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Sara Bober, *The University of Western Ontario*

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

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ATRIAL FIBRILLATION PROMOTION BY INTERMITTENT HYPOXIA IN THE RAT

Thesis format: Monograph

by

Sara Bober

Graduate Program in Physiology and Pharmacology

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

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

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ABSTRACT

Obstructive sleep apnea (OSA) is a significant risk factor for developing atrial fibrillation (AF) in clinical populations, but the underlying mechanisms are unknown. Intermittent hypoxia (IH), as elicited by nocturnal airway obstructive events in OSA patients, has been implicated as the mediator of OSA-related cardiovascular outcomes. However, the role of IH in OSA-related atrial arrhythmogenesis has not been reported. For the first time, this thesis demonstrates AF promotion in a rodent model of OSA using IH to mimic hypoxic events, and investigates the underlying vulnerable substrates of induced AF. Rats exposed to IH for 7 days had significantly enhanced AF vulnerability compared to control animals exposed to normoxic conditions using both programmed electrical stimulation and atrial burst pacing to evaluate AF susceptibility. Enhanced AF vulnerability was accompanied by a number of atrial substrate changes that have not been reported previously in an IH model of OSA, including (1) lowered atrial Cx 43 content, (2) heightened cholinergic sensitivity with increased muscarinic receptor protein expression, and (3) alterations in adrenergic function characterized by enhanced responses to propranolol and blunted responses to isoproterenol. These findings highlight a potential causal role for chronic IH in OSA-related AF susceptibility and in the formation of AF-promoting vulnerable substrates.

KEYWORDS

Intermittent hypoxia

Obstructive sleep apnea

Atrial Fibrillation

Autonomic receptors

Connexins

CO-AUTHORSHIP

Dr. J. Ciriello and Dr. J. Moreau provided the first 2 sets of rat hearts (29 in total) used for atrial mRNA isolation and real-time PCR analysis. These rats were previously frozen following exposure to (1) 1 day of IH (n = 6) or normoxic conditions (n = 7) (group identified as "1 day_i" in the results used for real-time PCR analysis) or (2) 95 days of IH (n = 8) or normoxic conditions (n = 8).

Otherwise, Sara Bober performed all IH exposures and other experiments conducted as part of this thesis under the supervision of Dr. Douglas Jones at the University of Western Ontario.

I dedicate this thesis to Michael Bober, and to Laura and Forselius Pahapill

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Completing this degree over the last two years has been a truly incredible journey. First and foremost, I owe this to my supervisor Dr. Doug Jones. Since day one you've shown me seemingly limitless patience and unconditional freedom to make decisions and mistakes, ultimately enabling me to become an independent scientist. From you I've learned that I can solve any problem on my own, one of your many attributes that amazes most people.

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LIST OF ABBREVIATIONS

AERP	atrial effective refractory period
AF	atrial fibrillation
AHI	apnea-hypopnea index
ANS	autonomic nervous system
APD	action potential duration
AVN	atrioventricular node
β1-AR	β1-adrenergic receptor
β2-AR	β2-adrenergic receptor
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase type II
Cx 40	connexin 40
Cx 43	connexin 43
DAD	delayed afterdepolarization
EAD	early afterdepolarization
ECG	electrocardiogram
FiO_2	fraction of inspired oxygen
GP	ganglionated plexi
HRA	high-right atrium
I_{CaL}	L-type calcium current
ICANS	intrinsic cardiac autonomic nervous system

IH	intermittent hypoxia
IK_{ACh}	acetylcholine-activated inward rectifying K+ current
IK_{M3}	M3 receptor mediated K+ current
LL1	limb lead 1
LRA	low-right atrium
M2R	muscarinic type 2 receptor
M3R	muscarinic type 3 receptor
MRA	mid-right atrium
NCX	sodium-calcium exchanger
ODDD	oculodentodigital dysplasia
OSA	obstructive sleep apnea
PES	programmed electrical stimulation
PKA	protein kinase A
PLN	phospholamban
PV-LA	pulmonary vein-left atrial
RYR2	ryanodine type 2 receptors
S1	basic drive train stimulus
S2	single extrastimulus
S1-S2	coupling interval of the S1 and S2
TBST	Tris-buffered saline with 0.01% Tween-20

1.0 LITERATURE REVIEW

1.1 Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia and a global health burden. The presently available therapeutic options carry considerable risks and are not effective for most patients, highlighting the need for new approaches to treatment. Rarely a primary electric disorder, AF most often arises as a manifestation of a host of predisposing diseases. The development of improved treatment modalities is limited by an incomplete appreciation of the mechanisms of AF, particularly with respect to its underlying risk factors, although the importance of the autonomic nervous system has been demonstrated in a variety of scenarios, both clinically and with the use of animal models. In addition to a number of conditions classically associated with AF, the common sleep breathing disorder, Obstructive Sleep Apnea (OSA), is being increasingly recognized as an important AF risk factor, but the underlying mechanisms are not known. Intermittent hypoxia (IH) is a critical pathophysiological component of OSA and is used to model the disease, primarily in rodents. The use of these models has implicated IH in mediating many OSA-related cardiovascular outcomes, but IH has not been used in investigations of atrial arrhythmias. IH also causes profound autonomic dysfunction found in the human disease which, given the role of the autonomic nervous system in predisposing the atria to AF, may be arrhythmogenic in the context of OSA.

This thesis focuses on the role of IH in predisposing the atria to AF (*in vivo*) through the creation of AF substrates. This first chapter will provide an overview of AF, highlighting the role of autonomic influences and connexins in AF pathophysiology. This is followed by a description of OSA, emphasizing the role of

IH and its effects on the autonomic nervous system and the use of IH models to elucidate mechanisms of OSA-related cardiovascular outcomes.

1.2 Atrial fibrillation

Atrial fibrillation (AF) is the most common cardiac arrhythmia encountered by the general practitioner (Benjamin et al., 1998). It is characterized by rapid, chaotic atrial activation, manifesting as an undulating isoelectric line in place of regular P waves on the electrocardiogram (ECG). This is accompanied by an “irregularly irregular” QRS pattern (an irregular QRS rhythm with no apparent pattern of irregularity, Krummen et al., 2006), as the extremely rapid atrial rates during AF exceed the impulse carrying capacity of the atrioventricular node (AVN), resulting in uncontrolled ventricular responses related to AVN hysteresis. Initially AF episodes are often self-terminating and limited in duration (less than one week), which is termed paroxysmal AF, but over time, the episodes can become longer, and AF forms will often progress from paroxysmal to persistent (episodes lasting longer than 1 week and are routinely successfully terminated by cardioversion), or permanent (AF is continually present and a strategy of rhythm control is not advisable) (January et al., 2014). AF is most often the result of one or more predisposing pathologies, and the progression to more advanced forms is often associated with advancement of these underlying diseases. The progressive nature of AF is also partially caused by AF itself, as rapid rates during AF cause alterations of the atria that favour chronicity of the arrhythmia (“AF begets AF”; Wijffels et al., 1996; Morillo et al., 1995; Thijssen et al., 2000).

1.2.1 Clinical relevance: The global AF burden

AF is a major health care burden, due to complications and deaths. The current evidence suggests that AF affects about 1% of the general population (Kannel et al., 1998). However, the prevalence of AF increases with age, affecting over 10% of individuals over the age of 75 (Nixon, 2011). The prevalence of AF more than doubled from 1993 to 2007 (Piccini et al., 2012), and is expected to continue to rise due to the aging of the population (January et al., 2014). In adults between the ages of 40 and 55, the lifetime risk for developing AF has been estimated to be 22-26% (Heeringa et al., 2006).

Largely as a risk factor for stroke and heart failure, AF is associated with significant morbidity and increases mortality in affected individuals across a wide range of ages independent of preexisting conditions (Benjamin et al., 1998). AF is the cause of 20-25% of all strokes (Miyasaka et al., 2005) and AF-related strokes are nearly twice as likely to be fatal than strokes of other origins (Lin et al., 1996). AF also increases the risk of developing heart failure three-fold (Camm et al., 2012) and worsens prognosis in patients hospitalized for heart failure (Schotten et al., 2011).

In spite of active research efforts, there continues to be a lack of satisfactory therapeutic interventions that improve prognosis in AF patients (Nattel et al., 2002b). One therapeutic approach is the use of antiarrhythmic drugs that restore and maintain sinus rhythm by altering cardiac electrophysiology. However, these drugs are not specific for atrial electrophysiology and increase the risk of life-threatening ventricular arrhythmias such as Torsade de pointes (Nattel, 1998; January et al., 2014). Direct current cardioversion (delivery of an electric shock synchronized with

the “R wave” of the heart) can also restore sinus rhythm, but AF reoccurrence is likely in most patients without attempts to maintain sinus rhythm (January et al., 2014; Lundstrom and Ryden, 1988). Other non-pharmacological approaches to AF treatment include targeted ablation or electrical isolation of arrhythmia generating tissue (often the pulmonary veins) and the reduction and fragmentation of atrial contiguous surface area (e.g. the MAZE procedure), the later of which is highly effective in AF prevention, but is a highly invasive procedure that is not applicable to most patients (Nattel et al., 2002b). Ablative therapies are primarily only relevant for patients with focal AF and the risk of recurrent AF after a single ablation procedure is still 47% in paroxysmal AF patients and 58% in non-paroxysmal AF patients at long-term follow-up, as highlighted by a recent meta-analysis (Ganesan et al., 2013). Thus, AF remains a challenge with respect to its treatment, and a growing problem for health care systems due to its growing prevalence, significant mortality and costs associated with its adverse outcomes.

1.2.2 Mechanisms of AF

1.2.2.1 Overview

AF involves three major processes: initiation of the arrhythmia, arrhythmia maintenance and progression toward more severe forms (Heijman et al., 2014). Each episode of AF requires a trigger for initiation, together with a vulnerable substrate for maintenance of the arrhythmia. In the context of AF, substrates refer to the underlying electrical or structural alterations of the atria that support arrhythmia vulnerability. Abnormal electrical wavefronts emerging spontaneously from regions such as the pulmonary veins are the most common source of AF triggers

(Haissaguerre et al., 1998; Jalife et al., 2009). These ectopic atrial foci are thought to arise primarily as a result of triggered activity, due to either early afterdepolarizations (EAD)s or delayed afterdepolarizations (DAD)s. Either way, the trigger alone cannot initiate AF; electrical wavefronts must also propagate through a suitable arrhythmogenic substrate characterized by reduced refractoriness, enhanced spatial heterogeneity of refractoriness, conduction abnormalities and/or structural heterogeneities (Jones et al., 2012; Nattel et al., 2002a), giving rise to re-entry and AF maintenance. Functional substrates can occur transiently in structurally normal atria, such as in paroxysmal AF or in AF induced in experimental animals (Jalife et al., 2009). However, as AF progresses to more severe forms (i.e. from paroxysmal to persistent and permanent AF) the atria develop substantially altered substrates involving both altered ion channel function and/or expression and irreversible structural changes (Iwasaki et al., 2011). This is due in part to atrial remodelling induced by AF itself (Thijssen et al., 2000). Once initiated, the rapid rates during AF (Nattel 2002a) cause progressive changes in the atria that facilitate maintenance of the arrhythmia and re-initiation should it terminate spontaneously or by interventions ("AF begets AF"; Wijffels et al., 1995; Morillo et al., 1995).

There are also a number of factors that promote the initial development of a vulnerable substrate necessary for re-entry and AF maintenance. AF is a highly heterogeneous condition, occurring most often as a consequence of a wide range of predisposing diseases. AF that occurs in the absence of any demonstrable disease has been classically called "lone AF" (Rosiak et al., 2010), but increasingly the use of the term has been discouraged in clinical practice (January et al., 2014). Although once thought to occur in roughly 30% of all AF patients (Wolf et al., 1991; Roy et al.,

2008), long-term data revealed that lone AF accounts for only 2% of all AF cases (Schoonderwoerd et al., 2008; Jahangir et al., 2007; Rosiak et al., 2010). Long-established AF risk factors that promote substrate vulnerability include hypertension, heart failure, valve disease and thyroid disease (January et al., 2014). There are also a number of newly emerging risk factors including congenital heart disease, predisposing gene variants, and, according to increasing evidence, OSA. Although new risk factors for AF continue to be identified, the mechanisms underlying their relations to AF are not well understood. One hope is that an improved understanding of AF mechanisms and its underlying its risk factors may enable discovery of novel AF therapies that target the specific problem in affected individuals.

The mechanisms of AF are somewhat controversial but it is widely accepted that two major pro-arrhythmic mechanisms are involved: triggered activity and re-entry. In the presence of a vulnerable substrate that is conducive to AF maintenance, triggered activity can initiate AF, followed by re-entry maintenance, or maintain AF as a driver when arising from an ectopic focus firing rapidly and repetitively. Alternatively, AF can be maintained by re-entry, in the form of a single localized re-entry circuit or multiple functional re-entry circuits. In multiple circuit re-entry, the irregular atrial activity that defines AF is a direct consequence of the primary arrhythmia mechanism. In AF driven by ectopic foci or a single re-entry circuit, irregular atrial activity is thought to be due to fibrillatory conduction of wavefronts spawned from the primary arrhythmia generator (the ectopic focus or primary re-entry circuit) due to spatially variable refractory properties of atrial tissue. The following sections will describe the mechanisms underlying triggered activity and re-entry, with specific emphasis on the relationships between basic arrhythmia

mechanisms, the initiation and maintenance of AF and the autonomic nervous system.

1.2.2.2 Triggered activity and AF initiation

Ectopic atrial foci that participate in the initiation and maintenance of AF are thought to be primarily due to triggered activity (Andrade et al., 2014). Triggered activity arises from depolarizing membrane potential oscillations that occur during or after normal action potentials, called afterdepolarizations. Afterdepolarizations that are large enough to reach threshold trigger new action potentials, which can in turn elicit more action potentials, resulting in self-sustaining runs of triggered activity. Depending on the phase of the action potential in which they occur, afterdepolarizations are classified as either “early afterdepolarizations” (EADs) or “delayed afterdepolarizations” (DADs). DADs are membrane potential oscillations that occur following repolarization of the action potential (phase 4) while EADs occur during the action potential plateau (phase 2) or during the late phase 3 repolarization. DADs are the most common contributors to focal ectopic activity in the atria (Heijman et al., 2012) and are favoured by conditions that promote intracellular calcium overload, such as β-adrenergic stimulation, hypertrophy and ischemia (Jalife et al., 2009). Excess diastolic calcium is handled primarily by the sodium calcium exchanger (NCX), which extrudes 1 calcium ion for 3 sodium ions, causing a net depolarizing current, the transient inward current, which underlies DADs (Heijman et al., 2014; Wakili et al., 2011).

EADs are favoured by conditions promoting action potential duration (APD) prolongation, such as with a loss of repolarizing outward K^+ currents or an increase

of inward currents (Jalife et al., 2009). The inward current most likely responsible for the generation of EADs is the calcium window current (Jalife et al., 2009). These currents occur when sufficient time has passed for the L-type calcium current (I_{CaL}) to recover from inactivation (such as during prolonged action potentials). As a result, any abnormal depolarizing current can activate L-type calcium channels from a closed state (Schotten et al., 2011; January and Riddle, 1989), resulting in a transient inward current that can lead to EADs.

