.

qPCR/RT-qPCR

RNA was extracted from isolated cardiomyocytes, and cDNA was synthesized as described previously (17). A brain sample was used as a positive control for VACHT, and a non--template reaction was used as a negative control. qPCR for atrial natriuretic peptide (ANP) and G protein-coupled receptor kinases 2 and 5 (GRK2 and GRK5) was performed as described previously (17). For primer sequences, see Supplemental Table S1.

Immunoblotting

Isolated adult cardiomyocytes were lysed using ice-cold modified RIPA buffer. Protein (80 µg) was separated using SDS-PAGE, and PVDF membranes were probed with anti-VAGHT antibody (1:200; Synaptic Systems, Gottingen, Germany), α-Actinin (1:2000; Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control.

Immunostaining

Adult cardiomyocytes were subjected to immunofluorescence protocol as described previously (12). Cells were incubated with one of the following antibodies: anti-VACHT (1:50; Synaptic Systems), anti-ChAT (1:100; Abcam, Cambridge, MA, USA), and antiFITC (1:2500; kindly supplied by R. Jacobs, University of Western Ontario, London, ON, Canada; ref. 18), anti-ANP (1:200; Abcam), or anti-GRK5 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunostaining. Alexa Fluor 488, 594, or 647-labeled secondary antibodies were used for detection.

Measurement of ACh secretion (fluorometric assay)

Transmitter release was measured using the Choline/ACH Quantification Kit (Biovision, Milpitas, CA, USA) as described previously (19). Briefly, cultured medium was collected from VACHT/mice and VACHT/mice cardiomyocytes and incubated with either 100 µM pyridostigmine bromide (Sigma-Aldrich; P9797) or 100 µM pyridostigmine bromide and 1 µM Y0543 (Sigma-Aldrich; V100) at 37°C for 2 h. Cultured medium was collected and centrifuged at 10000 rpm for 5 min at 4°C. The resulting supernatant was collected and filtered using a 0.2-µm Acrodisc Syringe Filter (Pall Life Sciences, Port Washington, NY, USA) and placed on ice. ACh concentration was determined using the fluorometric Choline/ACH Quantification Kit (λex=35 nm, λem=590 nm). Each sample was assayed in duplicate and experiments were conducted ≥4 times using separate cultures.

Measurement of ACh secretion (HPLC-electrochemical detection)

Cultured neonatal cardiomyocytes from VACHT/mice and VACHT/mice mice were treated with 100 µM pyridostigmine bromide (Sigma Aldrich; P9797) and incubated for 4 h at 37°C. It has been reported previously that both subacute treatment with pyridostigmine (20) as well as acute exposure to stress (21) can increase the transcription of acetylcholinesterase (AChE) in the brain. However, in vitro studies have confirmed that pyridostigmine is a very potent AChE inhibitor (KC50~0.35 µM for inhibition of erythrocyte AChE activity; ref. 22). As such, 100 µM pyridostigmine was used in our experiments to ensure the inhibition of all AChE activity throughout the experiment. Cultured media was collected and centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was filtered through a 0.2-µm Acrodisc Syringe Filter (Pall Life Sciences, Port Washington, NY, USA; PN 6052) and injected into an ESA Ulimate 3000 system.
with a Choulico electrochemical detector (Thermo Scientific, Waltham, MA, USA). The sequence was run using the following components and parameters: flow rate, 0.360 ml/min; injection volume, 20 μl; cell potential, 279 mV; column, MGL Capcell Pak C18 column (Shiseido, Tokyo, Japan; 92441); column temperature, 40°C; and Ag postcolumn solid-phase reactor (Thermo Scientific, 70-0606A).

Nitric oxide (NO) measurement

Neonatal cardiomyocytes from control and cardiomyocyte-specific VACHT-KO (cVACHT; VACHTmub1aC57B6J/mub) mice were used to measure NO production, as described previously (12). Cells were incubated with either carbachol (10 μM) or pyridoxime (100 μM). Images were acquired using the Leica SP5 II confocal system and analyzed using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry (IHC)

Hearts were excised and fixed using 4% PFA. IHC was performed on slices as described previously (25). Slices were incubated with either anti-VACHT (1:200; Synaptic Systems) or anti-CNTF (1:200) (18) to visualize intracardiac ganglia and terminals, respectively.

Heart rate and blood pressure recording

Both heart rate and blood pressure were recorded from conscious animals using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA), as described previously (34).

