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Cardioprotective Role of the Cholinergic System

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Graduate Program in Physiology and Pharmacology

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

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CARDIOPROTECTIVE ROLE OF THE CHOLINERGIC SYSTEM

(Thesis format: Monograph)

by

Mouhamed Dakroub

Graduate Program in Physiology and Pharmacology

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The process of aging is an irreversible continuum experienced by all individuals. A large number of physiological transformations occur to the cardiovascular system as one ages. These changes result in increased risk of developing cardiovascular diseases, many of which are frequently seen in geriatric populations. While the exact mechanisms of age-related cardiac dysfunction have not been established, abnormal cholinergic dysfunction has been implicated in the pathology of other age related diseases; therefore, we have hypothesized that age induced cholinergic dysfunction is detrimental to cardiac function and health. This study seeks to identify whether increased cholinergic signaling, either by transgenic overexpression of the vesicular acetylcholine transporter (VAcChT), or by drug intervention, decreases incidents of cardiovascular dysfunction with age. Our experiments suggest that cholinergic signaling is implicit in preventing age-related cardiac dysfunction. In addition, cholinergic signaling at the time of acute injury is also critical in preserving cardiac function post-injury.

Keywords

Aging

Cholinergic signaling

Heart failure

Vesicular acetylcholine transporter
Co-Authorship Statement

All experiments presented in this thesis were performed by Mouhamed Dakroub under the supervision of Dr. Robert Gros. Experiments were performed at the University of Western Ontario in the Department of Physiology and Pharmacology.
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AW</td>
<td>Anterior wall</td>
</tr>
<tr>
<td>ANF</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>ADBR1</td>
<td>Beta-1 adrenergic receptor</td>
</tr>
<tr>
<td>MHY7</td>
<td>Beta-myosin heavy chain 7</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>BChE</td>
<td>Butyrylcholinesterase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>ChR2+</td>
<td><em>ChAT-ChR2-EFYP</em> positive mouse</td>
</tr>
<tr>
<td>ChR2-</td>
<td><em>ChAT-ChR2-EFYP</em> negative mouse</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>cNOS</td>
<td>Constitutive nitric oxide synthase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>d</td>
<td>Diastole</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>CHT1</td>
<td>High-affinity choline transporter</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Gi</td>
<td>Inhibitory G-protein subunit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1-beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IR</td>
<td>Ischemic reperfusion injury</td>
</tr>
<tr>
<td>LVID</td>
<td>Left ventricular internal dimension</td>
</tr>
<tr>
<td>M2</td>
<td>Muscarinic receptor subtype 2</td>
</tr>
<tr>
<td>M3</td>
<td>Muscarinic receptor subtype 3</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NNCS</td>
<td>Non-neuronal cholinergic system</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>(PGC)-1alpha</td>
<td>Peroxisome proliferator-activated receptor-gamma coactivator</td>
</tr>
<tr>
<td>PW</td>
<td>Poster wall</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-time polyermase chain reaction</td>
</tr>
<tr>
<td>Gs</td>
<td>Stimulatory G-protein subunit</td>
</tr>
<tr>
<td>s</td>
<td>Systole</td>
</tr>
<tr>
<td>TNF (\alpha)</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>cVACHT-</td>
<td>(VAChT^{\text{lox}flox,\text{Myh6-cre}}) mouse line</td>
</tr>
<tr>
<td>cVACHT+</td>
<td>(VAChT^{\text{lox}flox,\text{Myh6-cre}}^{+}) mouse line</td>
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Chapter 1

1 Introduction

Cardiovascular disease (CVD) remains the leading cause of death in North America (Record N et al. 2015). Despite increased efforts by the public health sector to reduce the prevalence of these diseases, it has been proposed that by 2030, 40.5% of the North American population will have some form of CVD (Heidenreich et al. 2011). A plethora of potential risk factors have been established in attempts to help predict the potential onset of CVD in some individuals. Such factors include obesity, drug use, high-fat diet, and various other life-style related circumstances (Upadhyay 2015). The majority of these risk factors can be managed through life-style changes; however, one of the most critical factors remains inevitable: age (North and Sinclair 2012).

Aging is a continuous and irreversible process that affects every organ of the body (Aspinall 2013). Notably, it is common for elderly individuals to experience significant declines in cardiovascular function as a result of the aging process (Sahle et al. 2015). Cardiovascular aging includes changes in heart morphology, functionality, and increased risk for the development of cardiovascular diseases. As individuals age, changes in cardiac wall thickness, conduction, arterial stiffness, and oxygen consumption begin to occur. Many of these changes will continue to progress with age and are frequently implicated in the development of age-related cardiac decline (Gupta et al. 2015). Despite advances in medical treatments, mortality as a result of CVD is continuing to rise in the elderly population (Gedela, Khan, and Jonsson 2015). Currently, the mechanisms behind
why or how age-dependent cardiac abnormalities arise have yet to be elucidated.

Furthermore, very little knowledge exists about how to reverse age-related cardiac changes in attempts to prevent or delay the onset of CVD in the elderly. This study seeks to uncover information that will be crucial in understanding how to treat, delay, or even prevent cardiac aging and lower the chance of developing cardiac disease as a result of the aging process.

1.1 The Aging Process

From single-celled organisms to large mammals, almost all living beings experience the universal phenomena of senescence (Finch 1994). The biological process of aging is marked by the accumulation of irreversible and permanent physiological changes as one grows older. There have been many attempts to uncover the major pathological mechanism responsible for initiating the aging process, such as microbiome degeneration (Heintz and Mair 2014), accumulation of reactive oxygen species (Hekimi, Lapointe, and Wen 2011), and chromosomal telomere shortening (Xi et al. 2013). While these factors are likely important in certain age-related pathologies, no single factor has been isolated as the universal cause of aging. Attempts to discover a single genetic “switch” to initiate the aging process has proven that the process of aging is much more complex and likely arises as a result of thousands of different intertwined processes. Because of the complexity and scope of these cellular changes, it becomes crucial to study aging not by
using a universal approach, but rather study the age-related changes unique to groups of organs, tissues, and cells of the body.

Aging is often associated with declining function of the cardiovascular system (Lakatta 1990). In addition, cellular senescence is often a cause of diminished reparative and protective mechanisms throughout the body (Juhaszova et al. 2005). The convergence of declining cardiac function in addition to decreased reparative mechanisms results in a condition where the onset of CVD in the elderly is often associated with high mortality and morbidity (Roger et al. 2012). This association has considerable merit as one the leading cause of death in geriatric populations is heart failure (Roger et al. 2012). Although it has yet to be discovered why the heart progresses into a state of age-related dysfunction, it is apparent that the cardiovascular changes brought on by age increase the risk of developing CVD, potentially resulting in heart failure. Although the aging process alone does not cause heart failure, it does lessen the threshold for manifestation of CVD. The age-related changes in cardiovascular anatomy are likely adaptive in nature, brought on by lifelong stressors and reparative processes but necessary for maintaining proper physiological functioning (Gupta et al. 2015). However, overtime these structural changes in cardiovascular tissue are responsible for the alterations in cardiac function. The rate at which these changes develop varies between individuals; however, advanced progression in just one aspect increases the overall risk of cardiac dysfunction. (North and Sinclair 2012).
1.2 Cardiovascular changes with age

Cardiac aging is accompanied by variation in nearly all aspects of cardiac health. Structural, functional, protective, and reparative processes all make significant transformations as a result of the aging process (Gupta et al. 2015). Structural changes such as cardiac wall thickening, increases in fibrous tissues, and calcification may lead to functional changes in heart physiology that diminish the ability of the heart to respond to stressors and increased workload. Functional abnormalities may promote compensatory changes to overcome abnormal functioning that, as a result of diminished cardioprotective and reparative processes, may develop into cardiac complications and diseases (Cheitlin 2003). Prolonged dysfunction as a result of cardiac disease is often the cause of heart failure in the elderly.

1.2.1 Left ventricular wall thickening

The myocardial cells of the heart experience some of the most profound changes brought on by age. Older individuals experience increased myocyte apoptosis that results in a significant reduction in total myocyte number. This loss of functional myocytes is thought to be one of the main contributors to the decline of cardiac functionality with age (Cheitlin 2003). Additionally, older individuals are more susceptible to myocyte apoptosis due to loss of reparative processes and as a result are at high risk for further complications post-cardiac injury (Masoro and Austad 2010). Why apoptosis occurs in
aging cardiomyocytes has yet to be elucidated; however, it may be the result of a prolonged stressor, such as pressure overload or oxygen radicals (Twu et al. 2002) producing unreparable damage. As a result of apoptosis, the remaining myocytes increase in size to overcome the large loss of adjacent cells as a compensatory mechanism (Gupta et al. 2015). Increases in myocyte size are accompanied with fibrosis and calcification of the fibrous skeleton of the heart which further results in hypertrophy (Chetlin 2003). The end clinical consequence is an increase in ventricular filling pressure, diastolic dysfunction, and impaired diastolic filling resulting in cardiovascular dysfunction (Gupta et al. 2015).

### 1.2.2 Abnormal cardiac conduction

The conduction system of the heart begins with sinoatrial and atrioventricular (AV) node connected by the intermodal tract. Action potentials generated by pacemaker cells of the sinoatrial node travel throughout the atria primarily by cell-to-cell conduction. This propagation results in atrial contraction. Impulses propagate toward the apex of the heart through the HIS bundle into the left and right bundle branches before finally reaching the purkinje fibers in the apex of the heart (Betts et al. 2013). Impulse conduction by purkinje fibers results in depolarization of ventricular myocytes and thus contraction. Autonomic nerve activity heavily influences this process. Although abnormal propagation can occur at any point in the conduction pathway, dysfunction in the elderly is normally observed in the form of AV blocks and atrial arrhythmias (Gupta et al. 2015). Age-related apoptosis, calcification, or degeneration of nerves may all contribute to age-related abnormal
conduction. AV blocks are caused by the disruption of conduction between the atria and the ventricles. As a result, the ventricles begin to pace at their own intrinsic rate which rarely meets the demand of a non-resting heart. Age-related AV blocks and atrial arrhythmias may be the result of calcification or fibrofatty infiltration into tissues that contain the conduction pathways (Chow, Marine, and Fleg 2012). Abnormal conduction may result in a variety of clinical significance seen in the elderly, such as chest pain, exercise intolerance, and fainting (Stern, Behar, and Gottlieb 2003).

1.2.3 *Arterial stiffening and endothelial dysfunction*

Many vascular changes have been observed as a result of the aging process. Vessel wall thickening and increased dilatation are the predominant structural changes that occur within large elastic arteries (Lakatta and Levy 2003). Increased collagen and calcification in addition to reduced elastin within the arterial wall results in the thickening of the tunica externa and tunica media. These changes significantly decrease vessel compliance (Prisant 2005). Increased arterial stiffness, in addition to altered regulation of vascular tone, contribute to major risk factors for the development of hypertension (Gupta et al. 2015).

Endothelial dysfunction is also frequently observed in elderly patients (Wray et al. 2012). Under normal conditions, endothelial cells contain two forms of nitric oxide synthesizing enzymes (NOS): constitutive NOS (cNOS, also known as endothelial NOS (eNOS)) and inducible NOS (iNOS). cNOS functions to continuously catalyze the reaction responsible
for the production of nitric oxide (NO). Although cNOS mediated NO production is continuous, production can be upregulated by high levels of intracellular calcium (Xing et al. 2002). iNOS activation however is calcium independent and is mainly stimulated during inflammatory responses (Zamora, Vodovotz, and Billiar 2000). Newly synthesized NO rapidly diffuses into vascular smooth muscle cells where it can bind to and activate soluble guanylyl cyclase, catalyzing the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP predominately inhibits L-type calcium channels as well as causing cellular hyperpolarization through activation of potassium channels. This ultimately results in the relaxation of smooth muscle and subsequent vasodilation. Geriatric populations often demonstrate abnormal vasodilatory responses. Studies have demonstrated that as a result of the aging process, steady declines in NO production occurs, resulting in significant abnormal vasomotor tone (Torregrossa, Aranke, and Bryan 2011). Abnormal vasomotor tone often manifests as hypertension in the elderly and if not treated with antihypertensive medication, may result in CVD and mortality (Butt and Harvey 2015)

### 1.3 Heart failure in the elderly

By the age of 65, the heart has contracted and relaxed over 2 billion times. The amount of work performed by the heart is astounding when compared to any other muscle in the body. Despite this remarkable workload, most elderly individuals continue to have adequate heart function for many years after 65. However, progressive and inevitable
age-related alterations of the heart and vasculature will increase the risk of developing cardiovascular complications (Zaret et al. 1992). Heart function will eventually decline with age, and the risk for heart failure after the age of 65 increases drastically (Ahmed 2009).

Heart failure occurs when the heart is unable to sufficiently maintain blood flow to meet the normal demands of the body. Heart failure is not a disease, but rather a syndrome and is therefore caused by an underlying cause (Ahmed 2009). Coronary artery disease and hypertension are two of the most common underlying causes associated with heart failure in the elderly (Gottdiener et al. 2000). Age-related structural and physiological alternations, such as endothelial dysfunction, decreased elasticity, and increased calcification occur as a result of the normal aging process. Consequently, these changes predispose the elderly population to chronic diseases, such as hypertension, that if left untreated, may result in various CVD and potentially heart failure (Stern, Behar, and Gottlieb 2003).