1.2.2.3 Mechanisms of AF maintenance and re-entry

Re-entry arises when an electrical impulse persistently reactivates an area of tissue, often due to circular conduction around a circuit (circus movement re-entry). Re-entry circuits may form around fixed anatomic obstacles, such as those formed by the venae cavae, pulmonary veins or a region of inexcitability caused by scar tissue. Re-entry requires initiation by a trigger, often in the form of a premature ectopic beat, and depends on the occurrence of unidirectional block so that activation only occurs in one direction within the circuit (Jalife et al., 2009). Re-entry also requires that the conduction time around the circuit is longer than the refractory period to permit the recovery of excitability within the circuit. Therefore, a relatively long circuit, short RP and slow conduction velocity of the impulse make re-entry more likely. In other words, the wavelength (equal to the refractory period x conduction velocity) must be shorter than the path length of the circuit (Jalife et al., 2009). If the wavelength is greater than the length of a potential circuit, the impulse will traverse the circuit in a time shorter than the refractory period, forcing it to encounter its refractory tail and be extinguished (Nattel, 2002).

Re-entry occurring in the absence of a fixed anatomical substrate is termed functional re-entry, when a premature impulse encounters localized refractoriness and re-enters around this functional barrier (Heijman et al., 2012). For many years, the most widely accepted hypothesis to explain functional re-entry was the leading circle model of Allessie *et al.* (1973). More recently, the spiral wave hypothesis has become much more widely accepted. According to the leading circle model, re-entry circuits establish themselves in the smallest possible pathway that can sustain re-entry (i.e. In a pathlength equal to the wavelength). Shortened refractory period and reduced conduction velocity reduce wavelength, allowing a greater number of simultaneous re-entry circuits to be accommodated thus promoting leading circle re-entry. In contrast, according to the spiral wave model, re-entry circuits adopt the shape of a rotor that propagates around an excitable but unexcited core (Jalife et al., 2009).

Re-entry, substrate vulnerability and AF are favoured by short refractory periods, slow impulse conduction, structural heterogeneities and enhanced spatial dispersion of refractoriness (Jones et al., 2012). Refractory period is governed by APD, which is determined by the balance of inward and outward currents during the action potential plateau (Nattel et al., 2002a). Ion channel dysfunction characterized by increased plateau outward K^+ currents and/or reduced inward I_{CaL} accelerates repolarization, shortening APD and refractoriness, thereby facilitating re-entry (Schotten et al., 2011; Wakili et al., 2011). For example, AF-induced remodeling reduces I_{CaL} and causes pronounced shortening of APD and refractory period (Yue et al., 1997).

Cardiac conduction velocity is determined by (1) electrical coupling through gap junction channels and (2) the maximum upstroke velocity of the phase 0 inward Na^+ current (I_{Na+}). Gap junctions, comprised of transmembrane proteins called connexins, are the subcellular structures that permit electrical continuity between adjescent cells, and are critical for atrial impulse propagation. The heart expresses four primary connexin isoforms but in the atrial myocardium, the dominant isoforms are Connexin 43 (Cx 43) and Connexin 40 (Cx 40) are the dominant isoforms. Impaired gap junction coupling and connexin dysfunction, as occurs in animal models of AF-induced remodeling (Van der velden et al., 2000) and in human AF associated with fibrosis (Luo et al., 2007), reduce conduction velocity and promote re-entry. Cx 43 appears to play a particularly important role in AF. Lowered atrial Cx 43 is found in chronic AF patients (Kostin et al., 2002) and animal models of AF (Igarashi et al., 2012) and predisposes the atrium to AF (Thibodeau et al., 2010; Tuomi et al., 2011). Because altered connexin proteins are both observed widely in AF and also serve as important substrates promoting arrhythmia maintenance (in the case of Cx 43), connexins were a useful marker for AF substrate and inducibilty in this thesis.

Re-entry is also favoured by enhanced spatial heterogeneity of refractoriness, such as during vagally-mediated AF (Wang et al., 1996; Liu and Nattel., 1997). Dispersion of refractoriness introduces functional obstacles that promote fibrillatory conduction and stabilize the leading sources of re-entry circuits (rotors).

1.2.3 Role of the autonomic nervous system in the pathogenesis of AF

1.2.3.1 Atrial autonomic innervation

The autonomic nervous system of the heart includes both extrinsic and intrinsic components (Armour, 2004), both of which have been implicated in atrial arrhythmogenesis. Extrinsic sympathetic influences on the heart include both circulating catecholamines from the adrenal medulla and sympathetic efferent innervation originating from cervical, stellate and thoracic ganglia (Kawashima, 2005). The efferent parasympathetic nerve supply consists of vagal nerves that originate from medullary nuclei such as the nucleus ambiguus (Linz et al., 2013).

In addition to the extrinsic cardiac ANS, the heart is also innervated by an extensive intrinsic cardiac autonomic nervous system (ICANS). The ICANS forms a complex neural network consisting of ganglionated plexi (GP) housed within a number of interconnected epicardial fat pads (Armour et al., 1997). The GP contain both parasympathetic and sympathetic elements (Ardell, 1994) but acetyltransferase immunostaining of all neurons in guinea pig posterior GP indicates major cholinergic input to the myocardium (Mawe et al., 1996; Tuomi et al., 2010). The GP receive both sympathetic and parasympathetic extrinsic innervation and may modulate the interactions between the extrinsic and intrinsic cardiac autonomic nervous systems, acting as a “mini brain” on the heart (Hou et al., 2007).

1.2.3.2 Autonomic regulation of atrial electrophysiology and AF

During parasympathetic stimulation, acetylcholine released from cholinergic nerve terminals binds to and activates muscarinic receptors expressed by the atrial myocardium. Five muscarinic receptor subtypes, M1-M5, have been cloned. M1, M3

and M5 receptors are Gαq-coupled, while M2 and M4 receptors are coupled to Gαi/o (Jones et al., 2012). In the atria, M2 receptors are the most abundantly expressed (Krejci & Tucek, 2002) and, traditionally, have been considered the sole cardiac muscarinic receptor subtype. More recently, it has been established that atrial M3 receptors have numerous physiological functions (Shi et al., 1999b; Wang et al., 2007; Wang et al., 2004). The M2 and M3 subtypes activate distinct potassium currents: the acetylcholine-activated inward rectifying K+ current (IK_{ACh}) by M2 receptors (Reuveny et al., 1994) and the M3 receptor-mediated potassium current (IK_{M3}) by M3 receptors (Shi et al., 1999a, 1999c; Shi et al., 2004). M3 receptors may also contribute to the activation and desensitization of IK_{ACh} (Wang et al., 2007). Activation of IK_{ACh} facilitates an outward hyperpolarizing K^+ current that shortens APD and AERP, thereby facilitating re-entry and AF (Kovoor et al., 2001). M3 receptor-mediated activation of IK_{M3} results in membrane hyperpolarization and APD shortening (Shi et al., 2003; Shi et al., 1999a; Wang et al., 1999), which may facilitate re-entry in a manner similar to IK_{ACh}. Studies of transgenic mice have shown that both enhanced M2 (Posokhova et al., 2013) and M3 (Tuomi et al., 2010) receptor function in the atria are associated with enhanced susceptibility to electrically induced AF.

Owing to the heterogeneous distribution of vagal innervation, muscarinic receptors and/or IK_{ACh} (Lomax et al., 2003) in the atria, the effect of cholinergic stimulation on refractory period is spatially heterogeneous (Liu and Nattel, 1997). The spatially heterogeneous effect of acetylcholine on refractoriness has been shown to promote fibrillatory conduction and spiral wave re-entry in a detailed mathematical model of vagal AF (Kneller et al., 2002; Schotten et al., 2011).

Similarly, findings from optical mapping studies of sheep atria suggest that vagally-induced AF is maintained by a high-frequency mother rotor in the left atrium with fibrillatory conduction towards the right (Mandapati et al., 2000; Skanes et al., 1998; Mansour et al., 2001; Jalife et al., 1998; Jalife et al., 2009). Acetylcholine administration has also been shown to produce AF composed of multiple wavelets wandering through the atria in a chaotic pattern without a single source dominating the activation pattern (Schotten et al., 2011; Allessie et al., 1985).

Adrenergic regulation of atrial electrophysiology occurs primarily via β1- (β1-AR) and β2-adrenergic receptors (β2-AR), with β1-AR comprising 70-80% of all adrenergic receptors in the atria (Arora, 2012). Catecholamine binding to β-adrenergic receptors causes activation of adenyl cyclase, leading to cAMP production and subsequent protein kinase A (PKA)-mediated phosphorylation of several Ca^{2+} handling proteins and ion channels, including L-type Ca^{2+} channels, phospholamban (PLN) and ryanodine type 2 receptors (RYR2) (Bers, 2002). Adrenergic stimulation also increases Ca^{2+} binding to calmodulin, leading to activation of Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII), which phosphorylates many of the same substrates as PKA, thereby amplifying the adrenergic response (Chen et al., 2014). Phosphorylation of L-type Ca^{2+} channels by PKA increases Ca^{2+} influx via $I_{Ca,L}$ while Phosphorylation of PLN augments sarcoplasmic reticulum (SR) Ca^{2+} loading via dissinhibition of the SR Ca^{2+}-ATPase (SERCA2a) by PLN, responsible for reuptake of Ca^{2+} into the SR. As a result of RYR2 phosphorylation and greater SR Ca^{2+}, the β-adrenergic response increases RYR2 channel opening probability (Bers, 2002). Together these actions increase the systolic Ca^{2+} transient and promote abnormal spontaneous sarcoplasmic reticulum

(SR) Ca^{2+} release events (Ca^{2+} sparks; Marx et al., 2000; Ogrodnik and Niggli, 2010). Ca^{2+} sparks have been shown to promote DADs (Johnson et al., 1986; Wit and Boyden, 2007), suggesting a role for β-adrenergic activity in DAD-related triggered activity, although evidence for DAD-related triggered activity in AF is lacking. However, combined sympathovagal coactivation (during which APD is shortened by IK_{ACh} and the Ca^{2+} transient is enhanced) has been shown to cause late phase 3 EADs, triggered activity and AF initiation (Patterson et al., 2006; Burashnikov and Antzelevitch, 2003).

1.2.3.3 Autonomic activity and AF: review of the evidence

The importance of the autonomic nervous system (ANS) in the initiation and maintenance of AF has been demonstrated in many settings. In studying patients with paroxysmal AF, Coumel *et al*. (1996) noted two patterns: vagally mediated AF was common in young patients with structurally normal hearts while sympathetically mediated AF typically occurred in the presence of heart disease, and often during exercise or states of emotional stress. More recently, adrenergic triggers (associated with exercise or emotion), vagal triggers (mostly at night) and combined adrenergic and vagal triggers commonly preceded AF episodes in a large study of over 1,500 patients (De Vos et al., 2008). Studies involving direct nerve recordings have demonstrated that simultaneous sympathovagal activation (Tan et al., 2008; Ogawa et al., 2007) and intrinsic cardiac nerve activity (Choi et al., 2010) are common triggers of AF paroxysms in animal models. A high incidence of sympathovagal co-activation at baseline is associated with a high vulnerability to pacing-induced

sustained AF, suggesting that the ANS has a role in the development of persistent AF (Shen et al., 2011).

The pulmonary veins and pulmonary vein-left atrial (PV-LA) junction are heavily innervated by GP (Tan et al., 2006; Chou et al., 2005) and several studies have highlighted a role for these intrinsic nerves in promoting AF. Enhanced activity from the PV-LA junction GP has been implicated in triggering that arises from the pulmonary veins (Patterson et al., 2005; Patterson et al., 2006) and their ablation suppresses or eliminates focal AF from PVs (Lu et al., 2009). Stimulation of the PV-LA junction GP provides the substrate to convert PV firing to AF (Scherlag et al., 2005) which may involve EAD-related triggered activity induced by adrenergic stimulation combined with vagally mediated APD and refractory period shortening (Patterson et al., 2005).

1.3 Obstructive sleep apnea (OSA)

1.3.1 Definition of OSA

Obstructive sleep apnea (OSA) is a disorder in which episodes of pharyngeal collapse cause temporary cessations in breathing during sleep. These episodes can be complete (apneas) or partial (hypopneas), although both are sufficient to cause intermittent hypoxia (IH) and significant hypoxemia, as well as carbon dioxide retention. Apneas/hypopneas and their accompanying IH lead to arousals with sleep fragmentation, episodic intrathoracic pressure reductions from inspiration against an occluded airway and surges of autonomic activity with each episode. Excessive daytime sleepiness is the main symptom of OSA, but some patients also present with frequent snoring, choking or gasping during sleep, recurrent arousals from

sleep and/or impaired concentration (Parati et al., 2012). However, evidence suggests that a large proportion of OSA patients are asymptomatic (Duran et al., 2001).

OSA is identified based on symptoms and clinical findings, but a definitive diagnosis requires attended overnight polysomnography in a sleep laboratory. During polysomnography, sleep stages, heart rate and rhythm, limb movements, arterial oxygen saturation, and respiratory movements and/or respiratory effort are recorded. These parameters enable determination of the number of obstructive respiratory events (lasting >10s) per hour, which is used to determine the apnea-hypopnea index (AHI). OSA is defined as an AHI of at least 5 (Parati et al., 2012), and the severity of OSA is defined as: mild OSA, AHI of 5-15/h; moderate OSA, AHI of 15-30/h, or severe OSA, AHI >30/h; Parati et al., 2012. Reduction in blood-oxygen saturation by at least 90% has also been described as an important indicator of OSA severity as it is correlated with susceptibility to cardiovascular events (Nieto et al., 2000).

1.3.2 Clinical relevance of OSA

Data from large scale population studies in Wisconsin (Young et al., 1993), Pennsylvania (Duran et al., 2001) and Spain (Bixler et al., 1998) conducted using polysomnography suggest that OSA of at least mild severity (AHI > 5) affects 17-26% of men and 9-28% of women. However, these studies likely underestimated the true disease burden of OSA, as over 85% of patients with OSA remain undiagnosed (Young et al., 1997; Kapur et al., 2002). The prevalence of OSA increases with age in both men and women (Duran et al., 2001; Young et al., 2004). Obesity is also a

major OSA risk factor, as a 10% increase in weight corresponds with a 6-fold increased risk of developing OSA of at least moderate severity and a 32% increase in the AHI (Peppard et al., 2000a).

OSA has been linked to the development of a multitude of complications including cardiovascular, metabolic and neurocognitive consequences. In particular, the cardiovascular consequences of OSA contribute significantly to population morbidity and mortality. OSA has been recognized as an independent risk factor for hypertension (Peppard et al., 2000b; Nieto et al., 2002), stroke (Arzt et al., 2005), and coronary artery disease (Sorajja et al., 2008; Mooe et al., 2001). OSA is associated with increases in rates of cardiovascular morbidity and mortality independent of other risk factors (Marin et al., 2005; Yaggi et al., 2005; Campos-Rodriguez et al., 2012) and treatment of OSA with continuous positive airway pressure (CPAP) has been demonstrated to reduce cardiovascular risk (Marin et al., 2005; Campos-Rodriguez et al., 2012).

OSA is also an independent risk factor for AF (Mehra et al., 2006). The prevalence of OSA was 20% greater among AF patients compared to healthy individuals matched for age, sex, BMI, prevalent hypertension and heart failure (Gami et al., 2004). Similarly, the prevalence of AF was 5 times greater in adults with OSA than in those without the syndrome (4.8% vs 0.9%; Mehra et al., 2006). OSA is also associated with increased AF reoccurrence rates after cardioversion and ablation (Ng et al., 2011). The finding that treatment of OSA with continous positive airway pressure reduces the risk of recurrence of AF suggests a causal role for OSA in the pathogenesis of AF (Kanagala et al., 2003; Fein et al., 2013; Naruse et al., 2013), but the underlying mechanisms are unknown.