Electrocardiography (ECG)

Electrocardiograms were recorded using radiotelemeters, as described previously (3). HR was recorded continuously over 24 h to obtain baseline recordings. In addition, heart rates were recorded in the home cages immediately following i.p. injection of saline or following an acute exercise routine (ramp up from 5 m/min to 15 m/min over 60 s, followed by 180 s at 15 m/min).

Cardiomyocyte morphometry

In situ cardiomyocyte cell surface area was measured as described previously (3).

Reactive oxygen species (ROS) measurement

ROS levels were measured using the MitoSOX Red superoxide indicator (Invitrogen, Carlsbad, CA, USA), as described previously (17).

Protein oxidation measurement

Protein was isolated from whole hearts using ice-cold modified RIPA buffer, separated using SDS-PAGE, and transferred onto PVDF membrane. The levels of oxidatively modified proteins were analyzed in control and cVACHT hearts using the OxBlot Protein Oxidation Detection Kit (Millipore, Bedford, MA, USA) following the manufacturer’s directions.

Indirect calorimetry, activity, and inactivity

These experiments were performed using the Comprehensive Laboratory Animal Monitoring System (CLAMS) metabolic chamber (Columbus Instruments, Columbus, OH, USA), as described previously (25, 26). V0, 02, and food and water intake, and activity/inactivity were measured. Respiratory exchange ratio and energy expenditure/heat were calculated within the Oxymax software (Columbus Instruments). Periods of inactivity (sleep) were obtained using the sleep detection algorithms available within the Oxymax software.

Preparation of siRNA

Potential target sites within the VACHT gene were selected and then searched with the U.S. National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov) was used to confirm specificity for the transporter. The siRNA for VACHT was prepared as described previously by our group (27). The sense and antisense oligonucleotides of siRNA were 5’-GAGACGAGGCCACGAAGAAGCCTGT-3’ and 5’-ACAGTTTCGTCGCGCTGCGGATGCAT-3’, respectively. For siRNA studies, neonatal cardiomyocyte cultures were transfected at 4 with 100 nM of siRNA 4 h prior to measurements. The cells were then used for immunohistochemistry, immunoblotting, or qPCR analyses.

Cardiomyocyte isolation and Ca2+ recording

Adult cardiomyocytes were isolated and calcium transients were recorded as described previously (5, 17) using linescan imaging on a Zeiss LSM 510 Meta confocal microscope. Images were processed and Ca2+ recordings were analyzed using ImageJ software.

Hemodynamic measurements

Invasive left ventricular (LV) hemodynamic measurements were obtained under baseline as well as following administration of isoproterenol (ISO; 0.5 μg i.p.) using a Millar Mikrotip pressure transducer (Millar Instruments, Houston, TX, USA), as described previously (3). All the LV parameters were obtained using the PowerLab Chart Analysis software (AD Instruments, Colorado Springs, CO, USA).

Statistical analyses

Results for experiments are reported as means ± SEM. Student’s t-test, 1-way ANOVA with Tukey’s post hoc test, or 2-way ANOVA was used to assess statistical differences between experimental groups as required, using GraphPad (San Diego, CA, USA) or SigmaStat (Systat Software Inc., San Jose, CA, USA). Values of P < 0.05 were considered statistically significant.