Ventricular hypertrophy often occurs as a result of normal aging, and may predispose elderly individuals to cardiac dysfunction and heart failure (Jones et al. 1990). As the ventricular walls progressively thicken, the risk of diastolic dysfunction sharply increases (Nagueh et al. 2009). Myocardial fibrosis, an integral component of ventricular hypertrophy, appears to be the most important structural alteration in the development or progression of diastolic heart failure caused by ventricular hypertrophy (Gradman and Alfayoumi 2006). Increased stiffness as a result of fibrosis greatly decreases ventricular compliance and impairs relaxation, resulting in elevated left ventricular filling pressures and increased venous pressures. Interestingly, diastolic dysfunction as a result of age-
related ventricular hypertrophy is routinely discovered in elderly patients once they have been hospitalized due to pulmonary edema. Pulmonary edemas are often a result of increased venous pressure seen in elderly patients suffering from diastolic dysfunction (Fuster, Alexander R. Wayne, and O’Rourke 2001).

Cardiac conduction disturbances become more common and are treated in higher frequencies in the elderly population (Guize et al. 2006). Acute disruption of the cardiac conduction pathways may result in minor hemodynamic disturbances, such as short-lived episodes of bradycardia or tachycardia. However, major disturbances may result in more severe consequences, such as partial or complete heart block (Lilly 2007). AV heart block occurs when electrical impulses generated in the atria are unable to propagate into the ventricles. This results in disruption of cardiac contractions and subsequent inability of the heart to properly function (Schoenmakers, de Graaff, and Peters 2008). While third-degree heart block require medical attention immediately, second or first-degree block may appear to be relatively benign to the patient, but may severely impair proper heart function and can potentially result in heart failure if left untreated (Dhingra et al. 1974). Long Q-T syndrome is also frequently observed in the elderly population (Michael Reardon and Malik 1996) and is also a consequence of conduction disturbances. Patients with severely prolonged Q-T intervals experience abnormally longer periods of time between ventricular depolarization and subsequent repolarization. Abnormal Q-T intervals in elderly patients may result in severe arrhythmias and sudden death (Michael Reardon and Malik 1996).

Vasculature function also plays a central role in the development and progression of heart failure observed in the elderly population. Age-related endothelial dysfunction, NO
deficiency, and increased arterial stiffness may result in systemic and pulmonary vascular constriction and subsequent elevations in blood pressure (Marti et al. 2012). Abnormal endothelial-dependent NO vasodilation responses in the coronary arteries may result in insufficient myocardial perfusion resulting in worsening ventricular function and in severe cases, myocardial ischemia (Dusting 1996). In addition to cardiovascular injury, renal dysfunction routinely accompanies vascular abnormalities and is an important component in the cardiorenal syndrome of heart failure (Blair et al. 2007). Arteriolar vasoconstriction resulting in reduced filtration may result in vascular volume overload and increased cardiac-preload, hence accelerating the onset or progression of heart failure in the elderly (Marti et al. 2012).

In addition to thickening and stiffening of the vasculature, the four valves of the heart are also susceptible to age-related sclerosis. Aortic valve sclerosis is the most common valvular disorder and effects up to one third of the elderly population (Zaret et al. 1992). Increased calcification of the aortic valve may result in the progression and development of aortic stenosis. Calcified deposits begin to narrow and block the movement of blood out of the left ventricle. This condition is much more severe than aortic sclerosis and if not treated can result in heart murmurs, fainting, chest pain, and ultimately heart failure (Carabello 2002).

Although the development of just one of the pathological conditions above may not result in heart failure, severe or prolonged conditions may substantially increase the risk of development. Elderly individuals are at a much higher risk of developing these conditions and are thus at a much higher risk of suffering from heart failure.
1.4 Cholinergic Hypothesis of Aging

Similar to CVD, there is an unprecedented rise in the incidents of cognitive disorders in elderly populations (Ballesteros et al. 2015). A wide range of potential etiologies have been hypothesized as to why impairments in memory and executive functions seem to occur frequently in old age (Araujo, Studzinski, and Milgram 2005); however, it was not until 1982 that the cholinergic hypothesis of cognitive decline in aging was proposed by Bartus et al. 1982.

The cholinergic hypothesis of geriatric memory dysfunction was based on the model that aging causes alterations of the cholinergic system that results in cognitive decline (Bartus et al. 1982). Abnormal cholinergic activity, mainly as a result of decreased acetylcholine (ACh) release from cholinergic neurons play an important role in the development and progression of cognitive loss (Dumas and Newhouse 2011). Thus, by slowing the progression of cholinergic dysfunction with age, or by restoring proper cholinergic function, the severity of cognitive loss with age should be greatly reduced. The cholinergic hypothesis of aging has been supported by findings that patients suffering from cognitive impairment such as Alzheimer’s disease, show beneficial improvements in cognition when administrated cholinergic enhancing drugs (Hansen et al. 2008). Although various studies exist which support the cholinergic hypothesis of aging, it has yet to be discovered why cholinergic signaling becomes abnormal with advanced age. However, regardless of the potential etiology, it is apparent that restoration of cholinergic function is beneficial to those experiencing age-related cognitive decline.
Although the cholinergic hypothesis of aging was proposed as model of cognitive dysfunction, the principals of age-related decreases in cholinergic function also apply to peripheral tissues where both neuronal and non-neuronal cholinergic systems also exist and function. It has been demonstrated that cholinergic signaling in a variety of organs and tissues steadily declines as an individual ages. Age-related decreases in ACh release, and hence abnormal cholinergic signaling, have been implicated in the pathology and progression of a wide spectrum of diseases. Studies have demonstrated age-dependent reductions in cholinergic tone in tissues of the pituitary (Coiro et al. 1992), cortex (Shaw, Tafti, and Thorpy 2013), endothelium (Taddei et al. 2001), colon (Roberts, Gelperin, and Wiley 1994) and various other tissues of the body.

Although age-related declines in cholinergic signaling results in undesirable outcomes, cholinergic decline in cardiovascular tissues is of notable importance due to the resulting impairment of cardiac function. Investigations performed on rodent models have demonstrated that cardiomyocytes begin to excrete significantly less ACh as they begin to mature into older years of age (Rana et al. 2010). Similar results from clinical investigations performed on human patients demonstrating that significantly decreased amounts of ACh were being release from atrial tissues in patients >70 years of age (Oberhauser et al. 2001). Various physiological factors may be responsible for age-related decreases in cardiac cholinergic signaling. Studies on rodent models have demonstrated aging alone results in the down regulation of cholinergic enzymes responsible for the synthesis and release of ACh (Rana et al. 2010). Choline acetyltransferase (ChAT) is the enzyme responsible for synthesizing ACh by catalyzing the transfer of an acetyl group from the molecule acetyl-CoA to choline and thus
producing ACh. ACh must then be loaded into secretory vesicles by the actions of the vesicular acetylcholine transporter (VAcHT) (Prado et al. 2013) (Figure A). Both proteins are crucial for proper cholinergic signaling. Abnormal expression of both ChAT and VAcHT have been shown in aged rodent models suggesting aging alone may impair proper cholinergic signaling (Rana et al. 2010; Efange et al. 1997).

Regardless of the cause, the aging process results in a reduction in cholinergic signaling that has been implicated in the initiation and progression of a multitude of different diseases. Thus, cardiac cholinergic dysfunction may be an important factor for the development and progression of heart disease in the elderly.
Figure A – Acetylcholine synthesis, release, and degradation from a neuron terminal.

Diagram illustrating ACh synthesis and metabolism. Choline (Ch) is taken up into the synaptic vesicle by the actions of the high-affinity choline transporter (CHT1). Once inside the terminal, choline acetyltransferase (ChAT) adds an acetyl group to choline forming acetylcholine (ACh). ACh is then loaded into synaptic vesicles by the vesicular acetylcholine transporter (VACHT). Depolarization of the neuron causes release of ACh. ACh can be degraded by the enzyme acetylcholinesterase (AChE) back into choline and the process can repeat. Modified image. Original copyright Pearson Education Inc.
1.5 Proper autonomic control of the heart

In order to appreciate the harmful impact of abnormal cholinergic signaling to cardiac tissues, it is imperative to understand proper autonomic control of the heart. The cardiovascular system is continuously under precise homeostatic regulation. Regulation of the heart is coordinated by a variety of different mechanoreceptors, chemoreceptors, and endocrine hormones in the body; however, the autonomic nervous system remains the most prominent in controlling cardiac rate and contractility. The autonomic system is divided into the parasympathetic and sympathetic divisions which both innervate the right atria and sparsely innervate the ventricles. Autonomic innervation also occurs at the AV nodes, conduction pathways, and cardiac muscle (Klabunde 2012).

The parasympathetic system is responsible for decreasing cardiac chronotropic and ionotropic parameters. Parasympathetic ganglia may also be referred to as “cholinergic neurons” because these cells synthesize and release the cholinergic neurotransmitter ACh. Cholinergic neurons innervating the sinoatrial node release ACh that binds to subtype 2 muscarinic receptors (M2). Cholinergic neurons synthesize, store, and release ACh in a multi-step process controlled by a variety of cholinergic enzymes. The initial biosynthesis step is carried out by the enzyme ChAT which catalyzes the addition of an acetyl group to choline forming the neurotransmitter ACh. The choline substrate for ChAT is transported into the cell by the actions of the high-affinity choline transporter (CHT1). Once ACh has been synthesized, it is loaded into secretory vesicles by the vesicular acetylcholine transporter (VACHT). These secretory vesicles store ACh in nerve terminals and release it into the synaptic cleft upon depolarization. Once ACh is released
into the cleft, it can be hydrolyzed back into choline by the actions of acetylcholinesterase (AChE). This free choline can then be taken up by CHT1 where the synthesis and storage of ACh can repeat (Klabunde 2012). At the sinoatrial node, the release and binding of ACh to M2 receptors will cause the heart to revert back to sinusoidal rhythm and ultimately decrease heart rate and contractility. M2 receptor stimulation activates inhibitory G proteins (Gi) which inactivate adenylyl cyclase, inhibiting the conversion of ATP to cyclic adenosine monophosphate (cAMP). This decrease in intracellular cAMP will inactivate cAMP-dependent processes such as cAMP dependent protein kinase A (PKA) activation and phosphorylation of ion channels (Douglas, Baghdoyan, and Lydic 2001). M2 receptor stimulation results in hyperpolarization of pacemaker cells by altering potassium, calcium, and sodium currents. Ultimately, this intracellular signaling cascade causes the heart to decrease rate and contractility.

Opposite in function to the parasympathetic system is the sympathetic system. Sympathetic ganglia are also known as “adrenergic neurons” because they synthesize and release the adrenergic neurotransmitter norepinephrine (NE). Similar to cholinergic neurons, adrenergic neurons predominately innervate the sinoatrial node of the right atrium. These fibers synthesize and release the catecholamine neurotransmitter NE. At the sinoatrial node, NE binds predominately to the beta-1 adrenergic receptors (ADBR1) causing an increase in heart rate, contractility and increased cardiac output (Klabunde 2012). ADBR2 receptors are also involved in cardiac control but to a much lesser extent than ADBR1. Post-ganglionic adrenergic neurons synthesize NE from the amino acid L-tyrosine; however, the predominant mechanism of NE biosynthesis in adrenergic neurons
is due to the conversion of dopamine to NE by the actions of dopamine β-hydroxylase (Nagatsu, Levitt, and Udenfriend 1964). NE is then loaded into secretory vesicles by the actions of the vesicular monoamine transporter where NE will be stored until depolarization occurs. ADBR1 is coupled to stimulatory G proteins (Gs) which activate adenylyl cyclase to catalyze the conversion of ATP to cAMP. This intracellular increase in cAMP concentrations activates protein kinase A. This phosphorylates a number of targets such as L-type calcium channels to increase intracellular calcium concentrations promoting contractility. Gs activation also increases ion channel activity to increase heart rate. (Klabunde 2012)

1.6 Cholinergic dysfunction in heart failure

The pathophysiology of heart failure in the elderly is often characterized by abnormal autonomic imbalance resulting in profound impairment of cardiac function. Autonomic irregularities are often the result of increased sympathetic activity concurrently with decreased cholinergic signaling from the vagal nerve (Florea and Cohn 2014). This characteristic of heart failure is so commonly seen in the elderly population that blunting sympathetic activity with the use of beta-blockers has become the standard treatment for heart failure in the elderly (Ahmed 2009). Restoring proper autonomic balance, either through pharmacological intervention or vagal nerve stimulation has been shown to delay the onset of heart failure and greatly improve outcomes in patients with heart failure (Kember et al. 2014).
Decreased cholinergic signaling as a result of the aging process may play an important role in autonomic imbalance and potentially heart failure in the elderly. Age-related abnormal neuronal and non-neuronal release of ACh greatly impedes cardiac cholinergic signaling and thus results in autonomic imbalance. More so, due to the antagonistic relationship between autonomic branches, chronic impairments in cholinergic signaling may result in overactive sympathetic stimulation, ultimately increasing the risk of developing of heart failure (Triposkiadis et al. 2009). Prolonged sympathetic over activation is detrimental to proper excitation contraction coupling (Piacentino et al. 2003) and has even been implicit in the activation of cardiomyocyte apoptosis (Olivetti et al. 1997).