1.3.3 Intermittent hypoxia in OSA

OSA is a multicomponent disorder involving IH, sleep fragmentation, intrathoracic pressure swings and obstructed respiratory efforts. Together, these components contribute to the disease progression and development of OSA-related comorbidities (For review, see Dempsey et al., 2010). However, the widespread use of IH in animals to model OSA has enabled the recognition that IH is likely the most critical component underlying its cardiovascular complications (Dematteis et al., 2009).

1.3.3.1 IH model

Since their introduction over 20 years ago (Fletcher et al., 1992a, 1992b, 1992c), animal models employing IH have been used widely to investigate the pathophysiology of OSA and its consequences. Animals, typically rodents, are exposed to intermittent cycles of hypoxia-reoxygenation during their sleep cycles to emulate the oxygen desaturations caused by obstructive apneas. The IH stimulus is typically applied during the day, throughout the sleep cycle of nocturnal animals such as rodents, but with significant differences with respect to the duration of daytime exposure, number of cycles per hour and level of fraction of inspired O_2 (FiO_2). The IH stimulus may be applied up to 60 (Campen et al., 2005; Polotsky et al., 2006) or 120 (Fletcher et al., 1992c) times per hour, with FiO_2 levels typically in the range of 5-10% (Fletcher et al., 1992c; Soukhova-O'Hare et al., 2006). These oxygen desaturations are well correlated with those observed in OSA patients (Jun et al., 2010; Louis and Punjabi, 2009), and reliably produce the same physiological

perturbations that occur in the human disease, including profound effects on autonomic activity, heart rate and blood pressure.

1.3.3.2 Effects of IH on the autonomic nervous system and the heart

Hypoxia is sensed by specialized structures located in the carotid bodies known as peripheral chemoreceptors, which become activated during apneic episodes. The activation of these structures sets in motion a cascade of acute autonomic adjustments to maintain homeostasis during hypoxia, collectively referred to as the peripheral chemoreflex (For review, see Prabhakar, 2000). Paradoxically, chemoreceptor activation results in simultaneous activation of both vagal and sympathetic outflows to the heart in both dogs (Kollai and Koizumi, 1979) and rats (Boscan et al., 2001). The net response of the heart to chemoreceptor stimulation is profound bradycardia (Braga et al., 2008), indicating that the vagal effect overrides positive chronotropism of the sympathetic activation, and a positive inotropic response that is sympathetically mediated (Braga et al., 2007). In addition to these primary effects on cardiac autonomic activity, chemoreflex activation also causes sympathetically mediated vasoconstriction, resulting in acute surges in blood pressure (Haibara et al., 1995; Braga et al., 2008). These surges are detected by mechanosensitive arterial baroreceptors in the aortic arch, triggering activation of the associated arterial baroreflex which elicits sympathoinhibitory effects on the vasculature while further increasing parasympathetic activation of the heart (Braga et al., 2008). Thus, during IH and episodes of apnea the atria are bombarded by both sympathetic and parasympathetic activity.

Chronic repetition of these acute nocturnal hypoxic episodes leads to substantial autonomic dysfunction. As occurs in patients with OSA (Narkiewicz et al., 1999), peripheral chemoreflex sensitivity and the hemodynamic responses to peripheral chemoreceptor activation are potentiated in rodents exposed to chronic repetitive IH (Braga et al., 2006; Huang et al., 2009). At the same time, arterial baroreflex sensitivity and/or baroreflex control of sympathetic activity is reduced in OSA patients (Carlson et al., 1996; Narkiewicz et al., 1998) and following exposure to IH in animal models (Lin et al., 2007). As a result, subjects with repetitive sleep apneic events exhibit persistently elevated sympathetic tone and hypertension even during awake hours when there is an absence of apneas (Narkiewcz et al., 1999; Sajkov et al., 1994). Accordingly, chronic IH exposure leads to hypertension (Fletcher et al., 1992a; Fletcher et al., 1992c), elevated plasma catecholamines (Zoccal et al., 2007; Gonzalez-Martin et al., 2009; Peng et al., 2014), and elevated cervical (Greenberg et al., 1999), renal (Huang et al., 2009), splanchnic (Dick et al., 2007; Xing and Pilowsky, 2010), thoracic (Zoccal et al., 2008) and lumbar (Marcus et al., 2010) sympathetic nerve activity in rodent IH models of sleep apnea. The chronic effects of IH on parasympathetic nervous activity (PNA) are less well understood, but enhanced vagal efferent control of heart rate following chronic IH in rats (Gu et al., 2007) and mice (Lin et al., 2007) suggests that activity of peripheral vagal neurons become upregulated, to compensate for the loss of baroreflex control of the heart.

1.3.3.3 Implications for atrial arrhythmias

IH exerts a myriad of effects on the autonomic nervous system with potential implications for atrial arrhythmogenesis. During sleep, bouts of hypoxia cause repetitive oscillations in parasympathetic and sympathetic cardiac outflow that may predispose the atria to autonomically triggered arrhythmias. As previously mentioned, simultaneous sympathovagal activation (Tan et al., 2008; Ogawa et al., 2007; de Vos et al., 2008) and activity of intrinsic cardiac nerves (Choi et al., 2010) are common triggers of AF episodes. Moreover, AF can be initiated by stimulation of GPs (Scherlag et al., 2005), β-adrenergic agonists (Sharifov et al., 2004) and cholinergic agonists (Sharifov et al., 2004; Mandapati et al., 2000; Skanes et al., 1998; Mansour et al., 2001; Jalife et al., 1998; Allessie et al., 1985). In addition to having substantial acute effects on autonomic activity, repeated and chronic exposure to IH results in sustained autonomic dysfunction during wakefulness, which may give rise to atrial electrophysiological alterations and provide the necessary triggers and vulnerable substrate for AF. Abnormal autonomic function and/or changes in autonomic tone are associated with electrophysiological substrate development and AF in animal models (Jayachandran et al., 2000; Chang et al., 2001; Guasch et al., 2013) and human patients (Nguyen et al., 2009). The setting of autonomic dysfunction caused by IH may operate similarly to augment substrate vulnerability and AF susceptibility.

1.4 RATIONALE

Although substantial evidence from epidemiological and clinical studies suggests a causal role for OSA in the pathogenesis of AF, the use of animal IH

models to characterize this relationship is lacking and the underlying mechanisms have not been adequately investigated. IH has been implicated in mediating many consequences of OSA including substantial autonomic dysfunction and enhanced sympathetic drive, but the role of IH in predisposing to AF is not known. Given that IH bombards the atria with autonomic activity and augments chronic autonomic dysfunction, both of which have been implicated in AF promotion, IH may augment substrate formation and predispose the atria to AF.

1.5 HYPOTHESIS AND OBJECTIVES

We hypothesized that IH augments formation of electrophysiological and autonomic substrates for AF and increases susceptibility to electrically induced AF.

Specific objectives of this thesis were to determine in the rat:

1. the effect of IH on the expression of adrenergic and muscarinic receptor mRNA and protein in the atria;
2. the effect of IH on atrial connexin mRNA and protein expression;
3. the effect of IH on atrial effective refractory period (AERP) and the susceptibility to electrically-induced AF;, and
4. the role of the autonomic nervous system in mediating atrial electrophysiological alterations and enhanced AF susceptibility induced by IH.

2.0 MATERIALS AND METHODS

2.1 Animals

All procedures were in accordance with the Guidelines on the Care and Use of Laboratory Animals of the Canadian Council on Animal Care and the Animal Use Committee at the University of Western Ontario (Protocol #'s 2006-122, 2008-030 & 2014-053). A total of 149 adult, male Sprague-Dawley rats (Charles River, Canada) weighing 275 – 400 g were included in this study. Rats were housed at a temperature of 22 ± 1 °C with 60% relative humidity, under 12/12 hour light/dark cycle. Food and water were provided ad libitum, except during exposure to IH or normoxic conditions.

2.2 Intermittent hypoxia model of Obstructive Sleep Apnea

OSA was modeled by exposing rats to repetitive cycles of hypoxia-reoxygenation during their sleep cycles (9 am to 5 pm) to mimic that caused by obstructive apneas, as described previously (Moreau and Ciriello, 2013). For 8 hours per day (9 am to 5 pm), rats were placed in plexiglass® chambers containing 4 tubes in which they were allowed to move freely. In the IH chamber, computerized solenoid valves which regulated the inflow of pressurized air and nitrogen were programmed to produce cycles of 80 seconds of hypoxia (6.5-7% O_2) followed by 120 seconds of reoxygenation (21% O_2 ; Figure 1.1A). This particular regimen emulates a moderate form of OSA, based on the apnea-hypopnea index (AHI; Moreau and Ciriello, 2013; Parati et al., 2012). Fans pushed the gasses through a mixing chamber prior to entering the chamber containing the animal tubes (Figure 1.1B). Sensors on the chamber continuously monitored oxygen and carbon dioxide levels, which allowed the computer to ensure proper cycling and maintaince of

eucapnic conditions (<0.1% CO_2). Normoxic control animals were placed in identical chambers through which only pressurized room air cycled through the system. To observe acute, intermediate and chronic effects of the model, animals were exposed to IH or normoxic conditions for an overall exposure duration of 1, 7 or 95 days, respectively (Messenger et al., 2013; Moreau and Ciriello, 2013). There were no obvious differences in sleep patterns between the IH and normoxia-exposed rats in the chambers.

2.3 Animal subgroups

For real-time PCR analysis, atrial mRNA from IH and normoxia-exposed rats were compared immediately after 1 day (n = 7 per group), 16 hours after 1 day (n = 5 normoxic rats, n = 6 IH rats), 16 hours after 7 days (n = 8 per group) and immediately after 95 days of exposure (n = 8 per group). For western blot analysis, atrial protein levels from IH and normoxia exposed rats were compared immediately following 1 day (n = 7 per group), 16 hours after 1 day (n = 5 normoxic rats, 6 IH rats), immediately after 7 days (n = 7 per group) and 16 hours after 7 days of exposure (n = 7 per group). Rats exposed to IH (n = 27) or normoxia (n = 27) for 7 days underwent intracardiac electrophysiological studies immediately after exposure.

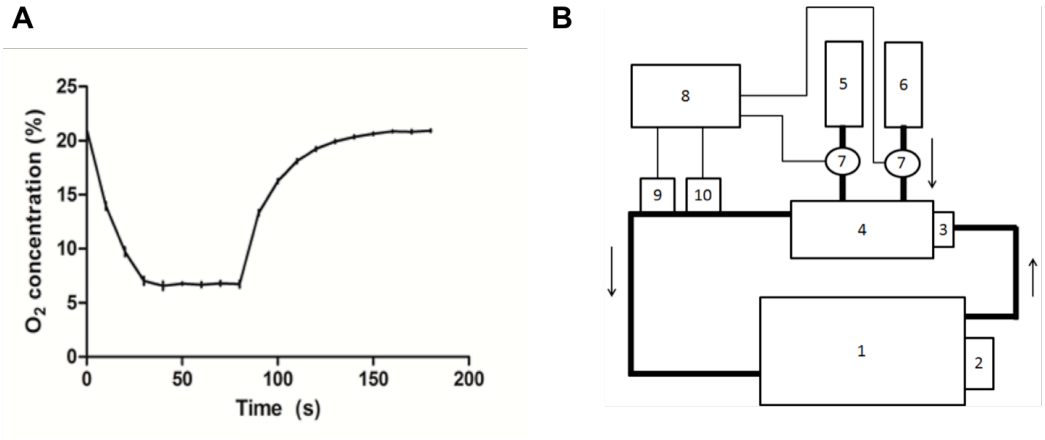

Figure 2.1 Intermittent hypoxia exposure model and chamber

(**A**) O_2 nadir (%) in the hypoxia chamber during a single cycle. (**B**) Schematic representation of the chamber used for IH exposures. Thick black lines represent tubing and thin black lines represent electrical connections. Arrows indicate direction of air flow. Animals are housed within the hypoxia chamber (1), which was attached to a zero-pressure escape valve (2) that prevented pressure changes within the chamber. Fans (3) promoted air flow from the animal chamber to the mixing chamber (4) which included baffles to encourage mixing of fresh gases with gases flowing through the system. The inflow of 100% compressed nitrogen (5) and pressurized room air (6) into the mixing chamber was controlled by solenoid valves (7) controlled by a computerized timing box (8) that received feedback from O_2 (9) and CO_2 (10) sensors. Taken with permission from Moreau, 2013.

2.4 Tissue collection

Rats subjected to IH or normoxic conditions for 1, 7 or 95 days were sacrificed and the hearts exposed using a thoracotomy approach. Right and left atria were excised and collected in Eppendorf tubes, rapidly immersed in liquid nitrogen and then stored at -80 °C until mRNA or protein analysis. Samples of total atria were partitioned into two halves to be further processed for isolation of RNA and protein.

2.5 RNA isolation and real-time PCR

Total atrial RNA was isolated using TRIZOL reagent (Life Technologies, Railey, UK) and RNA concentrations were determined with a NanoDrop2000 UV-vis spectrophotometer (ThermoFisher Scientific, Toronto, ON). RNA integrity was evaluated by visual assessment of ethidium bromide-stained agarose denaturing gels. cDNA was synthesized from extracted RNA using qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD). Oligonucleotide primer sequences for the M2 receptor, M3 receptor, Cx 40, Cx 43, β1-AR, β2-AR and ribosomal subunit 18S are shown in Table 1. Real-time PCR was carried out in triplicate parallel reactions on a Bio-Rad CFX384 (Bio-Rad, Hercules, CA) using the SsoFast EvaGreen Supermix system (Bio-Rad). Amplification was performed at 95ºC for 3 min, followed by 39 cycles at 95ºC for 15 s, 59ºC for 15 s and 72ºC for 15 s. Signal detection and analysis were performed using Bio-Rad CFX384 software (Bio-Rad). Amplification specificity was assessed based on the presence of a single, narrow melting curve peak for each assay. Fold differences, normalized to ribosomal subunit 18S were determined using the comparative ΔΔ CT method.

Table 2.1 Primer sequences used for real time PCR

Gene		Primer sequence
M2 receptor	Forward	5'-GCCCCAGCCATTCTCTTCTG-3'
	Reverse	5’-TATTCTGCTCTTGCTCGCCCG-3’
M3 receptor	Forward	5’-TGCCTGGGTCTCTTAATTCC-3’
	Reverse	5’-CTTCACATGGGATCTGGATG-3’
Cx 43	Forward	5’-TCCTTGGTGTCTCTCGCTTT-3’
	Reverse	5’-GAGCAGCCATTGAAGTAGGC-3’
Cx 40	Forward	5’-ATGGGTGACTGGAGCTTCCTGGGG-3’
	Reverse	5’-TCACACTGACAGGTCATCTGACCT-3’
β1-AR	Forward	5′-ACCCCAAGTGCTGCGATTTCGT-3′
	Reverse	5′-GCTCGCAGCTGTCGATCTTCTT-3′
β2-AR	Forward	5′-TTCTGTGCCTTCGCCGGTCTTCTT-3′
	Reverse	5′-ATGCCAGGGGCTTCCTCACAAA-3′
18S	Forward	5’-GTGACGGGGAATCAGGGTT-3’
	Reverse	5’-CCTTCCTTGGATGTGGTAGCC-3’

2.6 Protein isolation and western blot

Protein lysates were prepared in RIPA buffer (50 mM Tris, 150 mM sodium chloride, 0.1% SDS, 1% Triton-X 100, 0.5% sodium deoxycholate, 1mM EDTA, 1mM sodium orthovanadate, 1 mM sodium fluoride, 25 mM β-glycerophosphate, pH 7.5) with a protease inhibitor cocktail Tablet (Roche Applied Science; Laval, PQ). Tissue homogenization was performed on ice, with delivery of 3, 15-second bursts from a Kinematica polytron homogenizer set to 60% (Brinkmann Instruments; Rexdale, ON). Homogenates were then sonicated over 3 passages for 15 seconds each on ice (55%; Sonic Dimembrator Model 150; Fisher Scientific) and then centrifuged at 4°C for 30 min at 14 500 x g. Protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad; Hercules, CA).