RESULTS

Generation of cardiomyocyte-specific VACHT null mice

Previous experiments indicated that, in cultured neonatal cardiomyocytes, ACh secretion is dependent on the activity of VACHT (12), a transporter that is critical for ACh storage in nerve endings (28, 29). To selectively interfere with secretion of ACh from cardiomyocytes in vivo, we generated the VACHT-Myh6Cre/lox mice by crossbreeding Myh6-Cre mice [B6.129-Tg(Myh6-cre)21B122md/J; Jackson Laboratory] with VACHT floxed mice (ref. 13 and Supplemental.
Fig. S1A, B. The α-myosin heavy chain (αMHC; Myh6) promoter drives Cre expression exclusively in cardiomyocytes (30). Although previous reports indicated that Cre is expressed mainly in ventricles in Myh6-Cre mice, Cre expression in the atria has been observed during development (31). To confirm elimination of VACHt in cVACHt mice, we isolated adult cardiomyocytes and performed RT-PCR to detect VACHt transcripts (Fig. 1A). A band of 167 bp was detected in VACHt mice but not in cVACHt mice. Sequencing analysis confirmed that this band represented the VACHt sequence. Moreover, immunoblot (Fig. 1B) and immunofluorescence analysis (Fig. 1C) confirmed elimination of VACHt in isolated ventricular cardiomyocytes from cVACHt mice. Moreover, whole-mount fluorescent IHC in the atria of control mice revealed that VACHt is also expressed in sinoatrial (SA) nodal cells, as colocalization was observed between VACHt and HCN4, a marker for the SA node (Fig. 1D). Notably, VACHt staining in SA nodal cells was absent in cVACHt mice, indicating that VACHt was also deleted in these cells in cVACHt mice (Fig. 1D). Furthermore, VACHt expression in parasympathetic ganglia and in nerve terminals was not altered in cVACHt mice (Fig. 1D, E), indicating that we specifically eliminated non-neuronal VACHt in the heart. In support of these data, we used a reporter mouse line to demonstrate that Cre expression is absent in parasympathetic nerve terminals stained with an antibody against the high-affinity choline transporter (CHT; Supplemental Fig. S1C). In addition, expression of CHT was not affected in isolated cardiomyocytes from cVACHt mice (Supplemental Fig. S1D). However, cVACHt cardiomyocytes appeared to have greater levels of ChAT expression (Supplemental Fig. S1E), an observation similar to that previously reported in VACHt-KO mice, which reflects, in part, a rearrangement of the cholinergic gene locus following Cre-mediated recombination (29).

Genetic removal of VACHt from cardiomyocytes inhibits ACh release

Secretion of ACh from cultured neonatal cardiomyocytes was analyzed using a fluorometric assay to detect choline/ACh levels in culture media. The assay was performed in neonatal cardiomyocytes after 4 d in culture, to avoid potential contribution of parasympathetic neurons that could contaminate adult cardiomyocyte preparations. Using this method, we could confidently detect as low as 10 pmol/well for ACh (Supplemental Fig. S2A). ACh release from control cardiomyocytes could be easily detected in the presence of picrotoxin, a cholinesterase inhibitor (Supplemental Fig. S2B) used to preserve secreted neurotransmitter. In the presence of VES, a specific inhibitor of the VACHt, ACh release from these cells was significantly diminished (Supplemental Fig. S2B). In agreement

Figure 1. Selective elimination of VACHt in cardiomyocytes of cVACHt mice. A–G VACHt expression was determined by PCR (A, Br., brain, N.C., negative control), immunohistochemistry (B), and immunofluorescence (C) in adult cardiomyocytes. Scale bars = 25 µm. D) Colabeling for VACHt and HCN4 in whole-mount atrial tissue from control and cVACHt mice. Asterisks indicate VACHt staining in parasympathetic nerve terminals. Scale bars = 25 µm. E) VACHt immunoreactivity in intracardiac parasympathetic ganglia from cVACHt mice. Arrows indicate positive staining for VACHt. Scale bars = 25 µm.
Inhibition of ACh secretion from cardiomyocytes alters heart activity.

cVACHT mice did not present any gross abnormalities in appearance or body weight (29.1±4.1 g vs 30.8±1.9 g in control vs cVACHT mice). Furthermore, these mice did not present alterations in several metabolic parameters, as measured using metabolic cages (Supplemental Table S2), which suggests that the cardiomyocyte changes observed were not deleterious to mutant mice under control conditions. Accordingly, we did not observe changes in blood pressure, as determined by tail cuff (Fig. 3A), nor in basal heart rate, as measured by radiotelemetry (EGC, Fig. 3B). In contrast, cVACHT mice showed a significant increase in heart rate when analyzed using the noninvasive tail-cuff system (Fig. 3A). This increase in heart rate was not due to the presence of Cre recombinase, as it was not observed in Mthb-cre mice when compared to WT mice under the same conditions (Supplemental Fig. S3A). These results suggest a possible role for cardiomyocyte-derived ACh in the regulation of heart rate.