1.7 Cholinergic system as a target in age-related cardiac dysfunction

Age-related decreases in cholinergic signaling have been implicated as a major risk factor in the development and progression of cardiac disease (Wu et al. 2014). As such, restoration of proper cholinergic signaling has been demonstrated to be beneficial in treating patients suffering from heart failure. Increasing cholinergic signaling may be accomplished in a variety of distinct ways, each proving to be advantageous to cardiac health (Gavioli et al. 2014).

Acetylcholinesterase and butyrylcholinesterase (BChE) are the central enzymes responsible for ACh degradation (Minic et al. 2003). Both types of closely related
cholinesterases exist within cardiac tissue and in plasma, making them potential therapeutic targets for cardiac disease. Clinical parasympathomimetic compounds, such as neostigmine or pyridostigmine, are able to bind to the activation sites of both subtypes of cholinesterase enzymes, resulting in enzyme inhibition. Cholinesterase inhibition of AChE and BChE occurs in a reversible and competitive manner, thus resulting in increased extracellular levels of ACh and hence increased cholinergic signaling (Masuda 2004). Clinical investigation into the use of cholinesterase inhibitors as a treatment for cardiac dysfunction has begun to accumulate promising results. Studies have demonstrated that administration of pyridostigmine protects against myocardial ischemia and improves peak exercise performance in patients suffering from coronary artery disease (Castro et al. 2004). Additionally, studies have shown long term treatment with cholinesterase inhibitors reduces cardiac remodeling, improves vagal control of the heart, and reduces ventricular arrhythmia and dysfunction in animal models of heart failure (Handa et al. 2009). While large bodies of evidence exist in favor of the use of cholinesterase inhibitors in treating and preventing heart failure, the mechanism of action resulting in these beneficial effects has yet to be established. In the elderly, it is likely that restoration of cholinergic signaling results in the reestablishment of balanced autonomic signaling and thus proper cardiac function.

While inhibition of cholinesterases has been shown to be cardioprotective in the elderly, the opposite phenomenon has been shown to be true. Elevated levels of cholinesterase enzymes resulting in increased degradation of ACh may lead to abnormal cardiac function. Several studies have demonstrated an inverse relationship between circulating serum levels of BuChE and cardiovascular mortalities in elderly individuals (Calderon-
Margalit et al. 2006). Furthermore, patients already suffering from CVD experience increased morbidity, mortality, and decreased response to treatments when high levels of BuChE activity is detected (Goliasch et al. 2012).

Restoration of cholinergic tone may also be achieved without the use of pharmacological intervention. The vagus nerve is the tenth cranial nerve that innervates the heart and modulates parasympathetic activity. Vagus nerve stimulation can be achieved with the surgical implantation of a neurostimulator that uses electrodes to sense heart rate and accordingly generate pulses to the right vagus nerve by a multi-contact cuff electrode (Klein and Ferrari 2010). Electrode stimulation results in the depolarization of the efferent vagus nerve and subsequent release of ACh onto the heart. This results in active cholinergic signaling that is immediately apparent by observations of decreased heart rate, similar to normal parasympathetic activation (Buckley, Shivkumar, and Ardell 2015). Clinical investigation of vagal nerve stimulation in 23 heart failure patients demonstrated increased exercise tolerance, improved left ventricular ejection fraction, improved Minnesota Living with Heart Failure Quality of life score, and various other improvements in hemodynamic properties (Klein and Ferrari 2010). Similar to cholinesterase inhibition, the mechanism of vagal stimulation’s beneficial effect has yet to be elucidated. Nonetheless, increases in heart rate variability (an indirect measure of cardiac cholinergic signaling) reveal vagal nerve stimulation increases parasympathetic activity to the heart and may restore proper autonomic balance (Clancy et al. 2014). A variety of potential mechanisms have also been proposed, such as ACh-mediated anti-apoptotic mechanisms, increased NO production, and anti-inflammatory mediators are
potentially involved in the cardioprotective effects achieved by restoring cholinergic signaling through the use of vagal stimulation (Klein and Ferrari 2010).

1.8 Non-neuronal cholinergic system

Traditionally, cardiac cholinergic signaling was predominately associated with autonomic control through the parasympathetic branch of the autonomic nervous system. Classical views of ACh in cardiac signaling were mainly associated with the cholinergic ganglia innervating cardiac pacemaker regions where ACh released from nerve terminals predominately bound to muscarinic receptor subtype 2. However, the classical view of cholinergic signaling has dramatically evolved as a result of the discovery of the non-neuronal cholinergic system (NNCS). Cholinergic enzymes once thought to only exist in cholinergic tissues have been discovered to not only be expressed in non-neuronal cells, but have also been found to be functionally active. Pivotal proteins unique to the cholinergic system, such as VAChT, CHT1, and CHAT have been discovered to be expressed in hepatocytes, leukocytes, and cardiomyocytes (Beckmann and Lips 2013). Furthermore, various studies have demonstrated the importance of NNCS dysfunction in a wide-range of diseases (Wessler et al. 2003). In consideration of the recent discoveries of the role of NNCS in health and disease, it is as expected that the NNCS plays a crucial role in maintaining proper cardiac function with age.

Previous studies have found the existence of the cholinergic machinery responsible for the synthesis, storage and release of ACh from cardiomyocytes (Abramochkin et al.
2012). However, whether or not cardiomyocyte derived ACh was necessary for proper cardiac function was yet to be established. Various research groups attempted to provide insight into this question; however, some of the most convincing data was generated from experiments using mice with knockouts in the cholinergic genes encoding VACht and ChAT. Transgenic mice with knockouts in VACht (cVACht) or cardiac ChAT (cChAT) have severe cardiac abnormalities, indicating the importance of the NNCS in cardiac health. cVACht mice have no changes in physical appearance such as body weight; however, under stress exhibit large increases in heart rate due to decreased cholinergic signaling. Furthermore, cVACht hearts undergo cardiac remodeling and hypertrophy with decreased left ventricular function. The results obtained from cVACht mice demonstrate the importance of a functional NNCS in preventing the onset of cardiac abnormalities (Roy et al. 2013).

The role of the NNCS in age-related cardiac diseases has yet to be elucidated; however, results from cVACht studies suggests abnormalities in NNCS signaling may cause severe cardiac dysfunction. Abnormal cholinergic signaling observed in elderly patients has been mainly attributed to decreased vagal activity; however, dysfunction in NNCS signaling may also play an important role in cholinergic dysfunction seen with age. Studies conducted on rodent models have detected age-dependent decreases in cholinergic enzyme expression in cardiomyoctes (Rana et al. 2010) providing further evidence that age-related cholinergic dysfunction may be a result of both neuronal and non-neuronal abnormal cholinergic functioning.
1.9 Cholinergic anti-inflammatory pathway

Studies have demonstrated that heart failure is not a straight-forward syndrome of hemodynamic dysfunction, but rather orchestrated through complex interactions between various physiological systems. Recent studies have established the role of excessive pro-inflammatory cytokines being implicated in the pathogenesis of heart failure. Cytokines such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), and interleukin 1-beta (IL-1β) undergo increased expression and release from circulating leukocytes in the blood (Gullestad et al. 2012). Pro-inflammatory cytokines remain at the site of injury and confer protection to the host by prolonging the immune response and allowing for proper recovery. Beneficial pro-inflammatory responses normally resolve in 24-72 hours and are confined to the site of injury. Pro-inflammatory responses are necessary for proper recovery when responses are limited in duration and location; however, long term inflammatory signaling or systemic expression of inflammatory cytokines may result in sustained damage to tissues and organs (Dinarello 2000). In cardiac tissue, chronic expression of these cytokines results in severe detrimental changes to cardiac function. TNFα signaling promotes cardiomyocyte hypertrophy and adverse ventricular remodeling resulting in cardiac dysfunction. Furthermore, chronic TNFα signaling results in cardiac myocyte apoptosis further impairing cardiac function (Hilfiker-Kleiner, Landmesser, and Drexler 2006). Elderly heart failure patients often have elevated inflammatory cytokines in circulating leukocytes as well as in the myocardium (Gullestad et al. 2012).
The cholinergic anti-inflammatory pathway is thought to be the body’s braking mechanism to this inflammatory response. The celiac plexus located in the abdomen is comprised of the celiac plexus proper, a region containing the celiac ganglia which the vagus nerve innervates and where the splenic nerve originates. The splenic nerve carries on to the spleen which terminal nerve endings innervate. Activation of the vagus nerve results in ACh release which is able to bind to alpha-7 subtypes of nicotinic acetylcholine receptors (α7nAChR) of the splenic nerve. Activation of splenic α7nAChR results in down regulation of the production of TNFα and other pro-inflammatory cytokines, thus resulting in the suppression of the inflammatory response (Rosas-Ballina et al. 2008). Leukocytes are also responsible for the synthesis and excretion of pro-inflammatory cytokines and like the spleen, also contain α7nAChR that once activated, inhibit the production of these cytokines (Pavlov et al. 2003).

As previously discussed, cholinergic dysfunction is frequently observed in the elderly. Abnormal cholinergic signaling as a result of decreased ACh excretion will result in reduced cholinergic anti-inflammatory activation and thus may potentially exacerbate cardiac dysfunction. Insufficient vagal nerve activation may result in increased splenic production of pro-inflammatory cytokines resulting in overactive or prolonged inflammatory responses. Inadequate anti-inflammatory response is of notable importance in patients already suffering from heart failure. Prolonged chronic pro-inflammatory cytokines have been demonstrated to accelerate the progression of cardiac disease and increase mortality and morbidity (Anker and Haehling 2004). It is therefore likely that decreased cholinergic signaling not only directly impairs cardiac function through
autonomic signaling, but may also play an indirect role through anti-inflammatory effects.

1.10 Rationale and hypothesis

Heart failure is a syndrome predominately of the elderly. Aging causes a myriad of cardiac changes that predispose the elderly to numerous cardiovascular complications. The development of cardiac disease in the geriatric population is often labelled with a grim outlook because of pre-existing declines in cardiac function in addition to the diminished ability of the heart to recover after injury. Treatment has often focused on modulating hemodynamic parameters with the use of drugs in order to temporarily regain heart function to extend life. Traditionally, beta blockers are often administered in order to reduce the workload on the heart and temporarily delay the progression of heart failure. Recent studies have demonstrated heart failure in the elderly is frequently accompanied with increased sympathetic activation and decreased cholinergic activation, yet despite these findings, very little treatment options exist to restore cholinergic activation. Rather, treatment options have remained relatively unchanged and clinical intervention remains predominately geared toward reduction of sympathetic activation. Furthermore, despite the large accumulation of evidence towards cholinergic dysfunction being implicated in age-related heart failure, very little work is being conducted investigating cholinergic restoration as a preventive measure to age-related heart failure.
The overall aim of this thesis was to determine whether restoration of cardiac cholinergic signaling would improve, delay, or prevent age-related cardiac dysfunction. Furthermore, we have investigated whether increased ACh levels during acute cardiac injury acts as a cardioprotective mechanism in both young and old mice.

Previously, we have demonstrated that abnormal cardiac cholinergic signaling results in detrimental cardiac remodeling and function. As such, we have hypothesized that increased levels of cardiac cholinergic signaling will improve the outcomes of age-related cardiac decline.
Chapter 2

2 Methods

2.1 Animals

*ChAT-Channelrhodopsin2-EFYP*

*ChAT-ChrR2-EFYP* (ChR2⁺) [B6.Cg-Tg(Chat-COP4*H134R/EYFP)6Gfng/J; The Jackson Laboratory] mice were bred and maintained as hemizygous as previously described (S. Zhao et al. 2011). *ChAT-ChrR2-EFYP* negative (ChR2⁻) littermates were used as controls. Briefly, ChR2⁺ mice possess a bacterial artificial chromosome (BAC) that contains a copy of the cholinergic locus under the control of the ChAT promoter. Expression of the BAC can only occur in cholinergic cell types. Only cholinergic cells express the transcription factors necessary for ChAT promoter activation. To eliminate the overexpression of ChAT due to the presence of the BAC, the exons coding for the BAC ChAT gene were disrupted by the insertion of the gene encoding the channelrhodopsin 2 protein followed by a stop codon. Although the researchers were able to disrupt the exons coding for the ChAT gene in the BAC, they did not account for the VAChT gene which is contained within the cholinergic locus and upstream of the disruption site. As a result, BAC expression caused the transcription and synthesis of the VAChT gene in cholinergic cells. Therefore, the additive effect of both endogenous and BAC expression of VAChT resulted in an overexpression in cholinergic cells.
VAChT^{flox/flox,Myh6-cre+}

VAChT^{flox/flox,Myh6-cre+} (cVAChT) mice were generated similar to as previously described (Guzman et al. 2011). Briefly, transgenic mice expressing cre recombinase under the control of the cardiac alpha myosin-heavy chain promoter [B6.FVB-Tg(Myg6-cre)2182Mds/J; The Jackson Laboratory] were bred to VAChT-floxed mice. Offspring were then bred to generate cVAChT mice. VAChT^{flox/flox,Myh6-cre-} (cVAChT⁻) littermates were used as controls. Because cre recombinase was under the control of the cardiac alpha myosin-heavy chain promoter, cVAChT mice have a deletion of the VAChT gene only in cardiac tissue.