Samples containing 20-30 µg protein were heated at 75ºC for 5 minutes prior to being loaded onto 10% polyacrylamide gels and subjected to electrophoretic separation by size in the Mini-Protean II cell from Bio-Rad. Dose-response curves of various protein amounts (0.1-33 µg) were generated to ensure that the chosen amount of total protein was in the linear range of detection. Resolved proteins were transferred onto polyvinylidene fluoride (0.45 µm) membranes (Millipore; Billerica, MA) in a Mini Trans-Blot Transfer Cell (Bio-Rad). Buffer containing 5% skim milk, Tris-buffered saline with 0.01% Tween-20 (TBS-T; 20 mM Tris, 0.5 M NaCl, 0.1% Tween-20; pH 8.0) was used to reduce nonspecific binding and for the dilution of primary and secondary antibodies. Using routine procedures, (Moreau and Ciriello, 2013) membranes were probed overnight with primary antibodies at 4ºC, including rabbit anti-connexin 43 (1:10,000; C6219, Sigma-Aldrich, St. Louis, Missouri), rabbit anti-connexin 40 (1:8000; AB101929, Abcam Inc., Cambridge, MA), rabbit anti-β1-adrenergic receptor (1:2000; PA1-049, ThermoFisher Scientific; Rockford, IL), rabbit

anti-M2 muscarinic acetylcholine receptor (1:800; AB5166; Millipore; Billerica, MA), and polyclonal rabbit anti-M3R (1:1000; sc-9108, Santa Cruz Biotechnology; Santa Cruz, CA). After washing, membranes were probed again for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; CLCC27007, Cedarlane Laboratories, Burlington, ON) as the secondary antibody. Bands were detected using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific; Toronto, ON), visualized using a VersaDoc imaging system (Bio-Rad Labortories; Hercules, CA) and analyzed using QuantityOne version 4.6.6 software (Bio-Rad Laboratories; Hercules, CA). Membranes were exposed for 10-40 seconds. This software highlights saturated pixels, which enabled us to ensure that exposure never reached saturation.

To control for differences in protein loading, following detection of the initial protein, membranes were incubated with stripping buffer (200 mM Glycine, 6.9 mM SDS, 0.01% Tween-20, pH 2.2) and immunoblotting was performed again, using mouse anti-β-tubulin (1:2000, T8328; Sigma-Aldrich, St. Louis, Missouri) as the primary antibody followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:8000, 170-6516, Bio-Rad Laboratories; Hercules, CA) as the secondary antibody with chemiluminescence detection as described above.

2.7 Preoperative procedures

A subset of rats underwent *in vivo* intracardiac electrophysiological studies following exposure to IH (n = 27) or normoxia (n = 27) for 7 days. At the end of the exposure period, rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and fixed in a supine position over

a heated water blanket. A small animal rectal probe (YSI-402; Yellow Springs Instruments, Yellow Springs, OH) was used for continuous monitoring of body temperature, which was maintained within the normal physiological range (36.5–38°C) by adding or removing heat from lamps and heated water-filled gloves.

2.8 Electrocardiogram (ECG) Recordings

Standard ECG limb leads I, II, III, aVR, aVL and aVF were recorded continuously throughout the study using four 25-gauge subcutaneous platinum electrodes (Grass Instrument, Quincy, MA) placed at the base of each limb. ECG signals were sampled at 1.5 kHz and filtered (0.05 – 100 Hz) with an ECG 100 preamplifier connected to an MP100 recording system (BIOPAC Systems, Biolynx, Montreal, PQ, Canada).

2.9 Intracardiac electrophysiological studies

A 2-Fr octapolar stimulation/recording/drug infusion catheter (CIB'ER Mouse, NuMED, Hopkinton, NY) was inserted through the right jugular vein and advanced into the right atrium. The catheter was placed at the site where the amplitude of the atrial deflection exceeded that of the ventricular deflection in the intracardiac electrograms recorded from the two proximal pairs of bipolar electrodes. Bipolar pacing used 2-ms pulses at twice the diastolic threshold, delivered through a Grass SIU5 stimulus isolation unit, connected to a Grass S88 stimulator, programmed with a custom-built timer, as previously described (Tuomi et al., 2010). Atrial effective refractory period (AERP) measurements were made using cycle lengths of 150 ms and 100 ms in a conditioning train of 8 basic drive stimuli (S1 x 8) followed by a

premature extrastimulus (S2). The S2 was delivered at decrements of 10 ms, followed by 1 ms decrements as AERP was approached. AERP was defined as the longest S1-S2 coupling interval that failed to elicit an atrial response.

Both programmed electrical stimulation (PES) and burst pacing (2 ms pulses at 50 Hz applied for 1 second, up to 10 times per atrial site) were used to determine susceptibility to atrial arrhythmia induction. AF was identified based on the presence of intra-atrial electrogram fractionation and characteristics of the surface lead electrograms: lack of regular P waves and “irregularly, irregular” ventricular responses.

2.10 *In vivo* electrophysiological study design

After performing electrophysiological studies at baseline as described above, pacing protocols were repeated following administration of muscarinic and adrenergic receptor agonists and antagonists. IH or normoxia-exposed rats were assigned to 1 of 3 groups, each receiving a different drug regimen. In IH (n = 10) or normoxia (n = 9) exposed rats assigned to group 1, electrophysiological measurements were made sequentially at baseline, then in the presence of isoproterenol, then again at baseline after a 40 minute washout period, followed by in the presence of propranolol, then isoproterenol. In group 2 (n = 8 per condition), measurements were made at baseline, followed by in the presence of carbachol, then at baseline after a 60 minute washout period, followed by in the presence of atropine, then carbachol. In IH (n = 6) or normoxia (n = 5) exposed rats assigned to group 3, the measurements were made at baseline, followed by in the presence of

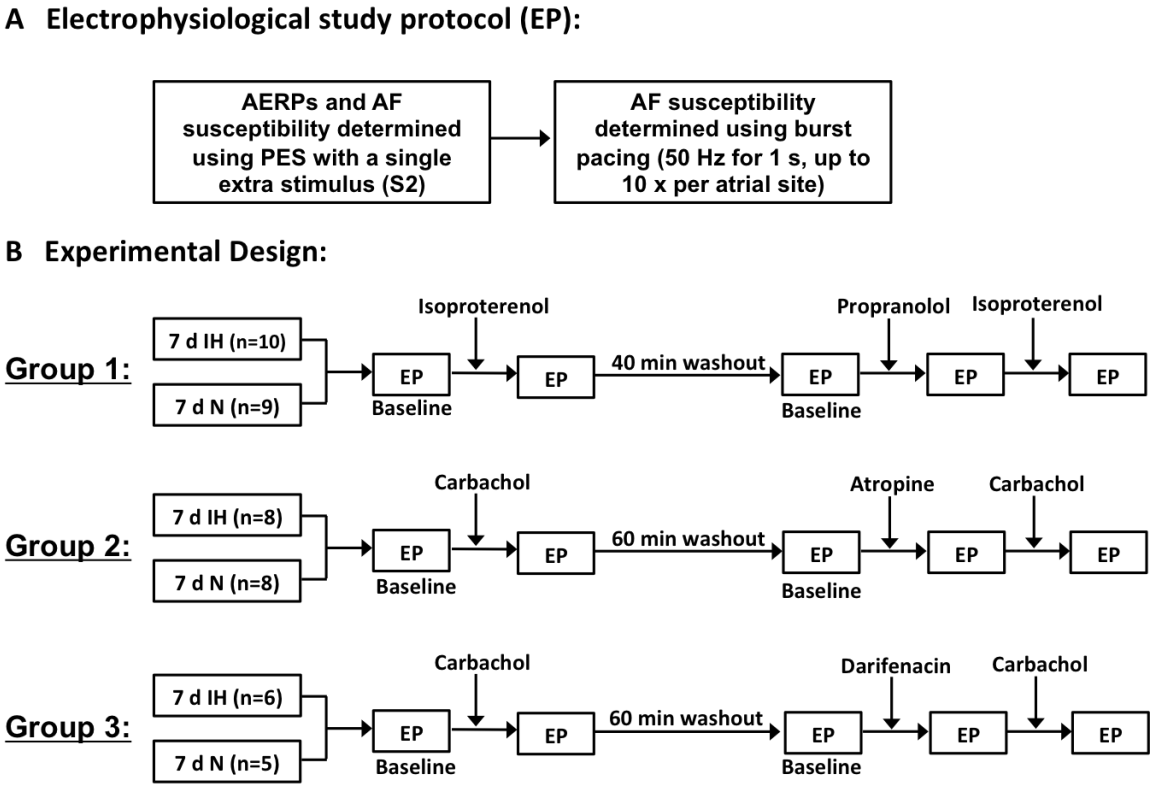

Figure 2.2 Electrophysiology protocol and experimental design

Flow chart showing the electrophysiological protocol (EP; panel **A**) and experimental design (panel **B**). AERP, atrial effective refractory period; AF, atrial fibrillation; PES, programmed electrical stimulation.

carbachol, again at baseline after 60 minutes for washout, followed by in the presence of darifenacin, then carbachol. The electrophysiological protocol and experimental design are shown in Figure 2.1.

2.11 Adrenergic and muscarinic receptor drugs

All drug doses and routes of administration were selected based on previous literature and the results of pilot studies undertaken to observe heart rate responses to cumulative doses within ranges expected to produce moderate (10-30%) changes in heart rate (please see the appendix for details). In individual rats, responses to at least two doses of each drug or dose-response relationships were observed (refer to the appendix). The M3 receptor-selective antagonist darifenacin hydrobromide (1.0 mg/kg; Cedarlane Laboratories, Markham, ON) was dissolved in a vehicle containing isotonic saline and DMSO (in a 1:1 v/v ratio) and administered intravenously. All other drugs, including the nonselective muscarinic agonist carbachol (0.05 mg/kg; Sigma, Mississauga, ON), the nonselective muscarinic antagonist atropine (1.0 mg/kg; Sigma), the nonselective β-agonist isoproterenol (0.1 mg/kg; Sigma) and the nonselective β-antagonist propranolol (10 mg/kg; Sigma) were dissolved in isotonic saline and administered intraperitoneally.

2.12 Statistical analysis

All values are expressed as mean ± standard error of the mean. For protein and gene expression analyses, comparisons between IH and normoxia exposed animals at the different time points were made using unpaired, two-tailed Student t-tests (GraphPad Prism 6; GraphPad Software, San Diego, CA). Discrete data were

analyzed by Chi squared analysis (GraphPad Prism 6; GraphPad Software, San Diego, CA). Drug and groupwise comparisons used two-way ANOVA using GraphPad Prism 6 software. In all comparisons, a *p-value* < 0.05 was considered statistically significant.

3.0 RESULTS

3.1 Expression of muscarinic receptors

Figure 3.1 shows that atrial M2 receptor protein was significantly higher immediately following both 1 ($P < 0.01$) and 7 ($P < 0.05$) days of IH exposure compared to normoxic controls. However, no significant differences were present between the IH and normoxia-exposed rats approximately 16 hours after the end of the exposure periods, suggesting that this effect is reversible at the 1 and 7 day time points. As shown in Figure 3.2, M2 receptor mRNA levels were not significantly different following IH exposure for 1, 7 or 95 days, indicating that elevated M2 receptor protein content in the IH rats was not due to increased gene expression; rather, reduced protein degradation and/or an increase in translation may be responsible for the changes we observed. Compared to normoxic controls, M3 receptors were also higher in 1 day IH-exposed rats at the protein level (Figure 3.3; $P < 0.01$) and at the mRNA level (Figure 3.4; $P < 0.05$).

3.2 Expression of adrenergic receptors

Rats exposed to 1 or 7 days of IH exhibit no change in β1-adrenergic receptor content at the protein (Figure 3.5) or mRNA (Figure 3.6) level. However, β1-adrenergic receptor mRNA was significantly lower in rats exposed to IH for 95 days compared with normoxic controls (Figure 3.5; $P < 0.05$). On the other hand, in the atria of IH-exposed rats, β2-adrenergic receptor mRNA levels were not significantly different from normoxic controls at any of the exposure lengths studied (Figure 3.7) and corresponding protein levels were not investigated.

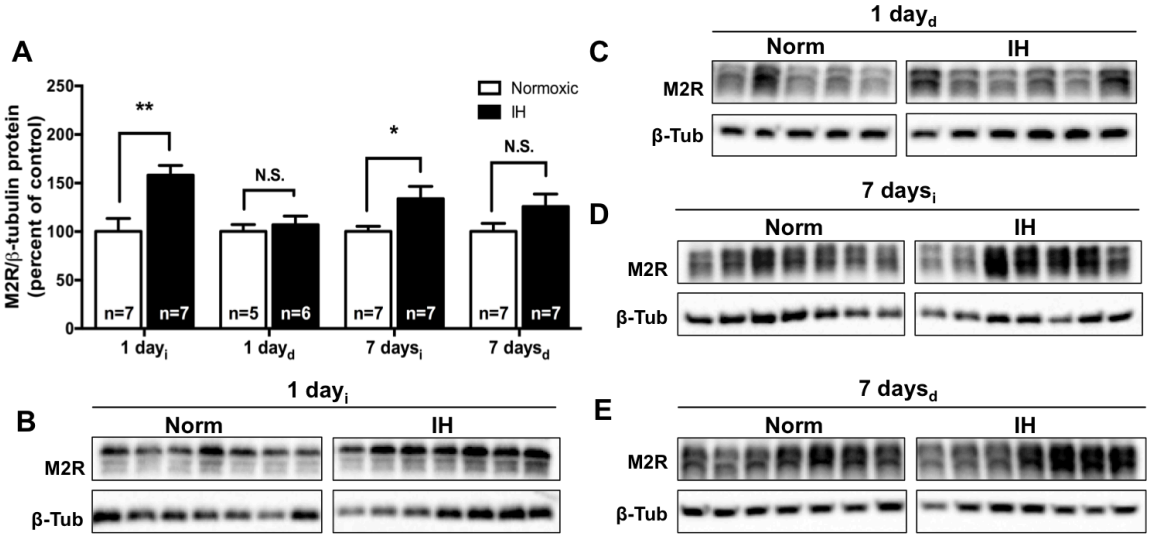

Figure 3.1 Effect of intermittent hypoxia on atrial M2 receptor protein expression

Bar graphs (**A**) and representative blots (**B-E**) showing M2 muscarinic receptor (M2R) protein expression as a ratio of β-tubulin in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1 or 7 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$ and 7 days$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$ and 7 days$_d$), and each time point was run on separate gels. Data shown are means ± SEM. *, $P < 0.05$; **, $P < 0.01$; N.S., not significant.