To further confirm that the increased heart rate observed in cVACHT mice via tail cuff was due to inhibition of the non-neuronal cholinergic system in cardiomyocytes, we generated cardiomyocyte-specific ChAT-KO (ChAT-Mutant) mice. This ChAT-deficient line has been previously characterized; when crossed with Cre lines, ChAT expression is abolished, which parallels elimination of ACh (15, 32). Tail-cuff analysis in these mice also revealed increased heart rate in mice lacking ACh production (Supplemental Fig. S3B). Tail-cuff measurements require physical restraint, which is stressful for mice, while ECG measurements are obtained under conditions similar to the home cage environment. Hence, we hypothesized that the difference in heart rate observed using these two techniques might reflect an imbalance in cardiac regulation in mutant mice. Specifically, that cardiomyocyte-derived ACh could play a physiological role in boosting the parasympathetic signaling required for heart rate recovery in response to stress. To test this hypothesis, we used ECG telemetry to record heart rates immediately

Figure 2. A) ACh release from neonatal cardiomyocytes isolated and cultured from control and cVACHT mice. n = number of separate cell isolations for each genotype. ***P < 0.001 vs. control. B) ACh release from control and cVACHT neonatal cardiomyocytes as detected through HPLC with electrochemical detection. n = number of separate cell isolations for each genotype. ***P < 0.001 vs. control. C) Bioassay to measure ACh release in cultured neonatal cardiomyocytes using DAF Fluorescence. Cells were treated with either carbacol (Carb.) to activate muscarinic receptors and increase NO levels or pridostigmine (Prid.), which prevents ACh, which can then activate muscarinic receptors and increase the production of NO (12). n = number of cells examined from 5 separate cell isolations/genotype. Scale bars = 25 μm. Data are represented as means ± SEM. *P < 0.05 vs. control.
after mice were stressed by an intraperitoneal injection of saline. We observed that heart rate recovery of cVACHT mice to baseline levels was slower compared to control VACHT mice (Fig. 3C). These data suggest that, following increased sympathetic demand due to stress regulation of heart function by the parasympathetic system relies, at least in part, on ACH secreted from cardiomyocytes. To further test this possibility, cVACHT and littermate controls implanted with ECG telemeters were submitted to an acute, low-intensity, treadmill exercise test. Immediately following this mild exercise routine, heart rates in control and cVACHT mice were recorded in their home cages. The results demonstrate that this exercise routine led to a significantly greater increase in heart rate in cVACHT mice as compared to control mice (Fig. 3D). Moreover, heart rate recovery to preexercise levels took significantly longer in cVACHT mice when compared to control mice (Fig. 3D). These results suggest that cardiomyocyte-derived ACH may normally offset sympathetic activity, helping to reduce increased sympathetic demand.

cVACHT mice display cardiac hypertrophy and molecular remodeling

The results above suggest that control of heart rate, particularly under stress, is affected in cVACHT mice potentially due to decreased cholinergic signaling. Previous experiments indicated that in vitro inhibition of either VACHT activity or ACH synthesis increased hypertrophy in cultured neonatal cardiomyocytes treated with sympathetic agonists (12). To determine whether cardiomyocyte-secreted ACH plays a role in cardiac remodeling in vivo, we analyzed heart weight in 5-month-old mutant mice. As shown in Fig. 4A, cVACHT mice showed increased heart size. Cardiac hypertrophy was due to remodeling of cVACHT myocytes, which exhibited a significant increase in surface area when measured both in vitro (Fig. 4B) and in situ (Fig. 4C). Notably, the presence of Cre recombinase alone did not lead to either cardiac or cardiomyocyte hypertrophy (Supplemental Fig. S4A, B).

To further test whether the observed hypertrophic response was indeed dependent on VACHT expression and activity, we cultured WT neonatal cardiomyocytes and treated them with a pharmacological inhibitor of VACHT activity (VES), or with siRNA against VACHT (Supplemental Fig. S4A). VACHT knockdown was confirmed through qPCR and immunoblotting, which revealed an 80 and 60% decrease in VACHT mRNA and protein levels, respectively. Both of these treatments led to a significant increase in cardiomyocyte size after 48 h in culture. Furthermore, cardiomyocytes treated with an inhibitor of CHT1, HCG, which blocks ACH synthesis, presented similar hypertrophy (Supplementary Fig. S4C), thus confirming a hypertrophic response was due to inhibition of cholinergic signaling at the level of the myocytes.