Animals were cared for in compliance with animal protocols at Western University (#2008-127). Animal maintenance and experiments were performed to the standards set forth by the Canadian Council of Animal Care guidelines. All attempts to reduce animal suffering were performed. Only male mice were used for experimentation.

2.2 Real-time polymerase chain reaction (qPCR)

Real-time polymerase chain reaction was performed as described previously (Roy et al. 2012; Roy et al. 2013). Briefly, atrial and ventricular tissue were isolated and total RNA was extracted using the Fatty and Fibrous Tissue RNA Extraction Kit (Bio-Rad Laboratories, Mississauga, Canada) following the manufacturer’s protocol. Total RNA from either atrial or ventricular tissue was eluted in 80 µl of Elution Solution provided in
the extraction kit. Prior to analysis, samples were tested for RNA quality and quantity via microfluidic analysis using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). All samples were to exceed an RNA integrity number of 8.0 to be used for the reverse transcription reaction. Five-hundred ng of total RNA was used to synthesize 20 μl of cDNA as per the manufacturer’s protocol outlined in the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Streetsville, Ontario). cDNA was then loaded onto a CFX-96 Real Time System (Bio-Rad Laboratories, Mississauga, Ontario) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Mississauga, Ontario). The following settings were programmed into the system: 95 ºC for 10 seconds, annealing and extension at 60 ºC for 30 seconds. A non-template reaction was used as a negative control. To quantify the relative amount of gene expression, DDCT method using β-actin was used to normalize expression.

2.3 High performance liquid chromatography & electrochemical detection of ACh

Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.25 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3, 11mM glucose) was prepared 2 hours prior to tissue collection and continuously adjusted to maintain a constant temperature of 37ºC and pH 7.4. 1 hour prior to tissue collection, the solution was aerated with 95% O2 and 5% CO2. Whole hearts were collected from wild type and ChR2+ mice and cleaned thoroughly of any connective tissue. Atrial tissue was cut away from the ventricles and both tissues
were placed into separate tubes containing 1ml of Krebs-Henseleit solution. 100 µM of pyridostigmine was added to each tube and mixed thoroughly. Tissues were left to incubate for 3 hours at 95% O2, 5% CO2, and 37°C. Once 3 hours had elapsed, tissues were discarded and the remaining solution was centrifuged at 13,200 RPM for 10 minutes at 4°C. HPLC analysis was conducted on the extracted supernatant as described previously (Roy et al. 2013). Briefly, the supernatant was collected using a 0.2 µm filtered syringe and loaded into the autosampler of an UltiMATE 3000 HPLC system with a Coulomem electrochemical detector (Dionex, Oakville, Ontario) using the following programming: Flow rate: 0.300 ml/min; injection volume: 20 µl; Cell potential: 275 mV; column: MGII CAPCELL PAK C18 column (Shiseido, Grand Island, NY; Cat. No.= 92461); Column temperature: 40 ºC; ACh post-column solid phase reactor (Thermo Scientific, Waltham, MA; Cat. No.=70-0640A).

2.4 Electrocardiography

Electrocardiogram measurements were obtained using a telemeter radio frequency transmitter with leads positioned in a lead II configuration as previously described (Lara et al. 2010; Roy et al. 2013). Briefly, telemeters were mounted in a dorsal position subcutaneously. Recordings were initiated when home cages were placed on Data Sciences International telemetry system (Transoma Medical, St. Paul, MN) recording platforms controlled by Dataquest A.R.T. software (Transoma Medical, St. Paul, MN). 7 days post-implantation, heart rates were recorded continuously over a 24 hour period to
obtain baseline recordings. Additionally, heart rates were recorded following acute treadmill exercise as described in 2.5.

2.5 Treadmill exercise tolerance

Mice were made to run on a 800 Series Rodent Treadmill (IITC Life Sciences, Woodland Hills, CA) as described previously (Kolisnyk et al. 2013). Prior to experimentation, mice were trained for 4 days for a period of 5 minutes per day to familiarize the mice with treadmill running. Initially, mice were trained at a speed of 8 meters/minute; however, on each subsequent day the speed was increased up to a final speed of 12 meters/min on the final day of training. Post-training, mice were run at an initial speed of 12 meters/min and the treadmill speed was increased to 20 meters/min over a 15 minute acceleration period. For endurance testing, mice were run until 60 minutes of time had elapsed or until the mice had reached exhaustion and were no longer able to keep up with the treadmill speed. Exhaustion was determined when the mice were no longer able to keep up with the treadmill speed or accumulated 3 low-voltage shocks as a result of stepping off the treadmill.
2.6 Metabolic assessments

Food and water intake, oxygen consumption, carbon dioxide production, respiratory exchange ratio and physical activity were simultaneously measured as described previously (Guzman et al. 2013; Kolisnyk et al. 2013). Briefly, mice were housed individually in metabolic chambers (2730 cm³) and connected to the Comprehensive Lab Animal Monitoring System controlled by Oxymax software (Columbus Instruments, Columbus, OH). Mice had unrestricted access to powdered standard rodent chow and water. Metabolic chambers were maintained at 24 ± 1°C and 0.5 L/min airflow volume. Measurements were taken every 10 minutes for 24 hours after a 16 hour habituation period elapsed. Measurements dependent on movement were obtained using the Infrared Opto-M3 Activity Monitor controlled by Oxymax software (Columbus Instruments, Columbus, OH). All measurements were calculated using software algorithms within Oxymax software as previously described (Guzman et al. 2013).

2.7 Echocardiography

Echocardiograms were performed using the Vevo 2100 ultrasound imaging system (Visual Sonics, Toronto, Ontario) as described previously (Lara et al. 2010). Briefly, left ventricular dimensions (LVID), ejection fraction (EF), fractional shortening (FS), and wall thickness were determined. All measurements were obtained using M-mode in short axis view. Ejection fraction was calculated as: 

\[ EF (%) = \left( \frac{((LVID_d)^3 - (LVID_s)^3)}{((LVID_d)^3)} \right) \]
Fractional shortening was calculated as: \( FS(\%) = \frac{(LVIDd - LVIDs)}{LVIDd} \times 100 \). All measurements were averaged from 3 cycles of images.

### 2.8 Langendorff perfused heart

A custom built apparatus was constructed out of 4.0 mm silicon tubing, a glass heating jacket, and a Minipuls 2 peristaltic pump (Gilson, Middleton, WI) using similar specifications as described previously (Skrzypiec-Spring et al. 2007; Bell, Mocanu, and Yellon 2011; Ghelardoni et al. 2014). Briefly, the pump’s inlet tubing was submerged into 1L of filtered pH 7.4 Krebs-Heneseleit solution. The solution was drawn up by the pump at a rate of 2 ml/min and was passed into an OXYR-50 miniature gas exchange oxygenator (Living Systems Instrumentation, St Albans City, VT). The aerator was attached to a pressurized tank of 95% O₂ and 5% CO₂ at 15 PSI of pressure, allowing for gasses to dissolve into the solution passing through the aerator. The oxygenated solution was then pumped through the glass heating coil jacket connected to a thermocirculator (Harvard Apparatus, Holliston, MA). Prior to starting the experiment, the temperature of the effluent was measured and any adjustments were made to maintain the effluent at temperature of 37ºC. Krebs solution leaving the heating coil was then pumped through an in-line pressure transducer and recorded using a Perfusion Pressure Monitor 4 system (Living System Instruments, St Albans City, VT). The solution then moved through the attached needle cannulating the aorta of a heart (discussed in 2.9). Effluent exiting the heart from the coronary sinus ostium was collected into a container. A FTO3 Force-
Displacement Transducer (Grass Technologies, Warwick, RI) was placed 2cm beneath the apex of the heart and a 5.0 silk black braided suture (Ethicon, Markham, Ontario) was passed through the transducer and into the apex of the heart. One gram of tension was applied between the heart and force transducer. Contraction of the heart caused a displacement in the force transducer head resulting in a recording. In-line pressure and force displacement recordings were connected to a DI-720 Data Acquisition System (DATAQ Instruments, Akron, OH) and read outs were displayed and analyzed in WinDaq (DATAQ Instruments, Akron, OH) and LabChart (ADInstruments, Colorado Springs, CO) software, respectively.

Animals were injected with 600 IU/kg intra-peritoneal heparin 15 minutes prior to sacrifice. Hearts were collected from animals under anesthetization via inhaled isoflurane (2%) and euthanized via cervical dislocation. Hearts were removed from the chest with 2-3 cm of aorta still intact. The hearts were immediately placed in ice-cold Krebs–Henseleit solution at pH 7.4 which had been previously bubbled with 95% O$_2$ and 5% CO$_2$. The heart was immediately cleaned of connective tissue to expose the aorta. A 12 gauge bulb-feeding needle was then placed into the aorta and pushed through the aortic arch but stopped just before the aortic valve. Extra care was taken to ensure the feeding needle did not breach or damage the aortic valve. Silk suture was then repeatedly tied around the aorta to secure the heart to the needle. The feeding needle was then attached to the Langendorff apparatus which had been previously running oxygenated Krebs solution at a rate of 2ml/min and temperature of 37ºC. Removal, cannulation, and connection to the Langendorff apparatus was completed in under 5 minutes.
2.9 Ischemic reperfusion injury

Ischemic reperfusion injury was conducted as described previously (Venardos et al. 2009; Rossello et al. 2015; Venardos et al. 2015). Briefly, once hearts were secured to the Langendorff apparatus, a 20 minute “stabilization” period was elapsed to establish baseline heart rate and contractility. After stabilization, the heart was subjected to global ischemia when the peristaltic pump was shut off and flow ceased for 12 minutes, effectively stopping delivery of oxygen and perfusate to the heart. A 12 minute ischemic period was selected as a result of trials wherein 12 minutes resulted in significant but not excessive tissue necrosis. Finally, normal flow was returned to the heart for 60 minutes during the “reperfusion” phase.

2.10 Quantification of myocardial necrosis

Quantification of myocardial necrosis was performed as described previously (Hochhauser et al. 2013, 2; Y and Jc 2014). Briefly, hearts were transversely sliced into 5 horizontal sections of roughly equal thickness. Sections were then placed in 1% triphenyl tetrazolium chloride solution which had been previously prepared and warmed to 37°C. Sections were left in solution for a total of 15 minutes and were continuously disturbed every 1 minute to ensure even staining. Sections were then placed in 10% formalin solution for 10 minutes and washed with saline solution. Once staining was complete, sections were placed on a glass slide and images were obtained using a Nikon Digital
Sight connected to a Nikon SMZ800 microscope. Images were then imported into ImageJ software (National Institutes of Health, Bethesda, MD) and pale necrotic tissue was quantified as area of necrotic tissue as a ratio of the total area of tissue. Black and white images were generated using colour threshold filters in order to quantify necrotic area. Analysis was performed in a 2-person blinded protocol where measurements were performed in randomized order.

2.11 In-vivo cardiac arrest

In-vivo cardiac arrest was performed on mice under anesthesia (2% isoflurane) as previously described (Sharp et al. 2015). A small incision was made under the jaw to expose the right external jugular vein. A 0.4-mm diameter heparinized cannula was inserted into the jugular vein and 0.08mg/g KCl was slowly injected. Cardiac arrest was confirmed by observing the cessation of cardiac contractions normally seen through the rib cage. After 4 min, the mouse was resuscitated by injection of 1.5 µg warmed epinephrine solution followed by 200 BPM chest compressions. Resuscitation protocol was performed until heart contractions recovered or until 5 minutes of unsuccessful CPR.
2.12 Statistical analyses

Results for all experiments are provided as mean ± SEM. To calculate statistical differences between experimental groups, a Student’s t-test, one-way ANOVA with Tukey’s post-hoc test, two-way ANOVA, or chi-squared test were used. \( p < 0.05 \) was considered to be statistically significant. GraphPad Prism was used for all statistical analysis.
Chapter 3

3 Results

3.1 ChR2\(^+\) mice demonstrate increased cardiac cholinergic signaling independent of age.

We have utilized the ChR2\(^+\) mouse line for a large portion of our studies because of a unique characteristic: overexpression of the VAChT enzyme (Kolisnyk et al. 2013).