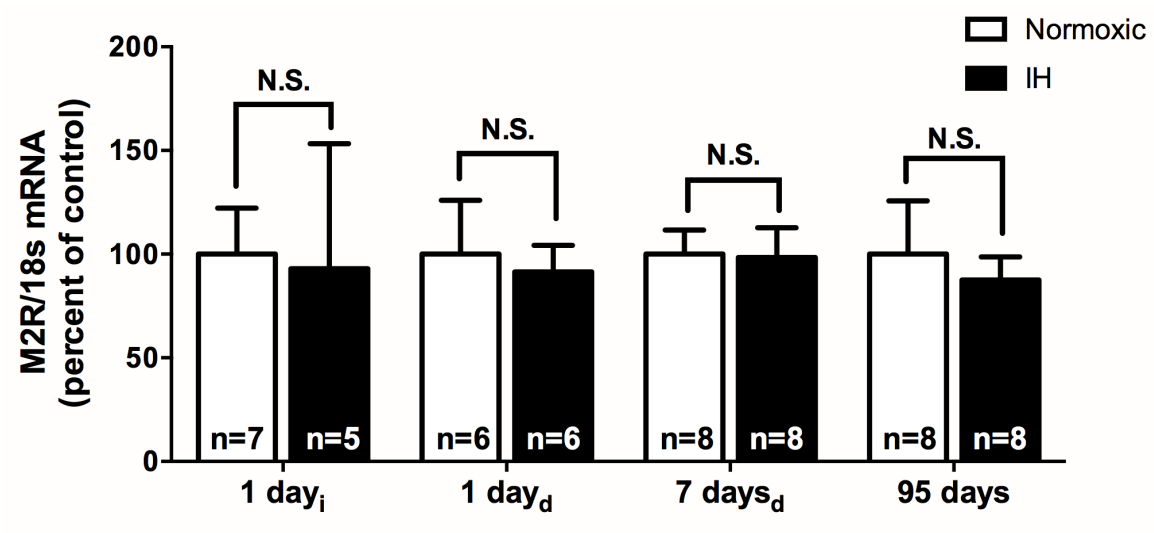

Figure 3.2 Effect of intermittent hypoxia on atrial M2 receptor mRNA expression

Bar graphs showing no difference in M2 muscarinic receptor (M2R) mRNA expression as a ratio to 18S in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1, 7 or 95 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$, 7 days$_d$ and 95 days$_d$). Data shown are means ± SEM. N.S., not significant.

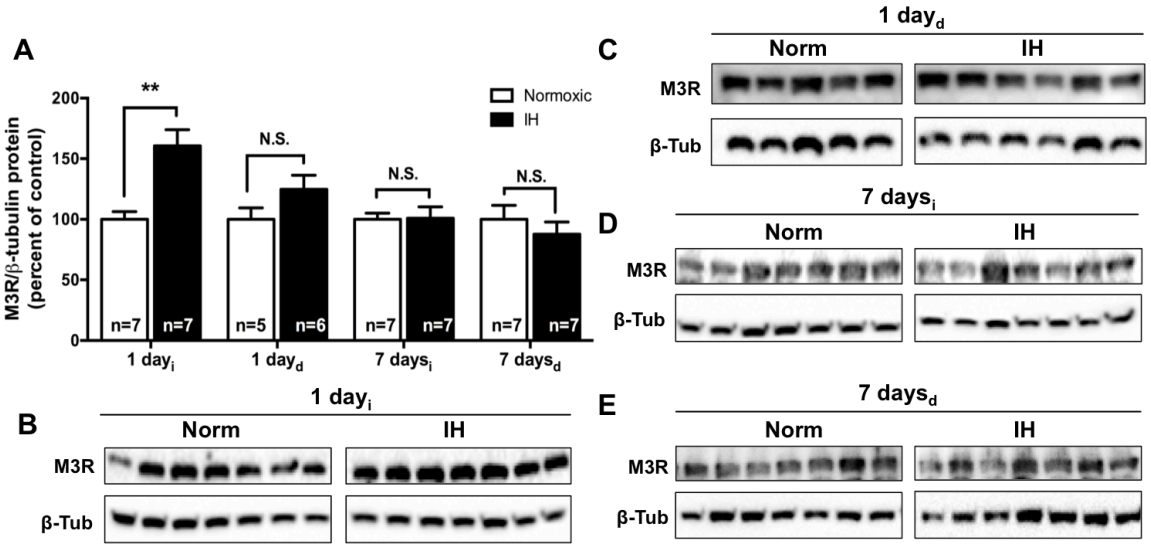

Figure 3.3 Effect of intermittent hypoxia on atrial M3 receptor protein expression

Bar graphs (**A**) and representative blots (**B-E**) showing M3 muscarinic receptor (M3R) protein expression as a ratio of β-tubulin in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1 or 7 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$ and 7 days$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$ and 7 days$_d$) and each time point was run on separate gels. Data shown are means ± SEM. **, $P < 0.01$; N.S., not significant.

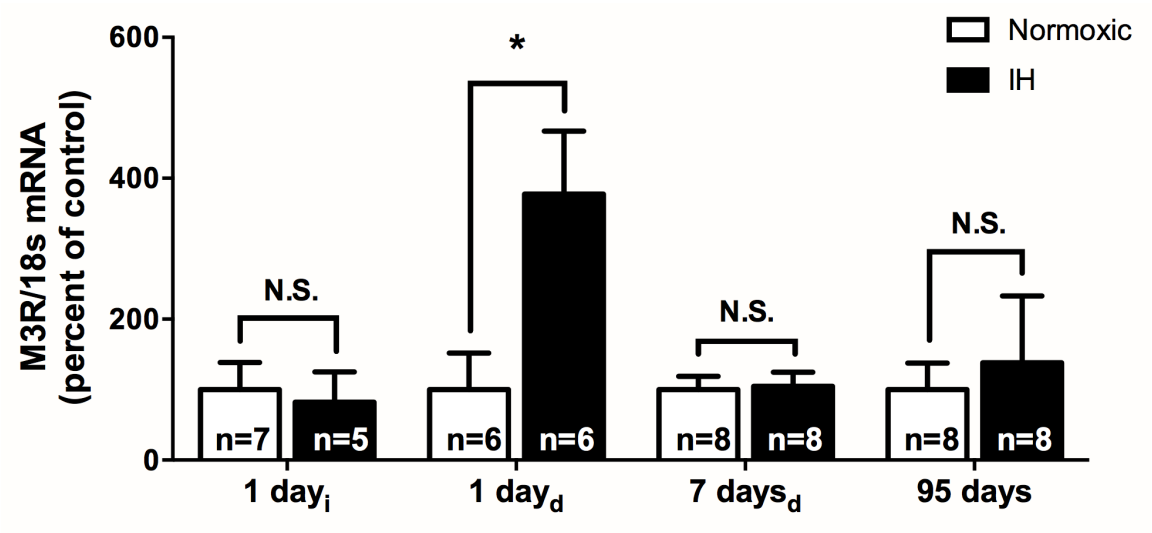

Figure 3.4 Effect of intermittent hypoxia on atrial M3 receptor mRNA expression

Bar graphs showing M3 muscarinic receptor (M3R) mRNA expression relative to 18S in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1, 7 or 95 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$, 7 days$_d$ and 95 days$_d$). Data shown are means ± SEM. *, $P < 0.05$; N.S., not significant.

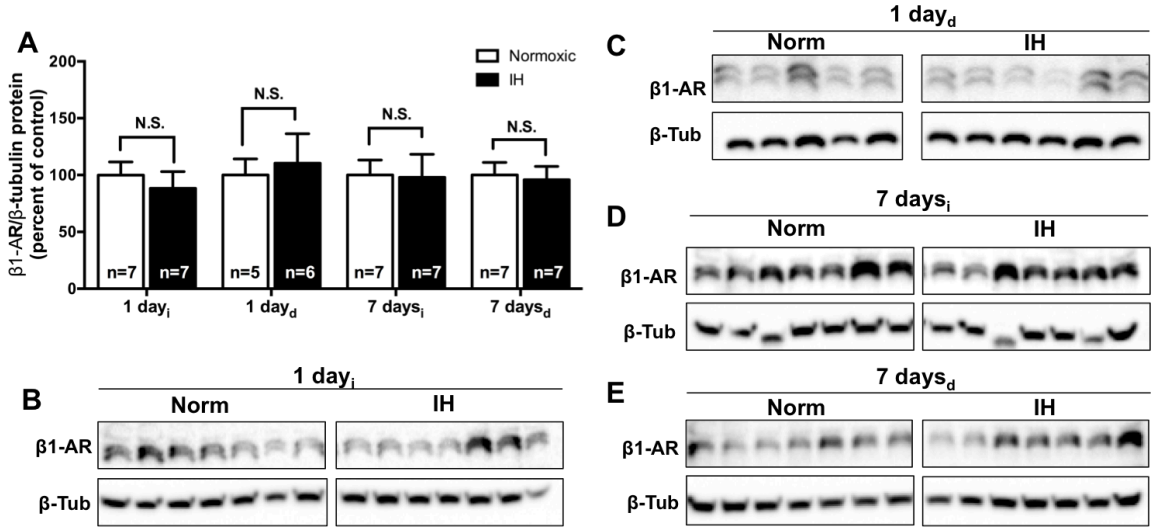

Figure 3.5 Effect of intermittent hypoxia on atrial β1-adrenergic receptor protein expression

Bar graphs (**A**) and representative blots (**B-E**) showing β1-adrenergic receptor (β1-AR) protein expression as a ratio of β-tubulin in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1 or 7 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$ and 7 days$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$ and 7 days$_d$), and each time point was run on separate gels. Data shown are means ± SEM. N.S., not significant.

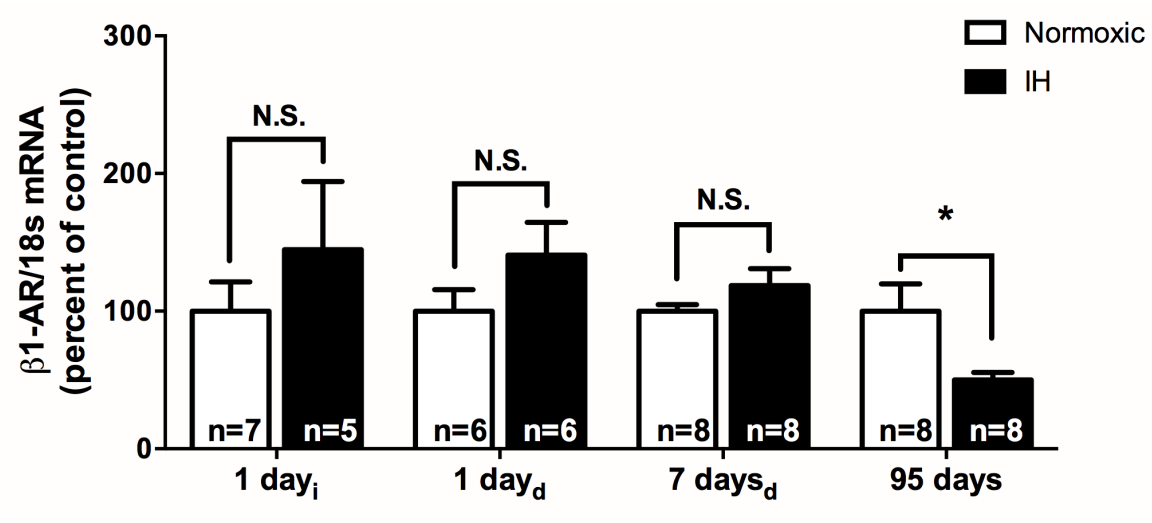

Figure 3.6 Effect of intermittent hypoxia on atrial β1-adrenergic receptor mRNA expression

Bar graphs showing β1-adrenergic receptor (β1-AR) mRNA expression as a ratio of 18S in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1, 7 or 95 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$, 7 days$_d$ and 95 days$_d$). Data shown are means ± SEM. *, $P < 0.05$; N.S., not significant.

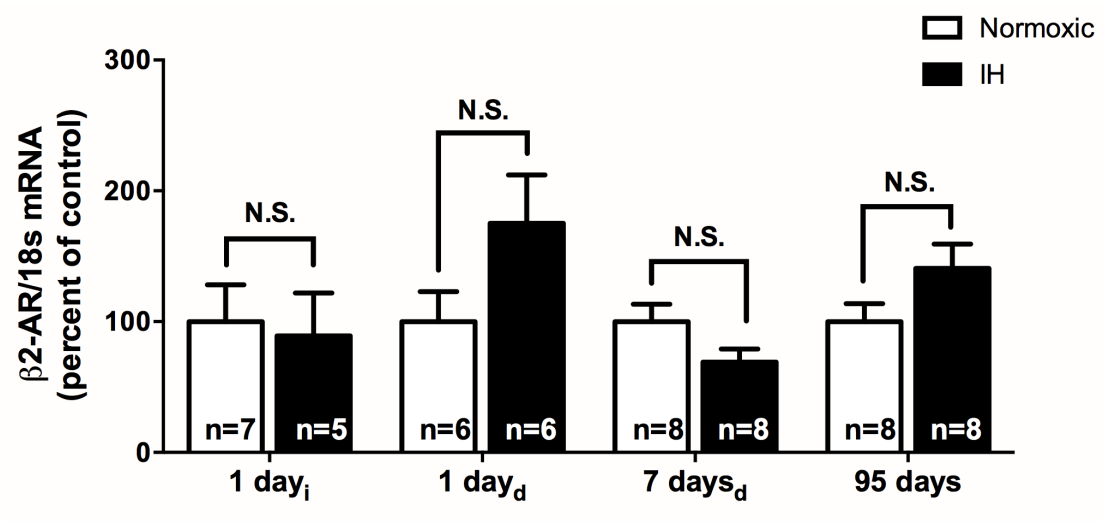

Figure 3.7 Effect of intermittent hypoxia on atrial β2-adrenergic receptor mRNA expression

Bar graphs showing β2-adrenergic receptor (β2-AR) mRNA expression as a ratio of 18S in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1, 7 or 95 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$, 7 days$_d$ and 95 days$_d$). Data shown are means ± SEM. N.S., not significant.

3.3 Expression of connexins

Figure 3.8 shows that protein expression of Cx 43 was significantly lower the next day in the atria of rats exposed to IH for 7 days compared to normoxic controls ($P < 0.05$). This is likely at least partially due to a reduction in gene transcription, since lowered Cx 43 protein was accompanied by similar reductions in Cx 43 mRNA content, as shown in Figure 3.9 ($P < 0.05$). With more prolonged exposures for 95 days, IH rats had significantly lower Cx 43 mRNA compared to normoxic controls (Figure 3.9; $P < 0.05$). IH exposure for 1 day and sacrificed immediately was paradoxically associated with transiently higher Cx 43 protein (Figure 3.8, $P < 0.05$), with no change in mRNA (Figure 3.9). This suggests that elevated Cx 43 protein content immediately following 1 day of IH was not due to increased gene expression; rather, reduced protein degradation and/or an increase in translation may be responsible. The opposing changes in Cx 43 protein at 1 versus 7 days indicates that the effects of IH in the atria may be biphasic, as has been demonstrated for numerous proteins in cardiac tissues exposed to IH (for review, see Yin et al., 2012). Levels of Cx 40 protein (Figure 3.10) and mRNA (Figure 3.11) were not significantly different in IH or normoxia exposed animals at any of the time points investigated.