Cardiac hypertrophy is associated with increased expression of fetal program genes, which contribute to cardiac remodeling (33). Expression of the markers of cardiac remodeling and stress, β-amyloid heavy chain (β-MHC) and ANP, was increased severalfold in cardiomyocytes from cVACHT mice (Fig. 5A). Immunostaining in isolated cardiomyocytes also revealed increased ANP protein levels (Fig. 5B, C), compatible with cardiomyocyte remodeling. Cardiomyocyte remodeling and reactivation of the fetal gene program are usually associated with increased cellular stress (34). Furthermore, hypertrophy induced through hyperadrenergic signaling has been coupled to increased ROS levels (35). If removal of VACHT from cardiomyocytes leads to imbalanced autonomic control of the heart, it is possible that mutant cardiomyocytes are under considerably more stress than control cardiomyocytes. We tested this possibility and found that cardiomyocytes isolated from cVACHT mice showed increased levels of ROS (as determined via nitro-
chondrial superoxide levels (Fig. 5D), suggesting that myocardial cells lacking the intrinsic cholinergic system display increased levels of stress. Furthermore, we determined that increased ROS levels in myocardial cells led to an increase in overall protein oxidation in the whole heart in eVACHt mice (Fig. 5D), as measured using the OxyBlot protein oxidation kit (Millipore), which can detect the levels of oxidized proteins.

Increased levels of mitochondrial ROS have previously been shown to increase [Ca^{2+}]_i in arterial myocytes, especially following induction of hypoxic stress (56). Therefore, we sought to determine whether the increased levels of ROS observed in eVACHt myocytes were accompanied by changes in calcium transients in ventricular cardiomyocytes. In agreement with this possibility, we detected an alteration in calcium handling in cardiomyocytes obtained from mutant mice with an increase in peak calcium (Ca^{2+}) transients (Fig. 5F, G). These results suggest that elimination of VACHt in cardiomyocytes leads to remodeling of cardiomyocytes, with overactivation of Ca^{2+} transients potentially altering cardiac function.

It is likely that inhibition of the nonneuronal cholinergic system leads to imbalanced regulation of cardiac activity. As such, we analyzed whether components involved in sympathetic signaling, and thus, regulation of cardiomyocyte function, were altered in eVACHt mice. GRKs have been shown to play important roles in cardiac hypertrophy in response to increased sympathetic activation (57, 58). Therefore, we examined the expression of GRK2 and GRK5, the two most predominantly expressed GRKs in cardiac tissue. Although GRK2 did not show any changes in expression, mRNA expression of GRK5 was significantly increased in eVACHt myocytes (Fig. 6A). Moreover, immunostaining analysis showed that cytoskeletal localization of GRK5 was altered in eVACHt cardiomyocytes, as mutated cells showed increased nuclear localization for GRK5 compared to control cardiomyocytes (Fig. 6B, C).

**Cardiomyocyte remodelling in eVACHt mice affects LV function**

Ventricular cardiomyocytes from eVACHt mice display remodeling and hypertrophy; therefore, we tested whether these alterations affected LV contractility. Invasive hemodynamic assessments were performed on anesthetized control and eVACHt mice. As shown in Table 1, the hemodynamic parameters were similar in both genotypes under baseline conditions; however, the LV hemodynamics were altered in eVACHt mice following a bolus dose of ISO, used to increase cardiac contractility. Interestingly, although heart rate was similar in both genotypes under baseline, the eVACHt mice showed a significant decrease in heart rate following ISO treatment. Furthermore, the maximum rate of LV pressure rise (peak ++dP/dt max) was significantly decreased, and the maximum rate of LV pressure fall (peak --dP/dt max) showed a tendency for decrease in eVACHt mice (p<0.05). In addition, the contractility index of eVACHt mice was different from control counterparts under baseline; however, a significant reduction was found in the contractility index following ISO treatment in eVACHt mice. These results suggest compromised LV function in the mutant mice, especially under increased cardiac demand, as induced through treatment with ISO.

**DISCUSSION**

Our experiments reveal a novel and unexpected way by which cardiovascular function is regulated. These data indicate that cardiomyocytes can secrete significant amounts of ACh in vivo via a VACHt-dependent mech-
Figure 5. Cellular stress in cVACHt cardiomyocytes. A) Expression of the cardiac stress markers, β-MHC and ANP, in control and cVACHt cardiomyocytes (n=number of mice). B) Measurement of oxidized protein levels in whole hearts from control and cVACHt mice (n=number of mice). C) Recruitment of ANP in adult cardiomyocytes from control and cVACHt mice. D) Measurement of ROS levels in isolated cardiomyocytes loaded with the MitoSOX superoxide indicator (n=number of cells; ≥3 mice/genotype). E) Measurement of oxidized protein levels in whole hearts from control and cVACHt mice (n=number of mice). F) Representative recordings of line-scan profile of Ca2+ transients in control and cVACHt myocytes. G) Summary of peak Ca2+ (n=number of cardiomyocytes; cells isolated from ≥3 mice/genotype). Scale bar = 5 μm. Data are represented as means ± SEM. *P < 0.05 vs control.