Previous work performed by Kolisnyk et al. 2013 confirmed 3 month old ChR2\(^+\) mice demonstrate nearly a 50-fold increase in VAChT expression in neuronal tissues; however, VAChT overexpression in peripheral tissues had not yet been quantified. To confirm whether VAChT was also overexpressed in cardiac tissues of ChR2\(^+\) mice, qPCR analysis was performed on atrial and ventricular tissue. ChR2\(^+\) mice demonstrated nearly a 3-fold increase of VAChT expression in both atrial and ventricular tissue of 3 and 18 month old mice (Fig 1a, Fig 1b).

Considering that the VAChT is directly implicated in ACh release (Prado et al. 2013), it was expected that overexpression of functional VAChT should result in larger amounts of ACh excretion from cholinergic tissues. This would ultimately result in ChR2\(^+\) mice possessing a cardiac hypercholinergic phenotype. To confirm whether increased expression of VAChT results in increased ACh release in cardiac tissues, ACh concentration was quantified using HPLC analysis of ventricular (Fig 2a) and atrial (Fig 2b) tissues of 3 month and 18 month old mice. At 3 months of age, atrial tissue of ChR2\(^+\)
hearts excrete nearly 3-times as much ACh than wild type mice. Interestingly, increased release of atrial ACh in ChR2⁺ mice continues to persist to 18 months of age. In contrast, wild-type mice experience age-dependent decreases in ACh release, as predicted by the cholinergic hypothesis of aging. No significant differences were found between ACh levels in ventricular tissue of wild type and ChR2⁺ mice; however, this result was not unexpected as very sparse neuronal innervation is present in ventricular tissue (Kent et al. 1974). It may be possible ChR2⁺ mice have increased storage of ACh in secretory vesicles as a result of the VACHT overexpression. Unlike atrial ACh storage vesicles, ventricular ACh stores may require stimulation in order to eliciting exocytosis. This in part may have contributed to the low release of ventricular ACh seen in our studies.

By and large, these results confirm that ChR2⁺ mice overexpress the VACHT gene, resulting in increased ACh excretion and thus possessing increased cholinergic signaling in cardiac tissue. In addition, increased excretion of ACh from cardiac tissue persists in ChR2⁺ mice despite declines observed in wild type mice which begin to experience age-related decreases in ACh release. The ChR2⁺ mice provide us with a unique approach to investigate the importance of proper cholinergic signaling as age progresses, without the use of parasympathomimetic drugs or vagal nerve stimulation.
Figure 1 – ChR2+ mice overexpress VAChT mRNA in atrial and ventricular tissue.

qPCR quantification of vesicular acetylcholine transferase (VAChT) mRNA expression (normalized to actin) in (a) ventricular and (b) atrial tissue of wild type and ChR2+ mice aged 3 and 18 months of age. For all groups $n = 6$. *$p < 0.05$, **$p < 0.01$. Data are expressed as a mean ± S.E.M.
Figure 2 – ChR2+ mice demonstrate increased release of ACh in atrial tissue.

HPLC analysis of ACh release in (a) ventricular and (b) atrial tissue of wild type and ChR2+ mice aged 3 and 18 months of age. For all groups n = 6. Means with different letters are statistically significant p < 0.05. Data are expressed as a mean ± S.E.M.
3.2 Metabolic parameters and physical characteristics

In order to elucidate metabolic changes that may have occurred as a result of chronic ACh overexpression, metabolic analysis was conducted on ChR2+ mice. Using the Comprehensive Lab Animal Monitoring System, we were able to analyze the metabolic activity of 18 month old mice through the measurements of parameters such as volume of O₂ (Fig 3c) and CO₂ consumed and exhaled (Fig 3b), respiratory exchange rate (Fig 3a), food (Fig 3d) and water (Fig 3e) consumed, sleep (Fig 3f), and movement patterns (Fig 3g, 3h) during light and dark time periods. Movement patterns were reported as ambulatory (Fig 3g) and total ambulatory movements (Fig 3h). Ambulatory movement is a quantification of movement which has resulted in the mouse physically changing positions in the home cage. Total ambulatory movements is a quantification of all movements such as tail and ear flicks as well as movements resulting in relocation inside the home cage. All factors measured were found to be statistically similar between 18 month old ChR2+ and wild-type mice. This data suggests moderately increased cholinergic signaling appears to be inconsequential to the metabolic parameters measured.
Figure 3 – Metabolic analysis in ChR2+ and wild type mice.

Metabolic assessment conducted using Comprehensive Lab Animal Monitoring System in wild type and ChR2+ mice. (a) Respiratory exchange rate. (b) Volume of CO₂ expelled. (c) Volume of O₂ consumed. (d) Food consumed. (e) Water consumed. (f) Time spent sleeping. (g) (h) Movement. For all groups \( n = 4 \). Data are expressed as a mean ± S.E.M.
In order to determine the effect of increased cholinergic signaling on development, bodyweight (Fig 4a), tibia length (Fig 4b), heart weight (Fig 4c) and heart weight normalized to tibia length (Fig 4d) were collected from 18 month old wild type and ChR2+ mice. Although the tibia length of both wild type and ChR2+ mice were found to be of similar length, ChR2+ mice exhibit lower bodyweight compared to age-matched wild type mice. This result suggests ChR2+ mice do not exhibit any growth retardation as a result of hypercholinergic signaling. However, decreased bodyweight with no change in tibia length suggests ChR2+ mice maintain a leaner body composition as they age.
Figure 4 – Bodyweight, heart weight, and tibia length of aged wild type and ChR2+ mice

(a) Bodyweight (in grams) (b) Tibia length (in millimeters) (c) Heart weight (in milligrams) (d) Heart weight normalized to tibia length in 18 month old wild type and ChR2+ mice. For all groups \( n = 10 \). *\( P < 0.05 \). Data are expressed as a mean ± S.E.M.
3.3 Increased cardiovascular endurance in ChR2⁺ mice

We have previously demonstrated that under resting conditions, hemodynamic properties of both young and old ChR2⁺ mice did not significantly differ from that of wild-type mice (unpublished data). However, because cardiac autonomic activity is predominately activated during times of demand and recovery, data obtained when mice are at rest is not sufficient to elucidate changes in cardiac function. In order to determine the consequence of hypercholinergic signaling on cardiac function, it was necessary to cause increased cardiovascular demand. Mice were exercised on animal treadmills and made to run until exhaustion. Once top speed was reached, animals were run until they were not able to sufficiently keep up with the treadmill.

18 month old ChR2⁺ mice were capable of running for significantly longer periods of time and nearly 100m further than age-matched wild-type mice (Fig 5a). Our study had similar results to Kolisnyk et al. 2013 who demonstrated that 3 month ChR2⁺ mice were also able to run for longer distances than wild-type mice of the same age. Regardless of age, ChR2⁺ mice appear to have increased treadmill endurance compared to wild-type mice. Large bodies of evidence for the positive correlation between exercise tolerance and cardiovascular health has been well established in literature (Sharman, La Gerche, and Coombes 2015). As such, because aged ChR2⁺ mice demonstrate increased exercise tolerance, they may have retained cardiovascular function despite the aging process. Additionally, because 3 month old ChR2⁺ also demonstrate increased exercise performance, elevated cholinergic signaling may also serve to improve cardiac function during exhaustive exercise regardless of age.
Although exercise tolerance is a reliable measure of cardiovascular health (Myers 2003), variations in heart rate during activity and recovery will provide further insight as to why aged ChR2+ mice appear to have retained cardiovascular health. To measure heart rate, 18 month old mice were subcutaneously implanted with ECG telemeter units and allowed to recover for 1 week prior to experiments. After recovery, mice were exercised on the animal treadmill and immediately placed back into their home cages where telemeter ECG signal recording commenced. Post-exercise, heart rate returned to baseline levels after a period of time that was similar for both ChR2+ and wild type mice. However, ChR2+ mice demonstrated overall smaller elevations in heart rate during the recovery period (Fig 5b), suggesting increased cholinergic signaling is able to reduce large elevations in heart rate post-exercise. Recent literature has demonstrated that abnormal heart rate recovery, including prolonged elevated heart rate post-exercise may indicate autonomic dysfunction and may potentially be a predictor for declining cardiovascular health (Caetano and Delgado Alves 2015; Aneni et al. 2014). The findings of this experiment help strengthen previous data suggesting that ChR2+ mice have better retained cardiovascular function than wild-type mice of the same age as a result of increased cardiac cholinergic tone.
Figure 5 – Treadmill exercise tolerance and heart rate recovery in ChR2+ mice.

(a) Total distance (meters) run on a treadmill until exhaustion for 18 month old wild type and ChR2+ mice. (b) Heart rate recovery post-exercise obtained using ECG telemeter units in 18 month old wild type and ChR2+ mice. Post-exercise ECG recordings begin at 5 minute mark. For all groups n = 4. *P < 0.05. Data are expressed as a mean ± S.E.M.
Exercise tolerance is a product of both cardiovascular and skeletal muscle fitness (Sumide et al. 2009). It was therefore necessary to determine whether increased exercise tolerance was a result of enhanced cardiovascular fitness, muscular fitness, or both. Because ChR2 mice systemically overexpress VAChT and subsequently have increased release of ACh in most cholinergic tissues of the body, observed increases in treadmill exercise tolerance could potentially be the result of increased ACh release at the neuromuscular junction. In order to assess muscular strength and endurance, aged mice were subjected to wire hang tests (Fig 6a) as well as grip force strength tests for both hind and forelimbs (Fig 6b). In each of these experiments, no significant differences were found between ChR2 and wild-type mice. Kolisnyk et al. has also demonstrated that 3 month ChR2 and wild-type mice also do not differ in muscular strength. These findings suggest that both aged and young ChR2+ mice demonstrate higher thresholds for exercise endurance as a result of improved cardiovascular health rather than improved muscular endurance.
Figure 6 – Grip force in wild type and ChR2+ mice

(a) Total time (seconds) that 18 month old wild type and ChR2+ mice were able to hang from an inverted and elevated wire mesh. (b) Comparison of grip force (newtons) for both forelimb and hindlimb strength of 18 month old wild type and ChR2+ mice. For all groups n = 5. Data are expressed as a mean ± S.E.M.
3.4 ChR2+ mice retain cardiovascular function with age

In order to assess heart functionality of aged mice, echocardiograms were performed on 3 and 18 month old ChR2 and wild-type mice. Ejection fraction (Fig 7a), fractional shortening (Fig 7b), and wall thickness (Fig 7c, 7d) were measured.

Ejection fraction is a measure of the fraction of blood expelled out of the left ventricle with each ventricular contraction and is a common measurement to aid in the assessment of heart health and function (Vasan et al. 1999). Ejection fraction values of 55-70% are considered adequate for proper cardiac function (Vinhas et al. 2013), 40-55% are below normal or at risk for the development of cardiac dysfunction, and less than 40% is an indication of heart failure (Yang et al. 1999). Eighteen month old ChR2+ mice have retained significant heart function (ejection fraction average of 54%) (Fig 7a) compared to wild type mice of the same age (ejection fraction average of 40.1 %). Three month old ChR2+ and wild-type mice have no significant differences in ejection fraction and both maintain healthy ejection fraction percentages as expected in young mice.

Fractional shortening is the ratio between the diameters of the relaxing left ventricle when in diastole, and when cardiac contractions occur during systole. A fractional shortening value of 26% is deemed to be healthy (Yang et al. 1999). No significant differences were found for both 3 and 18 month old ChR2+ and wild-type mice (Fig 7b); however, it is apparent that there is a distinct trend toward significance (p = 0.08) with ChR2+ mice having retained ejection fraction.
Wall thickness is used to assess heart hypertrophy and remodeling. All measurements were made during both diastole and systole. Anterior wall thickness and posterior wall thickness for 3 month (Fig 7c) and 18 month old mice (Fig 7d) were found to be of similar dimensions. However, it appears that as a result of aging, wild-type mice experience larger increases in wall thickness compared to ChR2+ mice that seem to retain relatively consistent wall dimensions throughout life.