3.4 Electrophysiological characteristics

After 7 days of exposure, P-wave duration, PQ interval and QT interval were not significantly different in IH rats compared to normoxic controls (Table 3.1). QRS duration could not be measured because QRS complexes were often “buried” within the T waves on the ECGs of rapidly beating rat hearts. However, the QT interval can still provide an index for ventricular conduction, since the QT represents the duration

of ventricular depolarization and repolarization. IH caused a trend to increase heart rate in IH rats (248±6 BPM versus 262±6 BPM) compared to normoxic controls was noted but this difference was not statistically significant (P = 0.14).

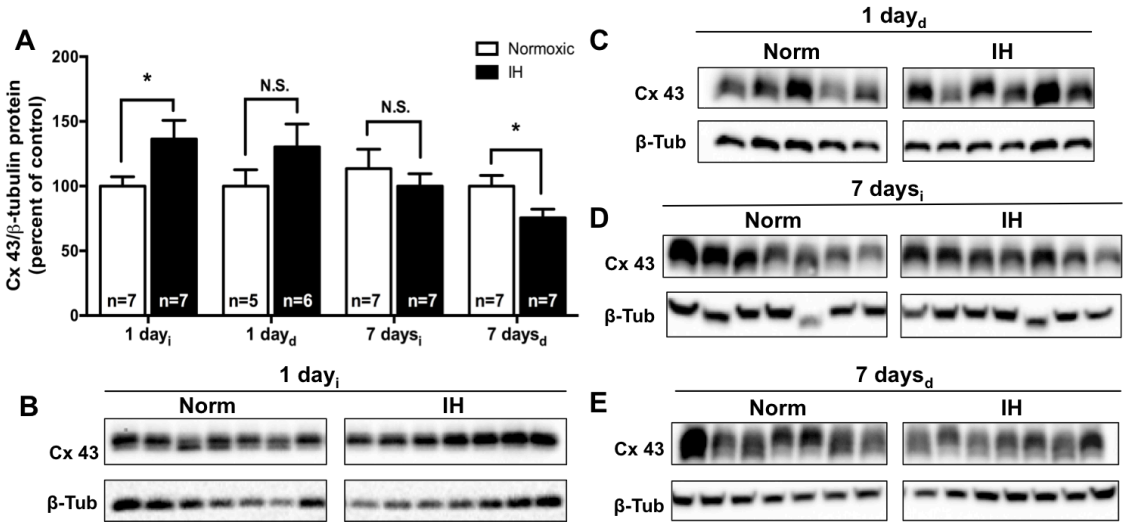

Figure 3.8 Effect of intermittent hypoxia on atrial connexin 43 protein expression

Bar graphs (**A**) and representative blots (**B-E**) showing Connexin 43 (Cx 43) protein expression as a ratio of β-tubulin in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1 or 7 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$ and 7 days$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$ and 7 days$_d$), and each time point was run on separate gels. Data shown are means ± SEM. *, $P < 0.05$, N.S., not significant.

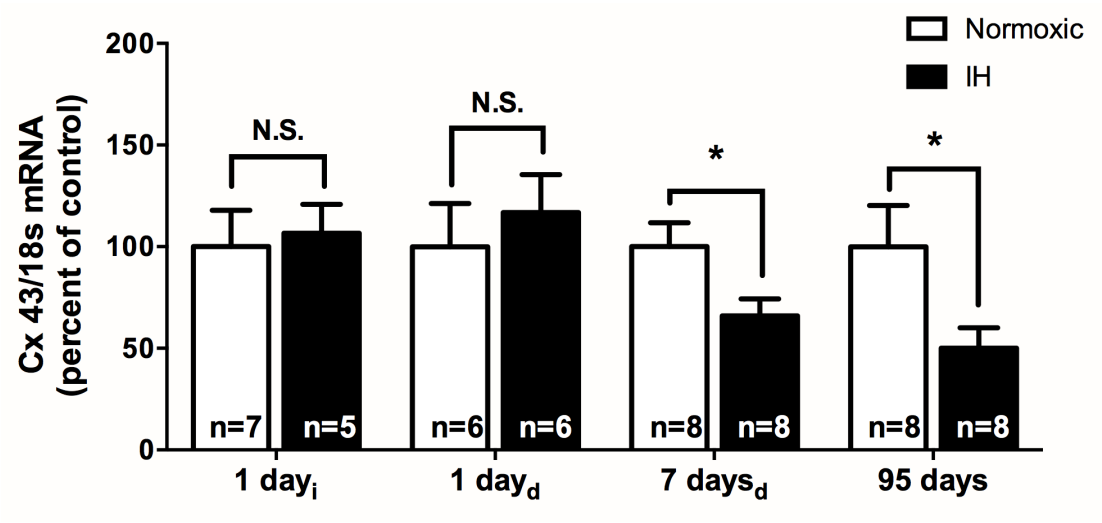

Figure 3.9 Effect of intermittent hypoxia on atrial connexin 43 mRNA expression

Bar graphs showing Connexin 43 (Cx 43) mRNA expression as a ratio of 18S in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1, 7 or 95 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$, 7 days$_d$ and 95 days$_d$). Data shown are means ± SEM. *, $P < 0.05$, N.S., not significant.

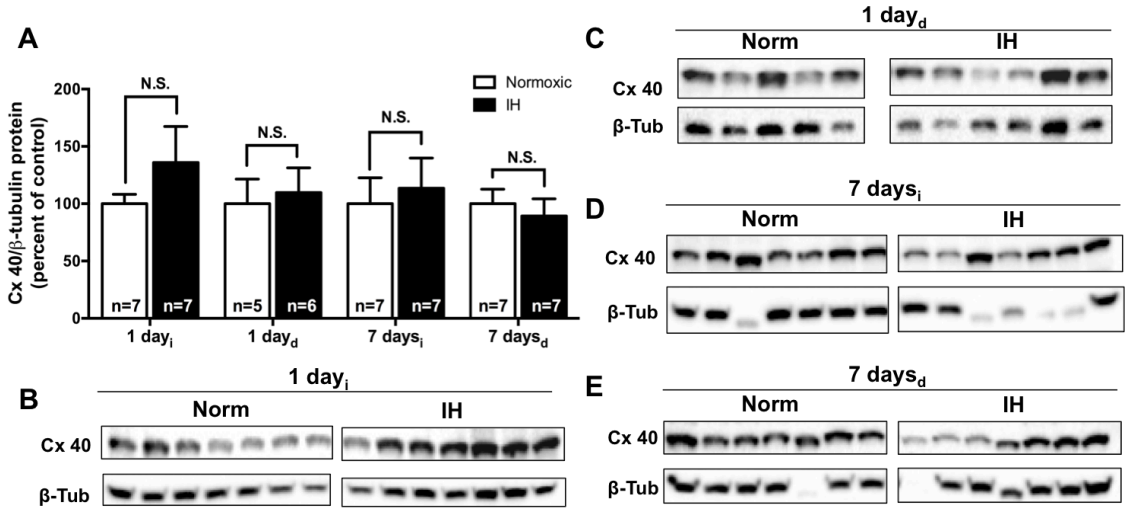

Figure 3.10 Effect of intermittent hypoxia on atrial connexin 40 protein expression

Bar graphs (**A**) and representative blots (**B-E**) showing Connexin 40 (Cx 40) protein expression as a ratio of β-tubulin in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1 or 7 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$ and 7 days$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$ and 7 days$_d$), and each time point was run on separate days. Data shown are means ± SEM. N.S., not significant.

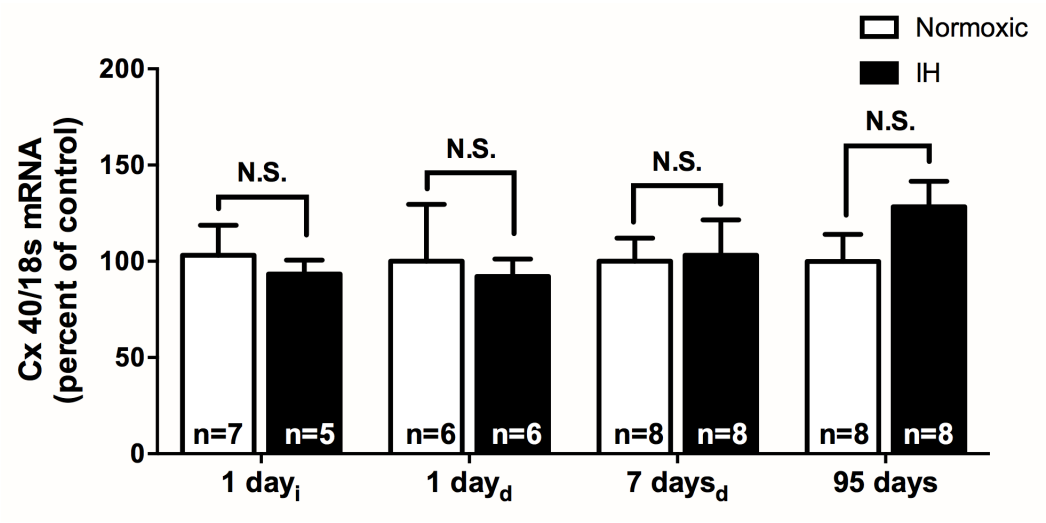

Figure 3.11 Effect of intermittent hypoxia on atrial connexin 40 mRNA expression

Bar graphs showing Connexin 40 (Cx 40) expression as a ratio of 18S in the atria of adult male rats exposed to intermittent hypoxia or normoxic conditions for 8 hours per day for 1, 7 or 95 days. Rats were sacrificed immediately after IH or normoxia exposure (1 day$_i$) or the day after exposure, following a 16 hour delay (1 day$_d$, 7 days$_d$ and 95 days$_d$). Data shown are means ± SEM. N.S., not significant.

Table 3.1: Electrophysiological characteristics measured from the surface lead electrograms recorded from rats exposed to 7 days of IH or normoxia.

	Normoxia		**Intermittent hypoxia**	
	Value (ms)	*n*	Value (ms)	*n*
P wave (ms)	19 ± 0	24	20 ± 0	23
PQ interval (ms)	49 ± 1	24	49 ± 1	23
QT interval (ms)	94 ± 1	24	96 ± 1	23
HR (BPM)	248 ± 6	24	262 ± 6	24

Values are means ± SEM; n, number of rats. BPM, beats per minute.

3.5 Atrial effective refractory periods

In both normoxia and IH-exposed rats, AERPs were spatially heterogeneous, with longer AERPs occurring in the high-right atrium (HRA) compared with the mid-right atrium (MRA) at basic drive train cycle lengths of 100 ms ($P < 0.01$; Figure 3.12A) and 150 ms ($P < 0.05$; Figure 3.12B). This finding is consistent with results of previous studies in which right atrial PES was performed in mice (Tuomi et al., 2010; Tuomi et al., 2011). Right atrial ERPs measured in IH-exposed rats at baseline were not significantly different from those of normoxic controls (Figure 3.12).

3.6 Effects of autonomic receptor drugs on AERPs

All measurements of AERP in IH- or normoxia-exposed rats in the presence and absence of carbachol, atropine, darifenacin, isoproterenol and propranolol are summarized in Table 3.2. Treatment with the nonselective muscarinic receptor agonist carbachol shortened AERP by 18-20% in normoxia-exposed rats and by 29-31% in rats exposed to IH (Table 3.2). With carbachol administration, IH rats had a lower average AERP than normoxic controls at both atrial sites and drive cycle lengths, but these differences were not statistically significant (Figure 3.13). Two-way ANOVA revealed a significant interaction between drug (carbachol) and treatment (IH versus normoxia), suggesting that rats exposed to IH may have enhanced cholinergic sensitivity. In contrast, the nonselective muscarinic receptor antagonist atropine caused similar increases in AERPs in all animals (Figure 3.14; Table 3.2). Following carbachol administration, AERP was measured again in the absence and presence of the selective M3 receptor antagonist, darifenacin, to

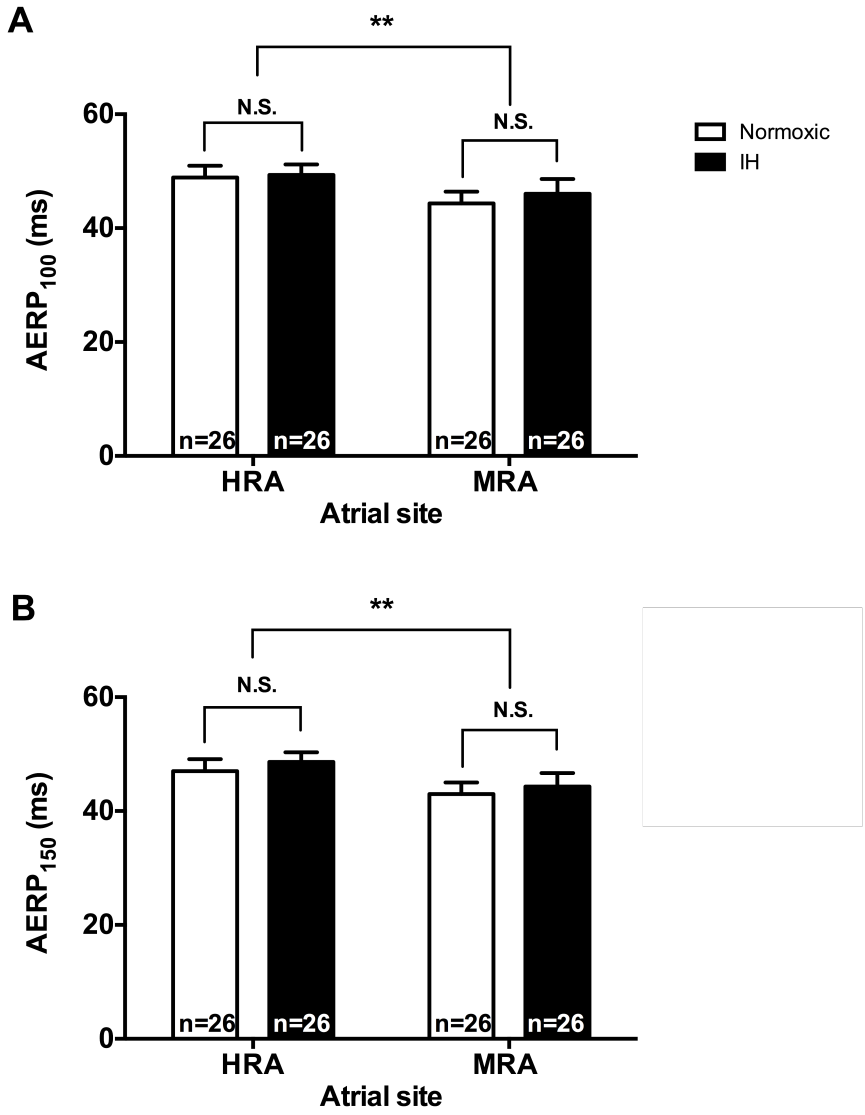

Figure 3.12 Effect of intermittent hypoxia on atrial effective refractory period

Atrial effective refractory period (AERP) measured using programmed electrical stimulation (PES) with (**A**) 100 ms and (**B**) 150 ms drive trains in rats exposed to IH or normoxic conditions for 7 days. AERP was measured in both the high-right atrium (HRA) and mid-right atrium (MRA). Values represent means ± SEM. **, $P < 0.01$; N.S, not significant.