...animal, similar to that observed in nerve terminals (39), cVACHt mice seem to lack the capacity to sustain normal levels of cholinergic signaling, implicating nonneuronal ACh as part of a physiological system that controls cardiac function. Lack of cardiomyocyte-secreted ACh leads to altered heart rate regulation under stress. The SA node controls heart rate; therefore, the alteration in heart rate we observed in cVACHt mice is likely related to the altered expression of VACHt in SA node myocytes observed in these mutants. Notably, measurements of heart rate in the absence of stress indicate that regulation of basal activity does not seem to depend on this cardiomyocyte cholinergic system. Hence, contribution of cardiomyocyte-derived ACh is revealed only with increased demand on the parasympathetic system, such as following exercise. Notably, an inverse relationship between decreased serum AChE levels and delayed heart rate recovery following exercise has previously been reported in healthy human subjects (40). This...

Figure 6. Cardiac remodelling in cVACHt cardiomyocytes. A) mRNA expression of GRK5 in isolated cells from control and cVACHt mice (n=number of mice). B) C) Immunostaining for GRK5 in isolated myocytes. Scale bar = 25 μm. Data are represented as means ± SEM. *P < 0.05 vs control.
previous work provides further support for the importance of peripheral ACh levels, including cardiomyocyte-derived ACh, in regulating cardiac function.

It is likely that non-neuronal ACh, secreted from cardiomyocytes, functions through similar second messenger systems as those activated when neuronal ACh binds to muscarinic receptors. Future electrophysiological studies will determine the mechanisms leading to alterations in chronotrophic responses observed following inhibition of non-neuronal cholinergic signaling in the specialized cardiomyocytes of the SA node.

It is well established that increased sympathetic signaling can lead to cardiac remodeling and increased cardiomyocyte stress in the working myocardium (41). Indeed, even though cVACH T mice presented normal regulation of heart rate under norepinephrine conditions, changes in heart rate, due to lack of cardiomyocyte-derived ACh, may cause long-term changes in heart function. It appears that lack of the intrinsic cholinergic system in cardiomyocytes leads to an increase in basal stress. This may play an important role in the regulation of cardiac function because it has previously been reported that increased stress can lead to an up-regulation of AChE transcription in the brain (42).

It will be important to determine whether cVACH T mice display altered AChE transcription in the heart, which may then serve to further exacerbate the stress response by increasing degradation of neuronal ACh.

Lack of cardiomyocyte-derived ACh was associated with ventricular cardiomyocyte hypertrophy, molecular remodeling, and increased oxidative stress, which argues for a widespread effect of cardiomyocyte-secreted ACh. These molecular changes were accompanied by alterations in left ventricular function and cardiac contractility under increased demand due to treatment with ISO. The observed hypertrophy may be related to the increased expression, as well as altered subcellular localization, of GRK5 observed in cVACH T cardiomyocytes. It has been reported that GRK5 overexpression leads to hypertrophy, which is dependent on its nuclear function (37). Conversely, GRK5 null mice display a delay in hypertrophy following transverse aortic constriction (43). Further studies will be required to elucidate potential GRK5-specific pathways affected in cVACH T myocytes that can lead to remodeling and hypertrophy. In addition, it will be important to determine whether the transcriptional activation of hypertrophic genes, including GRK5, are regulated by miRNAs as it has previously been shown that overexpression of the miR212/132 family of miRNAs leads to pathological cardiac hypertrophy (44). It will be interesting to determine whether lack of cardiomyocyte-

Figure 7. Non-neuronal release of ACh from cardiomyocytes. WT cardiomyocytes secrete ACh in response to increased physiological stress (e.g., exercise) in a VACH T-dependent manner to regulate heart rate. This response is blunted in mice lacking VACH T specifically in cardiomyocytes due to the lack of non-neuronal ACh release.
derived ACh leads to hypertrophy in a miRNA dependent mechanism.