While quantitative echocardiography is immensely useful for determining data such as ejection fraction and fractional shortening, it becomes difficult to visualize irregularities in the pattern of contractions without qualitative images. Observing video or still images of heart contractions obtained from echocardiograms is sufficient in showing these patterns. Figure 8 is a screen capture of the recordings of ChR2+ (top) and wild-type (bottom) hearts of animals aged 18 months. Cardiac contractions in wild type mice are frequently asymmetrical and have irregular movements of the posterior wall, suggesting early signs of cardiac dysfunction. ChR2+ hearts demonstrate symmetrical contraction in both the posterior and anterior wall with little irregularities and retained ejection fraction.
Figure 7 - ChR2+ resist age-related decline in cardiovascular function

(a) Ejection fraction and (b) fractional shortening percentages of 18 month old wild type and ChR2+ mice obtained using echocardiograms. Anterior wall (AW) and posterior wall (PS) thickness measured during systole (S) and diastole (D) of (c) 3 month and (d) 18 month old wild type and ChR2+ mice. For all groups \( n = 6 \). *\( P < 0.05 \). Data are expressed as a mean ± S.E.M.
Figure 8 – Echocardiogram image of aged wild type and ChR2+ mice

Echocardiogram images obtained using Vevo 2100 ultrasound imaging system. 18 month old wild type mouse (lower image) demonstrates significant cardiac dysfunction and asymmetrical contraction.
Our previous data suggests that wild type mice experience severe decreases in heart function as they age. Detrimental declines in heart function should be accompanied by increased expression of cardiac stress markers. Recent literature has used atrial natriuretic peptide (ANF) and β-myosin heavy chain 7 (MHY7) mRNA expression as indicators of heart damage and/or heart failure (Goetze et al. 2015). In order to determine expression levels of these markers, qPCR analysis was performed on 18 month old wild type and ChR2+ whole heart tissue. Wild-type mice have significantly increased expression of both ANF and MHY7 as determined by qPCR analysis (Fig 9). Increased expression of both these markers, in combination with our heart function data, suggests that aged wild-type mice experience cardiac stress caused by the aging process. In contrast, ChR2+ mice have significantly lower amounts of cardiac stress and retain large portion of their heart function with age.
Figure 9 – Cardiac stress marker expression in aged ChR2+ and wild type mice.

qPCR quantification of cardiac damage markers (a) atrial natriuretic factor (ANF) and (b) β-myosin heavy chain 7 (MHY7) in whole heart tissues of 18 month old wild type and ChR2+ mice. For all groups $n = 4$. *P < 0.05. Data are expressed as a mean ± S.E.M.
Proper cardiac function is necessary for the longevity of an individual. A large portion of deaths in the elderly population are a result of cardiovascular complications (Roger et al. 2012). Our previous data indicates that ChR2+ mice demonstrate decreased cardiac stress and overall retained heart function with age. Accordingly, we would therefore expect increased survival of ChR2+ into old age. A Kaplan-Meier survival curve was constructed for wild type and ChR2+ mice in order to quantify survival into old age (Fig 10). By 25 months of age, the survival rate of ChR2+ far exceeds that of wild type mice. Although definitive conclusions about the cause of death cannot be confidently discussed, it remains apparent that mice exhibiting systemic hypercholinergic signaling appear to survive much longer into old age than their wild-type counterparts.
Figure 10 – Survival of aged and wild type and ChR2+ mice.

Kaplan-Meier death curve for wild type and ChR2+ mice. For all groups n = 10. *P < 0.05.
3.5 Aged ChR2+ hearts retain majority of function post injury

Our previous data demonstrated that ChR2+ mice were able to survive longer with retained heart function and decreased cardiac stress. However, a significant portion of the elderly population have had one or more cardiovascular injuries or complications that greatly impact longevity (Gottdiener et al. 2000). It is therefore beneficial to analyze heart function post cardiac injury to assess whether hypercholinergic signaling in old age is able to provide cardioprotection against post-injury dysfunction.

In order to employ cardiac injury while simultaneously recording data on heart function, a Langendorff perfusion assay was constructed and utilized. Heart isolation severs neurons which innervate cardiac tissue and thus isolates the heart from central nervous system control. The Langendorff apparatus thus allows us to further elucidate the importance of the NNCS in age-related cardiac dysfunction.

Isolated hearts from wild type and ChR2+ mice were attached to the Langendorff perfusion assay as outlined in 2.8. Hearts were subjected to no-flow ischemia until perfusion was restarted. Hearts were then reperfused for 60 minutes post-ischemia. Heart rate and contractility were recorded during all phases.

Initially heart rate was comparable for both ChR2+ and wild-type mice of the same age group (Fig 11a, 12a). Three month old mice exhibit larger stable heart rate than 18 month mice; however, no significant differences exist within groups. During ischemia (marked by a red arrow), heart rate for all groups dropped to near 0 BPM. Concurrently, as heart
rate drops, contractility begins to increase before beginning to fall back to starting levels just prior to reperfusion (Fig 11b, 12b). This increase in contractility is presumably an intrinsic mechanism to maintain cardiac output when heart rate falls as a result of ischemia. Post ischemia (marked by a green arrow), heart rates for both ChR2+ and wild-type mice began to fall indicating declining function as a result of injury; however, ChR2+ hearts experience significantly less decline in heart rate compared to wild-type mice. By the end of the reperfusion period, it became apparent that ChR2+ mice retain the majority of their function compared with wild-type mice. In contrast, wild-type hearts significantly struggled 60 min post-ischemia and demonstrated major decreases in heart rate, indicating significant damage. Similar trends were also observed with contractility loss. Aged wild type hearts had nearly a 30% decline in contractility in contrast to ChR2+ hearts which had only a 9% decrease. This once again supports the notion that wild type hearts undergo significantly more damaged by ischemia/reperfusion injury than ChR2+ hearts. Another interesting observation is that even in the younger age group, ChR2+ hearts maintain the majority of their heart function post injury, indicating hypercholinergic signaling may also be implicit in cardioprotection at a young age as well. These findings suggest increases in cholinergic signaling are not only important in the age-dependent progression of heart failure, but also may be important in acute cardiac injury at any age.

Elderly individuals are at a much higher risk of cardiac arrest and are more likely to require cardiopulmonary resuscitation than any other age group. The time to recover spontaneous cardiac contractions is known as time to beating recovery (TBR) and has been positively correlated with decreased survival post resuscitation and overall cardiac
health prior to cardiac arrest (Hasselqvist-Ax et al. 2015). Eighteen month old ChR2\(^+\) hearts were able to recover from ischemia-induced cardiac arrest in a shorter amount of time than wild type hearts. ChR2\(^+\) hearts therefore demonstrated overall significantly less TBR (Fig 13b). In addition, once ChR2\(^+\) hearts recovered, they demonstrated significantly less episodes of arrhythmic events (Fig 14a) and when a rare episode did occur, it was very short lived (Fig 14b). These results further indicate that post injury, ChR2\(^+\) hearts are better able to recover and maintain heart function.
Figure 11 – Heart rate and contractility post ischemic-reperfusion injury in 18 month old aged mice.

(a) Heart rate and (b) contractility of 18 month old wild type and ChR2+ *ex-vivo* hearts during ischemia reperfusion injury conducted on a Langendorff perfusion assay. Red arrows indicate beginning of ischemia. Green arrows initiate beginning of reperfusion. For all groups *n* = 5. *P < 0.05. Data are expressed as a mean ± S.E.M.
Figure 12 - Heart rate and contractility post ischemic-reperfusion injury in 3 month old mice.

(a) Heart rate and (b) contractility of 3 month old wild type and ChR2+ *ex-vivo* hearts during ischemia reperfusion injury conducted on a Langendorff perfusion assay. For all groups $n = 5$. *$P < 0.05$. Data are expressed as a mean ± S.E.M.*
Figure 13 – ChR2+ hearts recover from ischemia induced cardiac arrest faster than wild type hearts.

(a) Time to beating cessation during ischemia of ex-vivo hearts conducted on a Langendorff perfusion assay. (b) Time from beating cessation to beating recovery of ex-vivo hearts during an episode of reperfusion conducted on a Langendorff perfusion assay.

For all groups $n = 5$. *$P < 0.05$. Data are expressed as a mean ± S.E.M.
Figure 14 – ChR2+ hearts demonstrate less frequent and shorter periods of arrhythmic events post injury.

(a) Number of episodes and (b) duration of arrhythmic events occurring during reperfusion post ischemic injury in 18 month old wild type and ChR2+ *ex-vivo* Langendorff hearts. (c) Original tracing of an arrhythmic episode during reperfusion of a wild type heart. For all groups *n* = 5. *P* < 0.05. Data are expressed as a mean ± S.E.M.
Our previous data suggests that ChR2\textsuperscript{+} hearts retained the majority of heart function post injury compared to age-matched wild type mice. To further illustrate increased viability of ChR2\textsuperscript{+} hearts post-injury, tetrazolium chloride staining was performed on heart slices to visualize areas of necrotic tissue post injury. Tetrazolium salts penetrate cardiac tissue and convert to formazin pigments that visibly stain tissue bright red. Formazin conversion will only occur in the presence of active cardiac lactate dehydrogenase which is only functional in healthy viable heart muscle (Ramkissoon 1966). Thus, areas of infarct or necrosis will stain pale white. Infarct size is expressed as a percentage of necrotic (pale) regions to entire slice surface area. ChR2\textsuperscript{+} hearts displayed significantly less total infarct size area compared to wild-type mice (Fig 15a). As expected, 18 month old wild type hearts displayed significantly larger necrotic tissue area than 3 month old wild type indicating age plays an important role in post injury heart viability. Interestingly, 18 month old ChR2\textsuperscript{+} hearts did not experience larger areas of necrotic tissue compared to 3 month ChR2\textsuperscript{+} hearts. These findings, in addition to heart functionality data suggest that ChR2 hearts are able to retain function and sustain less damage overall during episodes of ischemia.
Figure 15 – ChR2+ hearts contain less necrotic tissue post injury.

(a) Comparison of infarct size of 18 month old wild-type and ChR2+ heart slices after an episode of ex-vivo ischemia/reperfusion injury. (b) Staining was conducted using tetrazolium chloride solution. For all groups $n = 5$. Different letters represent statistically significant differences. Data are expressed as a mean ± S.E.M.
Ex-vivo experiments provide evidence that ChR2⁺ hearts experience significantly less tissue damage following acute ischemic perfusion injury while also retaining cardiac function. In order to supplement evidence regarding the cardioprotective role of the cholinergic system, we have utilized the in-vivo cardiac arrest model. Experimentation was initially performed on mice aged 16-18 months of age; however, low wild type resuscitation success rate impeded proper statistical analysis. To increase the chance of survival, younger 9 month old wild type and ChR2⁺ mice were used.

Wild type mice had decreased cardiac function post cardiac arrest as demonstrated by significant reductions in both ejection fraction and fractional shortening (Fig 16a, 16b). Additionally, time to resuscitation was measured from the initiation of epinephrine injection until spontaneous cardiac contractions were seen. Significantly longer periods of chest compressions were necessary for wild type mice to recover (Fig 16c). Despite making use of younger mice to increase survival, wild type mice continued to demonstrate much lower rates of successful resuscitation compared to age matched ChR2⁺ mice (Fig 16d).
Figure 16 – ChR2+ mice retain heart function after cardiac arrest

(a) Ejection fraction (%) and (b) fraction shortening (%) of 9 month old mice 7 days pre- and post-cardiac arrest injury. (c) Time to spontaneous cardiac contractions from initiation of resuscitation protocol. (d) Rate of successful resuscitations for wild type and ChR2+ mice. n = 15 for wild type and n = 12 for ChR2+. (e) Echocardiogram screen captures. For all groups in graphs (a) (b) (c) n = 4. *P < 0.05. Data are expressed as a mean ± S.E.M.
3.6 Inhibiting release of ACh during injury reduces the cardioprotective effects seen in ChR2+ mice

With the aim of determining the acute role of VACHT in *ex-vivo* cardioprotection, vesamicol was added into the Langendorff perfusate for a total working concentration of 5µM. Vesamicol is a potent inhibitor of VACHT activity and therefore impedes the release of ACh, resulting in impaired cholinergic signaling. Ischemia reperfusion protocol was followed as described above with the addition of vesamicol in the perfusate solution. ChR2+ hearts with vesamicol demonstrated a significantly larger amount of infarcted tissue compared to ChR2+ hearts without vesamicol (Fig 17), suggesting that the cardioprotective effects possessed by ChR2+ hearts are in part dependent on the acute activity of VACHT. Wild-type hearts treated with vesamicol do not show significant differences in infarct size compared to wild-type hearts without vesamicol.
Figure 17 – Vesamicol induced inhibition of ACh release results in increased damage post injury

Infarct size of 18 month old wild type and ChR2 heart slices after an episode of Langendorff ischemia/reperfusion injury (IR) with vesamicol in the perfusate. Staining was conducted using tetrazolium chloride solution. For all groups \( n = 4 \). *\( P < 0.05 \). Data are expressed as a mean ± S.E.M.
Inhibiting the activity of VACht with the use of vesamicol during acute cardiac injury significantly reduces the cardioprotective effects seen in ChR2+ hearts. In order to further elucidate the role of VACht in cardioprotection, we have utilized the cardiac VACht (VAChtMyh6-Cre-flox/flox; cVACht) knockout mouse model. cVACht mice provide us with the opportunity to perceive alterations in cardiac function and viability as a result of long term removal of cardiomyocyte VACht, rather than acute blocking with the use of vesamicol. Three month cVACht hearts were placed on the Langendorff perfusion system and ischemic reperfusion injury was conducted as previously described. cVACht hearts demonstrate profoundly greater damage than wild type and ChR2+ hearts, as determined by necrotic tissue post injury (Fig 18a). Very few cVACht mice are able to survive up to 18 months of age, therefore we were unsuccessful in repeating our aging experiments on 18 month cVACht mice. These findings suggest that cardiomyocyte VACht is necessary in proper heart function and longevity not only in acute injury, but in developmental aging as well.
Figure 18 – Cardiac VAChT knockout mice exhibit increased necrotic tissue post injury.