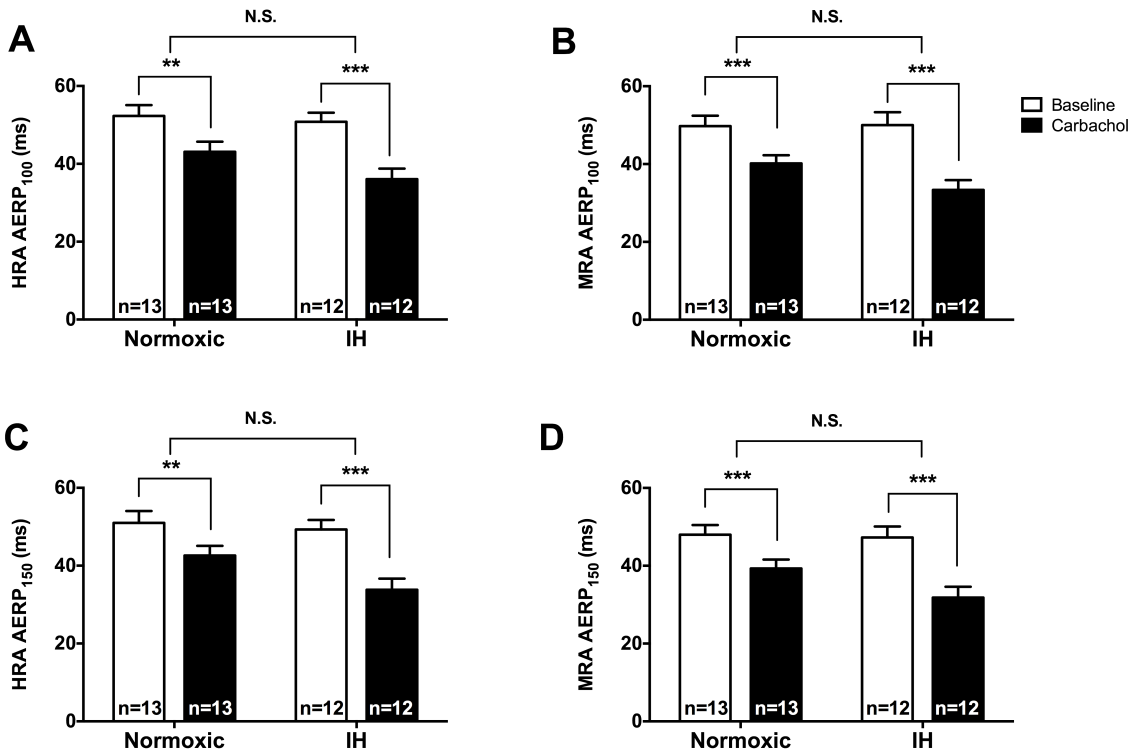

Figure 3.13 Effect of intermittent hypoxia on atrial effective refractory period in the presence and absence of carbachol

Atrial effective refractory period (AERP) measured using programmed electrical stimulation (PES) with (**A**, **B**) 100 ms ($AERP_{100}$) and (**C**, **D**) 150 ms ($AERP_{150}$) drive trains in rats exposed to IH or normoxic conditions for 7 days. AERP was measured in both the high-right atrium (HRA; **A**, **C**) and mid-right atrium (MRA; **B**, **D**) in the absence (baseline) and presence of carbachol. Two-way ANOVA and Bonferroni's multiple comparisons test revealed that carbachol significantly reduced AERP in all rats and that there was a significant interaction between drug and treatment on MRA ERP_{100} (**B**) and MRA ERP_{150} (**D**). Values represent means ± SEM. **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant.

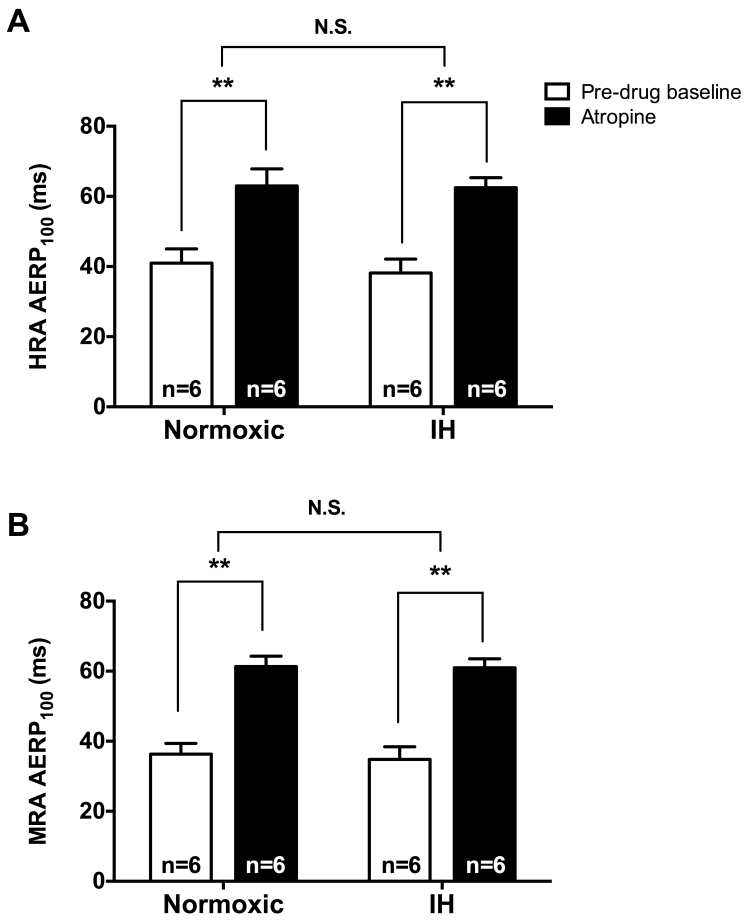

Figure 3.14 Effect of intermittent hypoxia on atrial effective refractory period in the presence and absence of atropine

Atrial effective refractory period (AERP) measured using programmed electrical stimulation (PES) with a 100 ms drive train ($AERP_{100}$) in rats exposed to IH or normoxic conditions for 7 days. AERP was measured in both the high-right atrium (HRA; **A**) and mid-right atrium (MRA; **B**) in the absence (pre-drug baseline) and presence of atropine. Pre-drug baseline values represent AERPs measured immediately before atropine injection, after a 60-minute washout period for carbachol. Atropine prolonged ERP at both atrial sites in all rats. Values represent means ± SEM. **, $P < 0.01$; N.S., not significant.

Table 3.2: Right atrial effective refractory periods (in ms) rats exposed to 7 days of IH or normoxia measured in the presence and absence of carbachol, atropine, darifenacin, isoproterenol and propranolol.

	Baseline				Carbachol				Atropine				Darifenacin			
	Norm		IH		Norm		IH		Norm		IH		Norm		IH	
	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*
HRA ERP_{100}	49±2	26	49±2	26	43±3	13	35±3	12	63±5	6	63±3	6	60±6	5	48±5	6
MRA ERP_{100}	45±2	27	46±3	26	40±2	13	32±3	12 ‡	61±3	6	61±3	6	56±5	5	48±4	6
HRA ERP_{150}	47±2	26	49±2	26	42±3	13	34±3	12					58±6	5	48±4	6
MRA ERP_{150}	44±2	27	44±2	26	39±2	13	31±3	12 ‡					56±5	5	48±4	6

	Baseline				Isoproterenol				Propranolol			
	Norm		IH		Norm		IH		Norm		IH	
	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*	Value (ms)	*n*
HRA ERP_{100}	49±2	26	49±2	26	34±2	8	41±1	7 ‡	40±2	9	51±4	9 †
MRA ERP_{100}	45±2	27	46±3	26	33±2	8	35±3	7 ‡	40±3	9	49±4	9 *
HRA ERP_{150}	47±2	26	49±2	26					35±1	6	45±2	7
MRA ERP_{150}	44±2	27	44±2	26					34±1	6	44±3	6

Values are means ± SEM; n, number of rats. HRA ERP, high-right atrial effective refractory period; MRA ERP, mid-right atrial effective refractory period. The subscripts 100 and 150 indicate basic drive cycle lengths of 100 ms and 150 ms, respectively. *, $P < 0.05$; †, $P < 0.01$ for intermittent hypoxia-exposed rats compared to normoxic controls within each drug group. ‡, significant interaction effect ($P < 0.05$) between drug and treatment.

determine the role of the M3 receptor in modulating AERP in IH and normoxia-exposed rats. We found that darifenacin caused similar increases in AERP in both groups (AERP increased by about 14 ms in IH-exposed rats and by 11 ms in normoxic control rats; Figure 3.15), suggesting similar contributions of the M3 receptor to AERP in the two groups. The nonselective β-adrenergic receptor agonist isoproterenol reduced AERP of the MRA from 41 ± 3 to 33 ± 2 in normoxic animals and from 41 ± 4 to 35 ± 3 in IH-exposed rats (Figure 3.16). Two-way ANOVA revealed a significant interaction between drug (isoproterenol) and treatment, suggesting that IH-exposed animals may be less sensitive to isoproterenol (Figure 3.16; Table 3.2). Figure 3.17 shows that treatment with the nonselective β-adrenergic receptor antagonist propranolol resulted in significantly longer AERPs in animals exposed to IH compared to normoxic control rats in both high ($P < 0.01$) and mid-right atrial sites ($P < 0.05$). Since AERPs were not significantly different prior to propranolol administration, this suggests that the effects of propranolol were greater in IH-exposed rats, possibly as a result of increased basal adrenergic activation.

3.7 Atrial fibrillation inducibility and effects of autonomic receptor drugs

Atrial burst pacing (Figure 3.18A) and PES (Figure 3.19) induced atrial arrhythmias with features that define AF: rapid, chaotic atrial activation patterns and irregularly irregular ventricular responses. All atrial arrhythmias with these characteristics were labelled as AF in this study, although a true diagnosis of AF requires high density mapping techniques and recording from both left and right atria (Tuomi et al., 2010). In normoxia-exposed control animals, AF was not inducible with PES (0 of 27, 0%). Burst pacing is a more provocative stimulus for arrhythmia

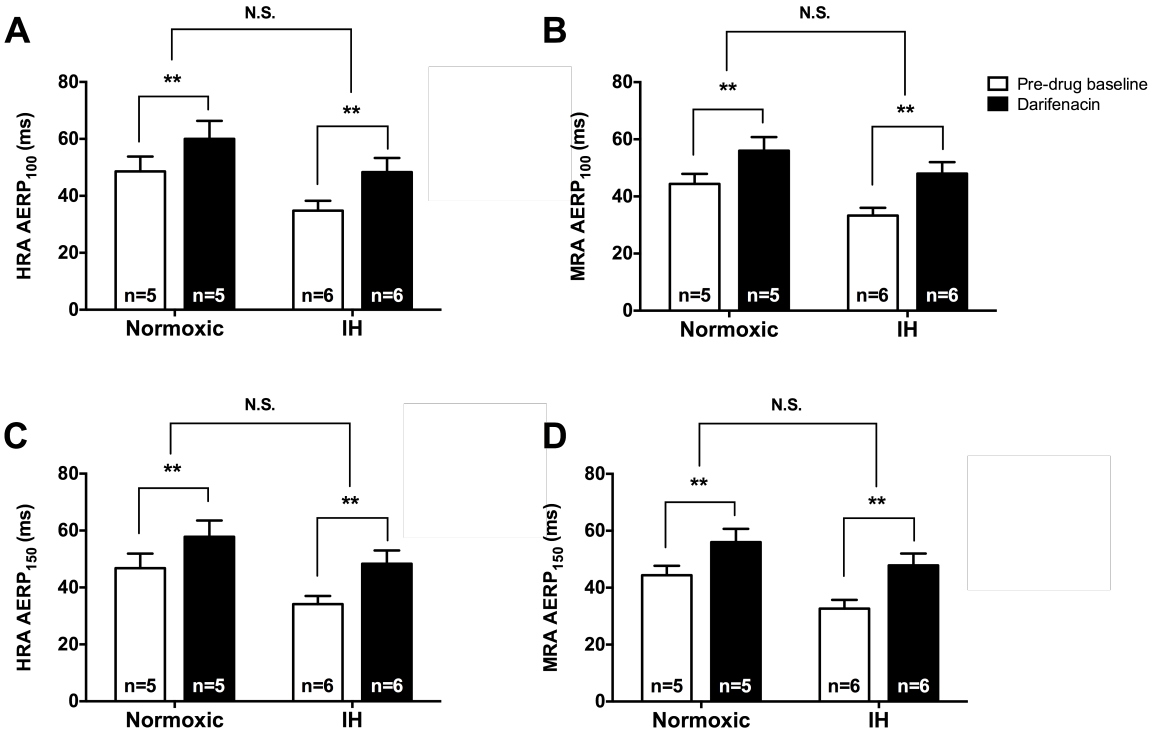

Figure 3.15 Effect of intermittent hypoxia on atrial effective refractory period in the presence and absence of darifenacin

Atrial effective refractory period (AERP) measured using programmed electrical stimulation (PES) with (**A**, **B**) 100 ms ($AERP_{100}$) and (**C**, **D**) 150 ms ($AERP_{150}$) drive trains in rats exposed to IH or normoxic conditions for 7 days. AERP was measured in both the high-right atrium (HRA; **A**, **C**) and mid-right atrium (MRA; **B**, **D**) in the absence (pre-drug baseline) and presence of darifenacin. Pre-drug baseline AERPs were measured immediately before darifenacin injection, following a 60-minute washout period for carbachol. Two-way ANOVA and Bonferroni's multiple comparisons test revealed that darifenacin prolonged ERP at both atrial sites in all rats. Values represent means ± SEM. **, $P < 0.01$; N.S., not significant.

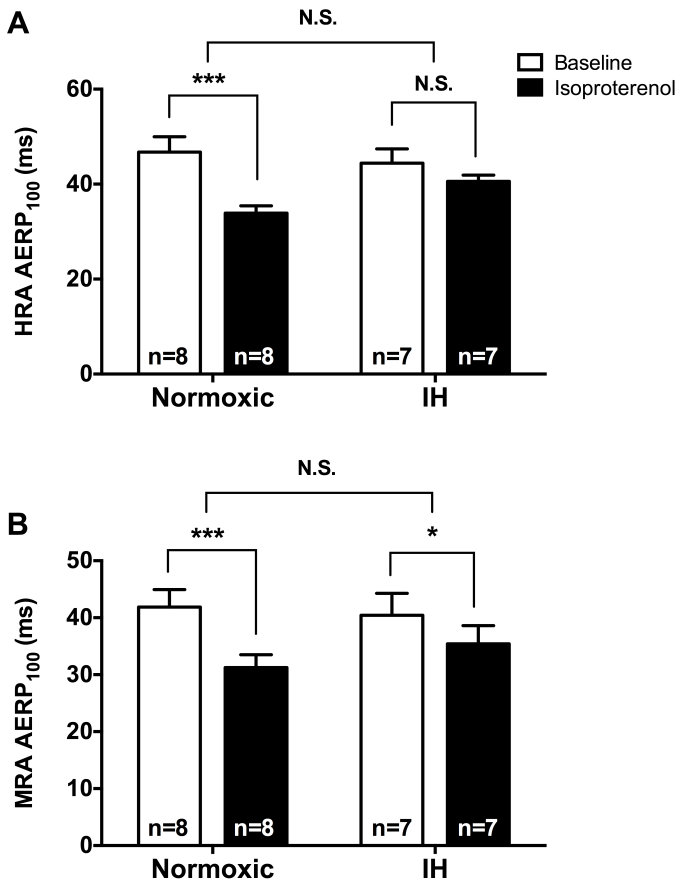

Figure 3.16 Effect of intermittent hypoxia on atrial effective refractory period in the presence and absence of isoproterenol

Atrial effective refractory period (AERP) measured in the high-right atrium (HRA; **A**) and mid-right atrium (MRA; **B**) in the absence (baseline) and presence of isoproterenol using programmed electrical stimulation (PES) with a 100 ms drive train ($AERP_{100}$) in rats exposed to IH or normoxic conditions for 7 days. Two-way ANOVA and Bonferroni's multiple comparisons test revealed that isoproterenol shortened ERP of the HRA (**A**) in normoxic, but not in IH rats, and there was a significant drug and treatment interaction in both high (**A**) and mid (**B**) right atrial sites. Values represent means ± SEM. *, $P < 0.05$; ***, $P < 0.001$; N.S., not significant.