Although the detailed mechanism that triggers the release of ACh from cardiomyocytes are not clear at the moment, a clear possibility might involve sympathetic signaling. Previous experiments in vitro suggest that adrenergic stimulation can induce the expression of choline genes in cardiomyocytes (12). Whether reciprocal interactions occur between sympathetic activation and cardiomyocyte-derived ACh is unknown. Interestingly, previous experiments have demonstrated that proteins involved with exocytic release of neurotransmitters, or their homologues, are expressed in vesicular compartments in cardiomyocytes (45). Hence, secretory vesicle compartments in cardiomyocytes are likely to be the source of ACh secretion (Fig 7). It should be noted we have previously established that VACHT is present in recycling vesicles in cardiomyocytes (12). Our observation that the cholinergic cardiomyocyte machinery seems to control heart rate after stress and exercise suggests that cardiomyocyte-derived ACh secretion may be regulated by sympathetic activity.

Interestingly, activation of the cholinergic anti-inflammatory pathway has been shown to play an important role in preventing the release of proinflammatory cytokines from circulating macrophages and thereby attenuating the effects of infection or injury (46). Immune cells can serve as a non-neuronal source of ACh, and its levels appear to be altered in a miR-122 dependent mechanism involving targeting of ACHe miRNA (47). Although the activity of the cholinergic anti-inflammatory reflex has not been measured here, it is expected that its function will be unravelled due to the specificity of the Cre line used.

Recently, non-neuronal secretion of ACh in a VACHT-dependent manner was demonstrated from pancreatic acinar cells, a process that regulates insulin secretion in humans (6). Here, we show that secretion of ACh from cardiomyocytes also has physiological relevance as this non-neuronal cholinergic system plays a previously unsuspected role in regulating heart rate as well as remodeling. Based on these results, it is necessary to reevaluate how the sympathetic-parasympathetic systems control heart activity to include this novel form of autocrine/paracrine communication from cardiomyocytes. We propose that secretion of ACh from cardiomyocytes enhances cholinergic signaling from parasympathetic neurons required to regulate cardiomyocyte activity (Fig 7). Cardiomyocyte-derived ACh augments extracellular levels of this neurotransmitter in synaptic junctions between parasympathetic terminals and cardiomyocytes during high demand; for example, in response to increased sympathetic activation due to physiological stress. This novel mode of communication may serve to offset constant sympathetic signaling avoiding increased cardiomyocyte stress and hypertrophic responses.

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REFERENCES


In the above publication, several contributions were made to see to its completion. My contributions to the paper involved both the direct, as well as the indirect measurements of ACh. More specifically, I was responsible for determining the amount of ACh released from both $\text{VACHT}^{\text{flox/flox}}$ and $\text{VACHT}^{\text{Myh-6-Cre-flox/flox}}$ neonatal cardiomyocytes under various pharmacological treatments using HPLC-ED and a choline/ACh fluorometric assay. Additionally, I was responsible for all the data acquired from $\text{VACHT}^{\text{flox/flox}}$ and $\text{VACHT}^{\text{Myh-6-Cre-flox/flox}}$ neonatal cardiomyocytes subjected to DAF-FM analysis.
Curriculum Vitae

William C. Fields

Education

2011-2013  Neuroscience Graduate Program; MSc Degree
            *University of Western Ontario*, London, Ontario, Canada

2007-2011  Biological and Medical Sciences, Honours Specialization
            Medical Sciences Graduate
            *University of Western Ontario*, London, Ontario, Canada

Relevant Work Experience

2011-2013  Psychology Teaching Assistant
            *University of Western Ontario*, London, Ontario, Canada
            
            As a TA I was responsible for holding office ours, marking assignments, and preparing, as well as hosting, exam review sessions

Awards and Honors

2011-2013  Schulich Graduate Scholarship
            *University of Western Ontario*, London, Ontario, Canada
            
            This is an award given to students with an academic average of 80+. This award pays the tuition fees for up to six terms of a Master’s Degree

2009-2011  Dean’s Honor List
            Award Recipient
            *University of Western Ontario*- Faculty of Science, London, Ontario, Canada
            
            This award is presented to those students who maintain a cumulative 80% average among all their courses for a given year.
2009  

**Triple-A Scholarship**  
Scholarship and Award Recipient  
*Phi Gamma Delta Fraternity*, Lexington, Kentucky, USA  

As the Fraternal Organization of Phi Gamma Delta prides itself on scholarship and pursuing higher aims, they award a $500.00 scholarship for those brothers who have attained at least a 3.0 in their pledging semester.