(a) Infarct size of 3 month old wild type, ChR2+, and cVAChT heart slices after an episode of ischemia/reperfusion injury. 3 month old wild type and ChR2+ data were presented previously in Figure 15. (b) Visualization of necrotic cardiac tissue post-injury. Staining was conducted using tetrazolium chloride solution. Different letters represent statistically significant differences. For wild type and ChR2+ groups $n = 5$. For cVAChT group $n = 4$. Data are expressed as a mean ± S.E.M.
3.7 Inhibition of ACh degradation during acute injury is cardioprotective in wild type mice.

ChR2+ mice demonstrate significant improvement in heart function and longevity with age. A key characteristic believed to be in part involved with age-related cardioprotection is increased cardiac cholinergic signaling mediated by increased release of ACh. Fortunately, many parasympathomimetic medications approved for clinical use exist today. One such drug is known is pyridostigmine, a reversible cholinesterase inhibitor. By introducing pyridostigmine into the Langendorff perfusate at various time points, we can determine if reducing the breakdown of ACh, and hence increased cholinergic signaling, may be important in acute injury reduction without the use of transgenic animals.

Ischemia-reperfusion injury conducted on a Langendorff perfusion assay was performed as previously described for 3 and 18 month wild-type mice hearts (Fig 19a, 19b). Pyridostigmine (100 µm final concentration) was added into the perfusate prior to injury (PYR) or after injury during the reperfusion phase (PYR Rep). Wild-type hearts treated with pyridostigmine either pre or post-injury demonstrated a reduction in infarct size compared with wild-type hearts that did not receive pyridostigmine treatment. Although 3 month old hearts treated with pyridostigmine exhibited significant reduction in necrotic tissue post injury, exaggerated differences in necrotic tissue post-injury become much more apparent in 18 month old wild-type hearts (Fig 19c).
Figure 19 – Pyridostigmine induced cardioprotection in wild type mice

Comparison of infarct size of *ex-vivo* wild-type hearts conducted on a Langendorff perfusion assay for (a) 3 month and (b) 18 month old mice. “PYR” hearts were perfused with pyridostigmine prior to injury. “PYR Rep” hearts were perfused with pyridostigmine post injury. For all groups *n* = 4. Different letters represent statistically significant differences. Data are expressed as a mean ± S.E.M.
Chapter 4

4 Discussion

Autonomic imbalance in parasympathetic and sympathetic signaling has been implicated in the pathogenesis of heart failure for many years. Despite this knowledge, many studies investigating this phenomenon have focused on modulating only sympathetic activity in attempts to delay or prevent the progression of heart failure. Very few studies have focused on the role of dysfunctional parasympathetic signaling as a catalyst to CVD. Furthermore, the recent discovery of the NNCS in cardiac tissue has exemplified the importance of proper non-neuronal cholinergic tone in cardiac health and disease. Recent literature has made it apparent that proper cholinergic signaling is necessary for healthy cardiac function. In the elderly, abnormal deterioration of cholinergic tone appears to frequently occur as a result of the aging process (Takei, Nihonmatsu, and Kawamura 1989). Age-related cholinergic dysfunction may prove to be an important factor in the decline of cardiac function seen in the elderly. In this section, we present data that demonstrates the importance of proper cholinergic signaling in age-related cardiac health, as well as its role in cardioprotection during acute-injury.
4.1 ChR2+ mice can be utilized as a model of cardiac hypercholinergic signaling

At present, very few studies exist which have utilized the ChR2+ mouse line for cardiac investigation. Rather, characterization of the ChR2+ mouse line has been aimed towards identifying neuronal alterations in cholinergic signaling. Extensive neuronal studies conducted in ChR2+ mice have uncovered the unique characteristic of VACHT overexpression in hippocampal and brainstem tissue of these mice (Kolisnyk et al. 2013). ChR2+ transgenic mice were designed to carry multiple exogenous copies of the cholinergic locus expressed via bacterial artificial chromosomes (BACs). The cholinergic locus within these BACs were modified in order to inactive the ChAT gene; however, the VACHT gene remained intact. The end result is ChR2+ mice overexpress VACHT in cholinergic neuronal tissues (Crittenden et al. 2014). Overexpression of VACHT has been demonstrated to enhance loading of excretory vesicles and increased evoked release of ACh (Song et al. 1997). In summary, studies have established that ChR2+ mice possess hypercholinergic signaling in the hippocampus and brainstem as a result of VACHT overexpression.

Expression of the cholinergic BAC is dependent on the presence of transcriptional factors found unique within cholinergic cell-types. As a result, only cholinergic cells are able to express the BAC, and hence possess overexpression of the VACHT. We have therefore hypothesized that because the ganglia innervating cardiac tissue is of cholinergic origin, these neurons should also be expressing the BAC. In addition, as a result of VACHT overexpression, cholinergic ganglia innervating cardiac tissues may be releasing larger
amounts of ACh if BAC expression is occurring in these cells. Furthermore, the discovery of cholinergic enzymes within cardiomyocytes has demonstrated the presence of a NNCS within the cardiac tissue. While it has yet to be established, it is possible that BAC expression in ChR2+ mice may also occur in cardiomyocytes, and hence result in VACHT overexpression and hypercholinergic non-neuronal cholinergic signaling. The end consequence of VACHT overexpression in ChR2+ mice is a hypercholinergic phenotype.

In order to determine whether VACHT overexpression was occurring in cardiac tissue of ChR2+ mice, analysis of atrial and ventricular tissue was conducted. We have been one of the first labs to demonstrate that ChR2+ mice overexpress the mRNA coding for VACHT in atrial and ventricular cardiac whole-tissue. Previous studies have established the relationship between increased VACHT expression and larger amounts of ACh excreted in neuronal cells (Nagy and Aubert 2012). It was therefore expected that ChR2+ mice exhibit increased ACh excretion as a result of this overexpression. We have confirmed that increased VACHT expression in atrial tissue results in increased excretion of ACh in ChR2+ hearts. Despite overexpression of the VACHT in ChR2+ ventricular tissue, no significant increases in ACh excretion were detected. It may hold that as a result of the sparse neuronal innervation present in ventricular tissue, much smaller quantities of ACh were being released from neuronal ganglia (Kent et al. 1974), thus potentially explaining the smaller amount of ACh detected despite an almost 3-fold increase in VACHT overexpression in ChR2+ ventricles.

Studies performed on geriatric populations have observed an age-dependent decline in cardiac cholinergic signaling (M. Reardon and Malik 1996, De Meersman and Stein
Interestingly, we have demonstrated sustained increased atrial ACh release in ChR2+ mice aged to 18 months. This data suggests ChR2+ VACHT overexpression and subsequent ACh release remains prominent even into old age. These findings allow us to utilize the ChR2+ mouse line in aged studies as a model of age-independent hypercholinergic signaling.

Like many other transgenic models of gene overexpression, ChR2+ mice exhibit unintended consequences as a result of VACHT overexpression. Although not immediately apparent, ChR2+ and wild type mice differ in cognitive function (Kolisnyk et al. 2013). Kolisnyk et al. demonstrated ChR2+ mice have impaired spatial memory, working memory, motor learning, and have deficiencies in cue-directed memory. These findings illustrate that abnormally overactive cholinergic signaling in neuronal tissue may negatively impact cognitive function (Thiel, Bentley, and Dolan 2002). However, it appears that a delicate balance exists in regards to the therapeutic effectiveness of modifying cholinergic signaling. Insufficient signaling is severely detrimental to cognitive function, yet overactive signaling may also produce unwanted side-effects. Therefore, small increases in cholinergic signaling is likely all that is necessary in order to provide the largest therapeutic benefit with minimal side effects. In terms of the potential clinical use of parasympathomimetics in treating cholinergic dysfunction, unwanted cognitive side effects may potentially limit the therapeutic use of these compounds to treat heart failure. However, many categories of cholinergic boosting drugs are peripheral-acting as a result of the inability to cross the blood brain barrier (Ogura et al. 2001). Use of peripheral-acting drugs may mitigate the reluctance to use
hypercholinergic drugs and solve the concern over potential cognitive defects when using these drugs.

Extra care must be taken when modulating cholinergic tone not only in cognitive diseases, but when being used for cardiovascular complications as well. Unnecessary elevations in cardiac cholinergic signaling has been shown to cause unwanted chronotropic effects such as bradycardia (Hernandez et al. 2009). However, ChR2+ mice do not exhibit detrimental bradycardia and therefore illustrate that a peripheral 3-fold increase of ACh appears to not result in negative cardiac consequences.

We have demonstrated ChR2+ mice do not exhibit any gross alterations in metabolism as a consequence of VAChT overexpression. Interestingly, aged ChR2+ mice maintain significantly leaner bodyweight despite similar caloric intake and activity as wild type mice. Various studies have established correlations between body weight and cardiovascular dysfunction, stating abnormal cardiac function may result in significant weight gain as a result of fluid retention and improper energy metabolism (Chaudhry et al. 2007). Additionally, cholinergic function has been implicated in the maintenance of leaner bodyweight. Patients receiving cholinesterase inhibitors for the treatment of a variety of different disorders often experience significant weight loss (Gauthier 2001). Rodent models of obesity also benefit from ACh stimulation of nAChR subunits found on various tissues (Somm 2014). It is therefore plausible that as a result of systemic increases in ACh excretion, ChR2+ are able to maintain leaner bodyweights as they age. Although wildtype and ChR2+ mice had no significant differences in activity, results were trending toward significance. This may be an indication that ChR2+ had increased
physical activity in their home cage and may in part play a role in the leaner bodyweight phenotype.

In summary, a 3-fold overexpression of the VACHT mRNA has been confirmed in atrial and ventricular tissue of ChR2+ mice. This overexpression results in an increased release of ACh in atrial tissue. Aged ChR2+ mice also maintain elevated cholinergic signaling in contrast to wild type mice which experience a decline in ACh. VACHT overexpression also does not appear to be metabolically detrimental to ChR2+ mice. Therefore, these data validate the use of the ChR2+ mouse as a model of age-independent cardiac hypercholinergic signaling.

4.2 Increased cholinergic signaling in old age is beneficial for cardiovascular function

Exercise tolerance has frequently been used as a reliable and non-invasive measure of cardiovascular health. A hallmark of abnormal cardiac function is the inability to perform aerobic exercise for extended periods of time (Piña et al. 2003). Heart failure patients frequently demonstrate the incapability of increasing cardiac output in times of demand mainly as a result of insufficient increases in stroke volume (Piña et al. 2003). Furthermore, exercise intolerance may be exacerbated in elderly individuals as a result of age-related cardiac dysfunction (van Mourik et al. 2012). We have demonstrated aged ChR2+ mice outperform wild type mice in exercise performance and endurance, suggesting retained cardiovascular function in aged ChR2+ mice. In addition, we have
revealed ChR2+ mice maintain lower heart rate immediately after exercise and during recovery. Sustained elevated heart rate post-exercise has been established as a powerful indicator of mortality in several studies (Nishime et al. 2000) (Javorka et al. 2002). Although consensus regarding why this phenomenon occurs has yet to be established, delayed heart rate recovery post-exercise may be a manifestation of abnormal cardiac cholinergic signaling which has been frequently implicated in cardiac disease and aging (Amorim et al. 1981). Sustained elevated heart rate after periods of exercise were demonstrated in aged wild type mice, suggesting possible cholinergic dysfunction in these mice.

Echocardiogram assessment of heart function demonstrated aged ChR2+ mice retained a large portion of cardiac function while wild type mice experienced detrimental declines. Previous studies have demonstrated a correlation between decreased vagal activity and declines in ejection fraction and fractional shortening, lending further support for the importance of cholinergic signaling in maintaining cardiac function with age (Hogue et al. 1995). Hypercholinergic signaling in ChR2+ mice appears to provide a cardioprotective mechanism against age-related declines in cardiac function. Various studies have demonstrated declines in cholinergic signaling with age, resulting in sympathetic over activation and eventual cardiac dysfunction (De Meersman and Stein 2007). Overexpression of VACHT provides ChR2+ mice with elevated cholinergic signaling throughout life and thus potentially preventing age-related autonomic imbalance. Studies have demonstrated that patients being administered parasympathomimetic drugs in order to restore proper cholinergic tone experience
significant improvements in cardiac function (Sant’anna et al. 2003). This provides further evidence that proper autonomic balance is crucial in elderly individuals.

Possibly the most convincing data for the importance of retained cardiac cholinergic signaling in old age is simply the observation that a large portion of ChR2+ mice survive when aged above 24 months. In contrast, survival of wild type mice begins to drop sharply after 17 months of age. Interestingly, cardiac cholinergic tone as a predictor of mortality has been found to be clinically relevant. Decreased cardiac cholinergic tone has been associated with increased mortality in elderly hospitalized patients (Tsuji et al. 1994), suggesting cholinergic tone may be very important in regards to cardiac health of the elderly.