2007-2008  

**Queen Elizabeth “Aiming for the Top” Scholarship**  
Scholarship Recipient  
*Government of Ontario*  

This award is provided by the Ontario Government to students who graduate their secondary education with an average of over 90%.

2007-2011  

**Millennium Bursary**  
Bursary Recipient  
*Government of Ontario*  

This bursary is given to those students who excel with their academics but concomitantly are in need of financial aid.

2007-2008  

**Kennedy Clipper Award**  
Scholarship and Award Recipient  
*Kennedy Collegiate Secondary School*, Windsor, Ontario, Canada  

This award of $1,000.00 is given to one male and one female of the graduating class. The recipient of this award is one who is an exceptional scholar, aids and provides to their community, and one who excels at extracurricular activities.

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**Publications**

**Extracurricular Involvement and Volunteering**

2010 & 2011  **Austen Berlet Campout for Mental Health**
Event Organizer
*University of Western Ontario*, London, Ontario, Canada

In honor of our late brother who was part of the fraternal organization of Phi Gamma Delta, this event is held annually in the spring in order to raise awareness about mental health and mental illnesses, as well as raise money for the Canadian Mental Health Association. After organizing the event in 2010 and 2011 over $8,000.00 and $20,000.00 was raised, respectively. The event requires but is not limited to, delegating tasks to several other committee chairs, dealing with several brokers and insurance agencies to obtain a five million dollar insurance policy, and acquiring bands and other forms of entertainment for the 24 hour event.

2010-2011  **Alzheimer’s Western**
Club Volunteer
*University of Western Ontario*, London, Ontario, Canada

This club at the University of Western Ontario was created in 2009-2010 in order to raise awareness about Alzheimer’s disease. As a member of this club, I have helped organize several different events, while additionally volunteering my time by working with those individuals and families affected by this disease. Working with such individuals has enabled me to be more patient, aided in my ability to be a more open minded individual and has increased my communication skills with others.

2010-2011  **“Get Swabbed” Drive for Bone Marrow**
Event Volunteer
*University of Western Ontario*, London, Ontario, Canada

In 2010, the first time that this event was held at UWO, the “Get Swabbed” Campaign identified donors for those in need of stem cell transplants. As a volunteer of this event, I was responsible for raising awareness about the importance of bone marrow and the need for more donors. Additionally, I had the responsibility of physically swabbing the inner cheeks of willing participants in
order to increase the ever growing database of bone marrow donors.

2009-2010

**Western Foot Patrol**
Walking Team Volunteer
*University of Western Ontario*, London, Ontario, Canada

Western Foot Patrol is a free service that promotes safety and deters crime on the UWO and affiliate college campuses. They seek to provide a safe and secure environment for students, faculty and staff and to raise awareness of personal safety issues, deter crime through visible patrols and to provide safe escorts to community members. As a member in my junior and senior years, I was responsible for escorting individuals across campus ensuring their safety and well-being, and additionally, was responsible for ensuring a safe campus and reporting any suspicious activity that may have been present.

2009-2011

**Phi Gamma Delta Fraternal Organization**
Executive Member; Academic Chairman and Athletic Chairman
*University of Western Ontario*, London, Ontario, Canada

In the spring of 2009, myself and 8 others, with the help of a field secretary from British Colombia, Canada, created the Lambda Omega Chapter of the Fraternal Organization of Phi Gamma Delta. In just over two years’ time, I helped to increase the number of academic members to over 50. In those two years I have headed both fraternal athletics and fraternal academics. In my first year I was the head of fraternal athletics and was responsible for organizing inter-fraternal and intra-fraternal sporting events. In my second year I was the academic chair which involved creating the Austen Berlet Scholarship Fund, creating an academic database of past lecture notes and exams, and ensuring that members maintained high academic averages through organizing library hours, private tutors, etc.

2006-2007; 2009

**Hotel Dieu Grace Hospital**
Emergency Room Volunteer
*Hotel Dieu Grace Hospital*, Windsor, Ontario Canada
Initially acquiring this position as a co-op in my senior year of high school, I have also volunteered in this position during the summer of 2009. In all, I have volunteered over 500 hours in the Emergency Room which involved aiding porters when needed, escorting patients to and from laboratory testing, stocking all patient rooms, and lastly, communicating with patients and their families. Emergency Room volunteering played an essential role in developing my ability to work independently while also enabling my ability to work in an extremely stressful environment.