Autonomic dysfunction as a result of decreased cholinergic signaling has shown to predict poor improvements in patients being treated for cardiac injury and heart failure (Compostella et al. 2014). We have demonstrated aged wild type mice express significantly elevated levels of ANF and MHY7, indicating critical cardiac stress. In contrast, aged ChR2+ mice demonstrated lower expression levels of ANF and MHY7. ANF seems to be upregulated in patients exhibiting cardiac stress and often serves as a marker for the progression of heart failure (Brandt et al. 1993). Interestingly, ANF seems to act as a sympathoinhibitory modulator of autonomic function, resulting in the decrease of sympathetic activation to the heart (Butler, Senn, and Floras 1994). These findings suggest the presence of increased ANF in patients with heart failure serves to suppress overactive sympathetic tone in order to prevent the progression of cardiac damage. Elevations in the expression levels of ANF have also been noted in the elderly and serve as indicators of potential cardiovascular comorbidity (Davis et al. 1996). Age-related
decreases in cholinergic tone, resulting in exaggerated sympathetic stimulation and cardiac dysfunction, may be implicated in the increased excretion of ANF from atrial tissue (Schiebinger, Baker, and Linden 1987) and may in part explain why hypercholinergic ChR2+ mice do not demonstrate age-dependent cardiac dysfunction.

4.3 Age-dependent decreases in cholinergic tone is detrimental to heart recovery post-injury

ChR2+ hearts were subjected to cardiac arrest and ischemic reperfusion injury on a Langendorff apparatus. ChR2+ hearts demonstrated only minor declines in heart rate and contractility and appear to maintain the majority of functionality post-injury. In addition, tissue assessment after injury revealed significantly smaller regions of infarct in both young and aged ChR2+ hearts. Various potential mechanisms have been proposed to explain why increases in ACh signaling appear to play a role in cardioprotection. The majority of proposed mechanisms are simply speculations and theories until further investigations are performed and confirmed by several independent groups. It does however remain clear that inhibiting either the synthesis or release of ACh from the NNCS of cardiomyocytes is severely detrimental to heart function, as demonstrated with ChAT knockout studies. ChAT knock-out cardiomyocytes have been shown to have lower levels of ATP, increased oxygen consumption with decreased utilization, and when ChAT knockout cells were deprived of oxygen, a significantly higher cell death was seen than in control cells (Kakinuma et al. 2012). Because ChR2+ cardiomyocytes have
hypercholinergic signaling either from neuronal or non-neuronal sources, ACh mediated signaling to cardiomyocytes may prevent or reverse the above from occuring. In contrast, age-dependent declines in cholinergic tone observed in wild type mice indicates potentially abnormal cholinergic tone and subsequent cardiac dysfunction as a result. These findings, in addition with our data, demonstrate the importance of cholinergic signaling in maintaining the ability to resist cardiac damage.

It is apparent that ACh signaling acts as a cardioprotective mechanism in cardiac tissue, yet very few studies have attempted to elucidate the specific signaling pathways involved. The mitochondria appear to play an integral role in the cardioprotective effects of cholinergic signaling (Oikawa, Iketani, and Kakinuma 2014). Growth and division of pre-existing mitochondria in cardiomyocytes occurs in order to respond to environmental stressors such as exercise, caloric restriction, and hypoxia injuries (Jornayvaz and Shulman 2010). In addition, inhibiting mitochondrial biogenesis leads to abnormal cardiomyocyte energy metabolism, energetic insufficiency and diastolic dysfunction (Rimbaud, Garnier, and Ventura-Clapier 2009). It has recently been proposed that ACh ameliorates cardiomyocyte injury by promoting mitochondrial biosynthesis and function. ACh binding to cardiomyocyte muscarinic receptors appears to confer cardioprotection by increasing mitochondria density, ATP synthesis, and up-regulation of genes involved in mitochondrial biogenesis. More specifically, ACh appears to increase the activity of AMP-activated protein kinase (AMPK) resulting in the overexpression of a key transcriptional activator of biogenesis known as peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha (Sun et al. 2013). The end result is upregulation of mitochondrial biogenesis which appears to confer cardioprotection.
Increased mitochondrial density increases respiratory rates in cardiac fibres, maintaining proper cardiac energetics and reducing oxidative stress (Rosca and Hoppel 2010). Disruption of any of the key steps involved in the mitochondrial biogenesis pathway results in severe molecular defects frequently observed in heart failure (Bayeva, Gheorghiade, and Ardehali 2013).

4.4 Restoration of cholinergic tone is cardioprotective in old age

Cardiac function after an episode of cardiac injury is a major contributing factor of prognosis (Ertl and Frantz 2005). Various circumstances, such as age, severity of inflammatory responses, and several other factors are determinants in how well the heart will continue to function after injury (Ertl and Frantz 2005). However, it still remains difficult to predict the potential severity of cardiac damage in patients who are at risk but are yet to experience an episode of acute cardiac distress. Indicators of cardiac viability often present themselves after injury, at which point the majority of damage has already occurred. For example, patients who develop left ventricular dysfunction or large areas of infarction after injury have poor clinical outcomes (Minicucci et al. 2011). It is therefore beneficial and highly desirable to prevent the progression and development of severe cardiovascular injury through the use of preventive medication in individuals who are at risk for developing these complications.
The beneficial effects of increased cholinergic signaling has been demonstrated in a variety of different animal models of heart failure. Nevertheless, transition from animal models to human therapeutics is often a long and complex process. However, many parasympathomimetic compounds have been used clinically for many years. The prevalent use of these compounds allow for retrospective analysis regarding the cardiovascular benefits of cholinergic-boosting drugs. A retrospective cohort study investigated the association between donepezil, a cholinesterase inhibitor often prescribed to Alzheimer’s patients, and cardiovascular mortality found that patients on donepezil experienced a significant reduction in cardiovascular mortality (Sato et al. 2010). Patients being treated with donepezil also had a significant reduction in plasma brain natriuretic peptide (BNP) levels. Plasma BNP concentration is often used as a marker of heart failure (Kubo et al. 2012). These studies indicate cholinesterase inhibitors seem to confer cardioprotection, especially in elderly patients.

Studies have also demonstrated restoration of cholinergic signaling in patients already suffering from heart failure benefit from treatment with cholinesterase inhibitors. Heart failure patients showed increased heart rate recovery and improved peak exercise performance after a single oral administration of 30mg pyridostigmine (Androne et al. 2003). In our study, we have demonstrated an acute dose of pyridostigmine produced a lower volume of necrosis in wild type hearts after cardiac arrest injury. In addition, we have demonstrated pyridostigmine confers cardioprotection whether given prior to or immediately after cardiac injury. Our findings, in addition to clinical studies, has demonstrated acute dosages of pyridostigmine resulting in increased cholinergic tone,
may provide cardioprotection to patients who have recently experienced myocardial infarction or cardiac arrest.

Although mitochondrial biogenesis provides a potential mechanism for ACh-mediated cardioprotection, the pathway relies on upregulation and expression of certain key gene components which may take days or even weeks to become expressed. Therefore, it is unlikely that mitochondria biogenesis is involved in immediate or short-term ACh-mediated cardioprotection. Instead, it is possible that increased activation of muscarinic subtype 3 (M3) receptors confers the short-term and acute cardioprotection demonstrated in our experiments. Activation of M3 receptors have been shown to immediately induce COX-2 activity resulting in the conversion of arachidonic acid to various prostanoids (Zhao et al. 2010, 3). Expression of prostaglandins in cardiac tissue has been shown to confer acute cardioprotection. Reductions in arrhythmic events, decreased infarct size, and overall improved hemodynamic properties have all been demonstrated (Needleman 1976). The likely mechanism for prostanoid induced cardioprotection may be conferred through inhibition of calcium influx, antagonism of adenylyl cyclase, opening of potassium channels, and attenuation of neutrophil infiltration (Bolli et al. 2002). In our studies, ChR2+ mice experienced fewer episodes of arrhythmias and decreased infarct size, which are similar outcomes to the cardioprotective effects seen with prostaglandin expression. A potential component of the ACh-mediated cardioprotection seen in ChR2+ mice may be increased expression of prostaglandin in cardiac tissue as a result of increased acute M3 signaling.

Interestingly, ACh appears to be associated not only in the release of pro-inflammatory prostaglandins through M3 signaling, but also in anti-inflammatory cytokine release.
Although these molecules confer opposite processes, both are necessary for proper cardiac function and repair (Ricciotti and FitzGerald 2011). Increased hypercholinergic signaling observed in ChR2+ mice may result in increased activation of \( \alpha_7 \)nAChR on circulating leukocytes and in the spleen. Activation of \( \alpha_7 \)nAChR results in reductions in the release of pro-inflammatory cytokines into the blood (Bencherif et al. 2011). Chronic pro-inflammatory responses in heart failure patients may play a role in the development and progression of the disease (Anker and Haehling 2004). Myocardial dysfunction in addition to pathogenic processes in other organs as a result of chronic inflammation severely impacts patient morbidity (Yndestad et al. 2006). Inhibiting or reducing the chronic pro-inflammatory response in patients suffering from cardiovascular disease may prove to be a key therapeutic component in the treatment of heart failure. Studies have demonstrated decreased inflammatory responses by simply administering cholinesterase inhibitors to increase circulating levels of ACh (Masuda 2004). Antagonism of \( \alpha_7 \)nAChR also significantly attenuates the cardioprotective benefits indicating the importance of the cholinergic anti-inflammatory pathway (Li et al. 2014). A component of the cardioprotective phenotype observed in ChR2+ mice may be a result of ACh activated \( \alpha_7 \)nAChR and subsequent reduction in inflammatory responses, therefore conferring cardioprotection to these mice.
4.5 Conclusion

With the use of the ChR2+ mouse line, we have demonstrated that increases in cholinergic signaling appear to confer cardioprotection in times of acute injury and age-related cardiac decline. While the exact mechanism of cholinergic cardioprotection has yet to be elucidated, it likely arises as a result of various independent pathways reliant on ACh as a major signaling molecule. Proper cholinergic signaling from the vagus nerve is likely to be crucial in maintaining autonomic balance to the heart and preventing overactive sympathetic activation frequently observed in elderly heart failure patients. In addition, proper vagal nerve activity is likely necessary to inhibit the overproduction of inflammatory cytokines which have been demonstrated to be highly detrimental to cardiac recovery. Finally, ACh produced by the NNCS present in cardiomyocytes has been demonstrated to be a key component in maintain proper cardiac function. Age-related declines in cholinergic tone and hence ACh signaling may impair any or all of these systems, resulting in a situation of cardiac dysfunction and eventual heart failure so frequently seen in geriatric populations. Our data has demonstrated the importance of maintaining proper cholinergic signaling with age. Abnormal cholinergic signaling brought on by the aging process may play a role in the high prevalence of heart failure seen in the elderly. In addition, we have demonstrated that with the use of cholinesterase inhibitors, increased cholinergic signaling may act as a cardioprotective mechanism. Our data suggests cholinesterase inhibitors could potentially be used as prevention or treatment for cardiac dysfunction as a result of aging. Restoration of proper cholinergic signaling may be a potential future therapeutic target in the treatment of heart failure and other CVD.
4.6 Future Directions

Although our data presents convincing evidence for the cardioprotective role of cholinergic system, the mechanism involved remain relatively unknown. It is likely ACh acts as an upstream signaling molecule that modulates a number of different physiological processes important to cardiovascular health. Future research should focus on elucidating the importance of the cholinergic anti-inflammatory pathway and restoration of autonomic imbalances in order to uncover the importance of each system.

4.6.1 Cholinergic anti-inflammatory pathway

The link between the cholinergic anti-inflammatory pathway and CVD occurs at the α7nACh receptor found in the spleen and circulating leukocytes. Stimulation of the α7nAChR by ACh results in suppression of inflammatory processes and cytokine release throughout the body (Forsythe 2015). It is possible that age-dependent declines in ACh, and hence reduced cholinergic stimulation to the spleen, can result in over-production of inflammatory cytokines. Chronic inflammatory responses are detrimental to patients suffering from CVD and thus plays an important role in cardiovascular health (Anker and Haehling 2004). Future studies should isolate the effect of the cholinergic anti-inflammatory pathway on CVD. ELISA assays could be run on plasma and tissue samples of aged ChR2⁺ mice in order to determine if increased ACh results in suppression of inflammatory cytokines such as IL-6, IL-10, and TNFα. Additionally,
antagonism of α7nAChR can be achieved with various compounds such as tetracaine and BTMPS (Peng et al. 2013). *In-vivo* cardiac arrest experiments can be repeated with α7nAChR antagonism in order to demonstrate whether the cholinergic anti-inflammatory pathway is critical in cardioprotection.

### 4.6.2 Restoration of autonomic balance

It has been established that autonomic imbalance often develops as a consequence of age (Katayama et al. 2015). The cardioprotective effects observed in aged ChR2+ mice may be in part a result of elevated ACh when levels normally begin to decline. Heart rate variability obtained using ECG telemeters is a reliable measure of cardiac parasympathetic activation. Performing heart rate variability analysis in conjunction with heart function echocardiograms may help deduce whether age-related decline in cardiac function is a result of parasympathetic imbalance. Furthermore, the same protocol could be repeated with pyridostigmine administration. If heart function improves as heart rate variability increases in wild type mice, it is likely that restoration of autonomic balance is a major mechanism behind cholinergic cardioprotection in the elderly.
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