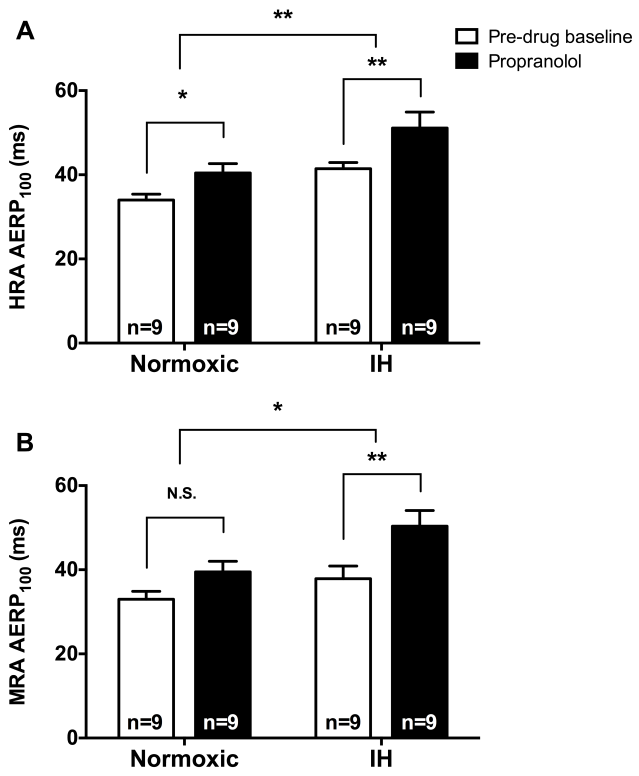

Figure 3.17 Effect of intermittent hypoxia on atrial effective refractory period in the presence and absence of propranolol

Atrial effective refractory period (AERP) measured using programmed electrical stimulation (PES) with a 100 ms drive train ($AERP_{100}$) in rats exposed to IH or normoxic conditions for 7 days. AERP was measured in the high-right atrium (HRA; **A**) and mid-right atrium (MRA; **B**) in the absence (pre-drug baseline) and presence of propranolol. Pre-drug baseline values represent the AERP measured immediately before propranolol administration, following a 40-minute washout period for isoproterenol. Propranolol prolonged ERP of the MRA in IH, but not in normoxic rats (**B**). Values represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$; N.S., not significant.

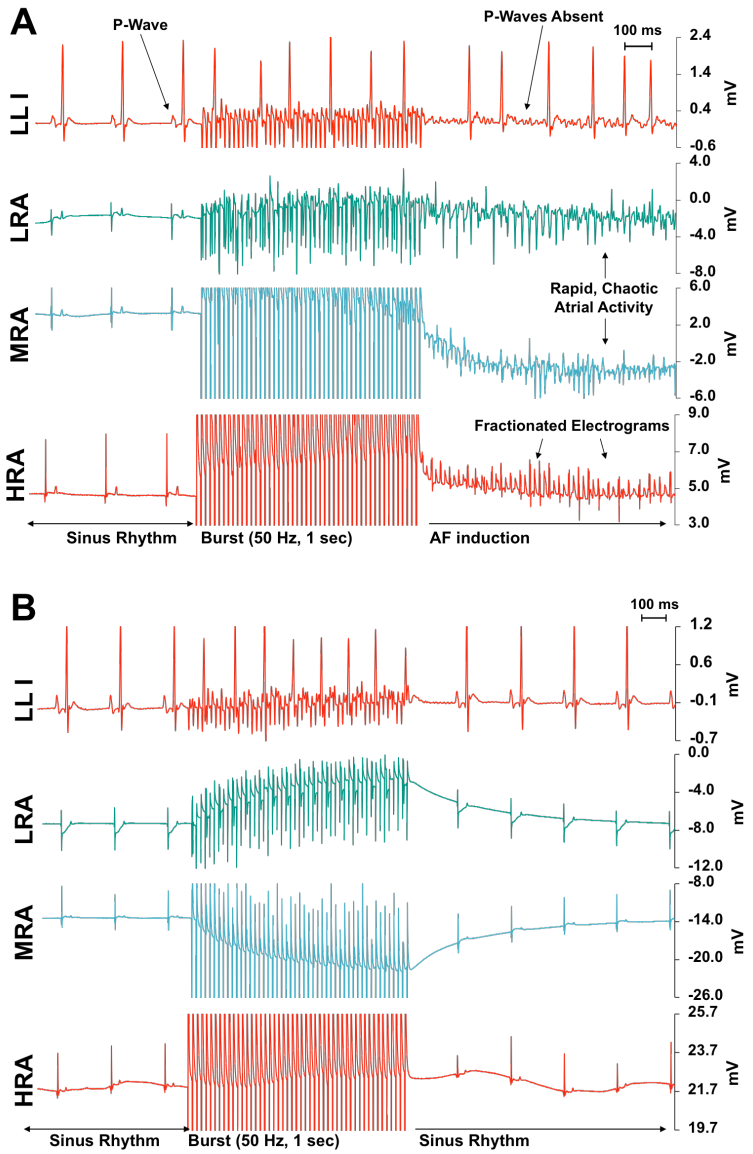

Figure 3.18 AF induction with atrial burst pacing

(**A**) Characteristic burst-induced atrial fibrillation (AF) in a rat exposed to 7 days of IH. Note the absence of P-waves and the irregular QRS responses on limb lead 1 (LL1), and the rapid, irregular atrial activation patterns and fractionation on the intra-atrial electrograms (LRA, low-right atrium; MRA, mid-right atrium; HRA, high-right atrium). (**B**) For comparison, a representative recording of failure to induce AF with burst pacing in a 7 day normoxia-exposed rat.

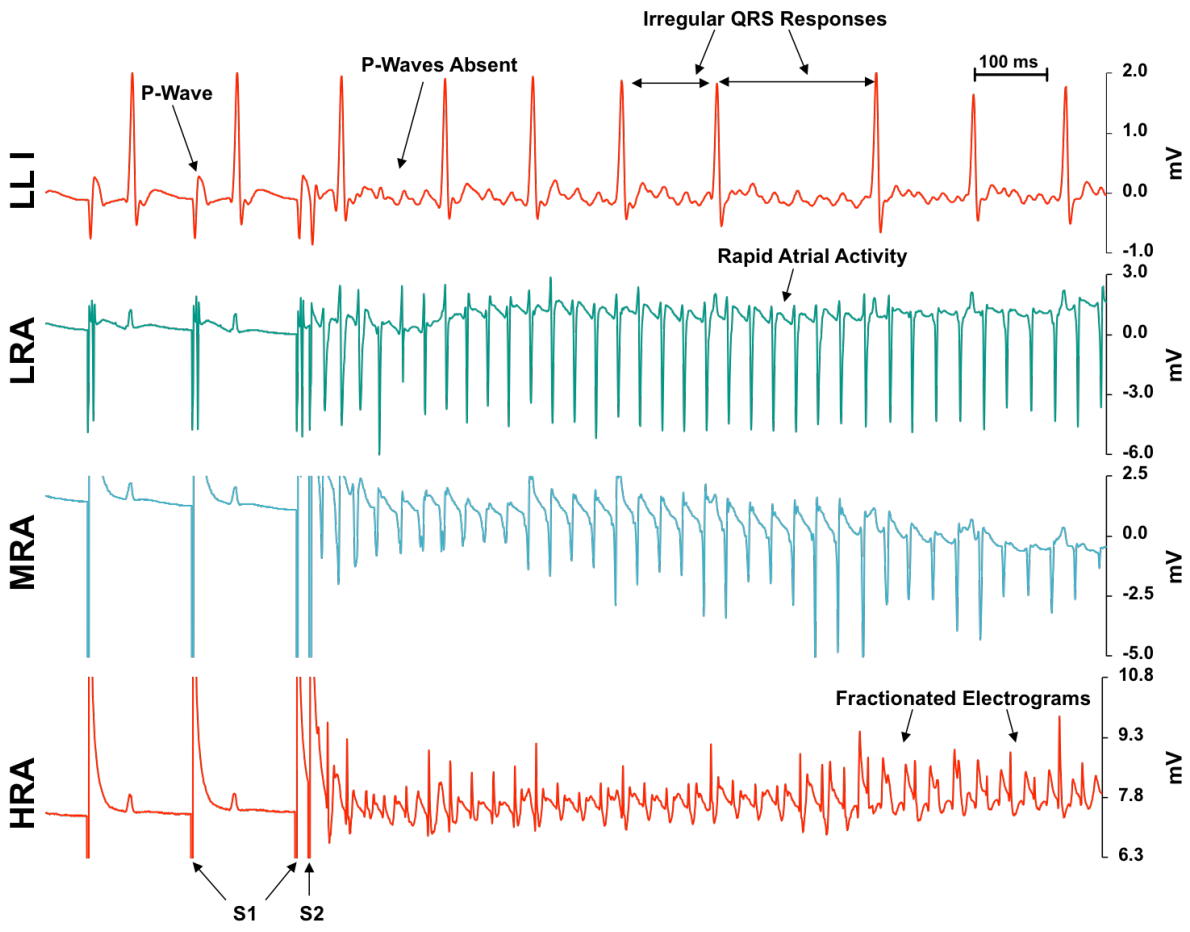

Figure 3.19 Characteristic recording of AF induced by PES

Characteristic recording of atrial fibrillation (AF) induced by programmed electrical stimulation (PES) with a single extrastimulus (S2) in a rat exposed to 7 days of IH. Note the rapid, irregular atrial activation patterns on the intra-atrial electrograms (LRA, low-right atrium; MRA, mid-right atrium; HRA, high-right atrium), in particular the different tachycardia rates in HRA compared to MRA and LRA. Irregularly irregular ventricular responses and the absence of P-waves can be seen on limb lead 1 (LL 1). Susceptibility to PES-induced AF indicates that IH-exposed rats have a highly vulnerable atrial substrate. Normoxia exposed rats were completely insensitive to arrhythmia induction with PES.

induction, but induced AF in only 4 of 27 (15%) normoxia-exposed rats. In contrast, IH-exposed rats were quite sensitive to AF induction with burst pacing (14 of 27, 52%) and were moderately susceptible to AF induction with PES (7 of 27, 26%). Chi-squared analysis revealed that IH-exposed rats were more sensitive to AF induction with both PES ($P < 0.01$; Figure 3.20) and burst pacing ($P < 0.01$; Figure 3.20) compared to normoxic controls. All rats that were susceptible to AF induction had short-lived, nonsustained AF (<10 seconds), with the exception of two rats in the IH group that were highly susceptible to PES-induced AF. One rat consistently had self-terminating AF episodes lasting approximately 3 minutes on average; the other remained in sustained AF for over an hour until exogenous termination was attempted with injection of atropine (1 mg/ml, i.p.).

Previous studies have used carbachol to enhance AF inducibility and duration (Tuomi et al., 2011; Tuomi et al., 2010) but surprisingly, carbachol did not significantly affect AF susceptibility in normoxia-exposed rats with either PES (0/13 without versus 0/13 with carbachol; Figure 3.21A) or burst pacing (1/13 without versus 2/13 with carbachol; Figure 3.21A). On the other hand, in IH-exposed rats carbachol significantly increased susceptibility to burst-induced AF (from 5/13 at baseline to 10/13 with carbachol, $P < 0.05$; Figure 3.21A). Carbachol increased the number of IH-exposed rats that were susceptible to AF induced by PES (2/13 without carbachol versus 6/13 with carbachol, $P = 0.089$; Figure 3.21A) but this difference was not statistically significant. Muscarinic receptor blockade with atropine completely prevented AF inducibility in IH-exposed rats with both PES ($P = 0.055$; Figure 3.21B) and burst pacing ($P < 0.001$; Figure 3.21B). Darifenacin also prevented AF inducibility in all IH rats with both pacing modalities (Figure 3.21C), but

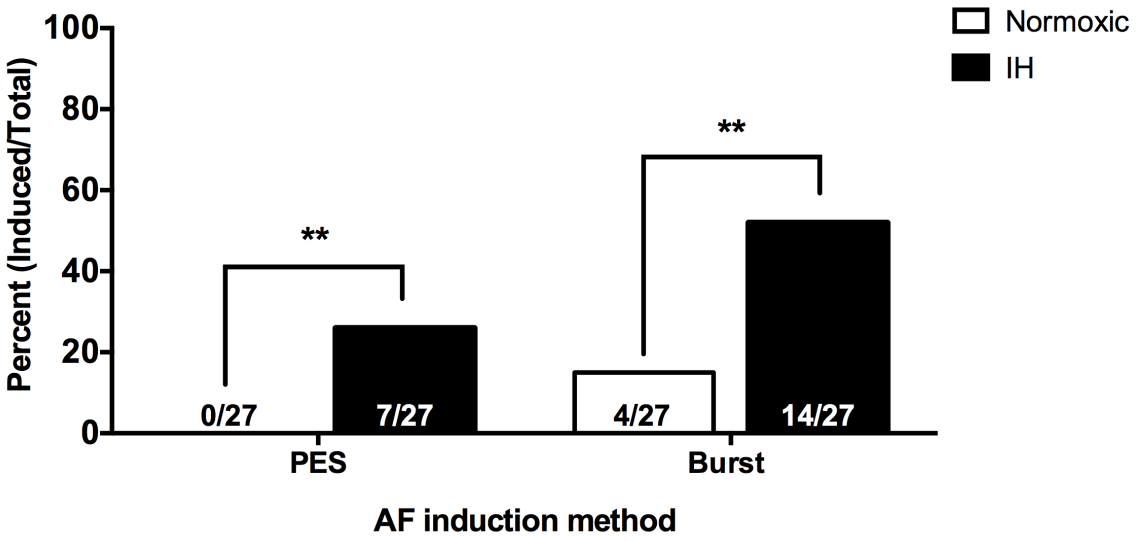

Figure 3.20 Effect of intermittent hypoxia on the inducibility of AF using different methods of stimulation

Incidence of AF induced with programmed electrical stimulation (PES) or burst bacing (burst) in rats exposed to IH or normoxic conditions for 7 days. IH increased susceptibility to AF induction with both pacing modalities compared to normoxic controls. **, $P < 0.01$.

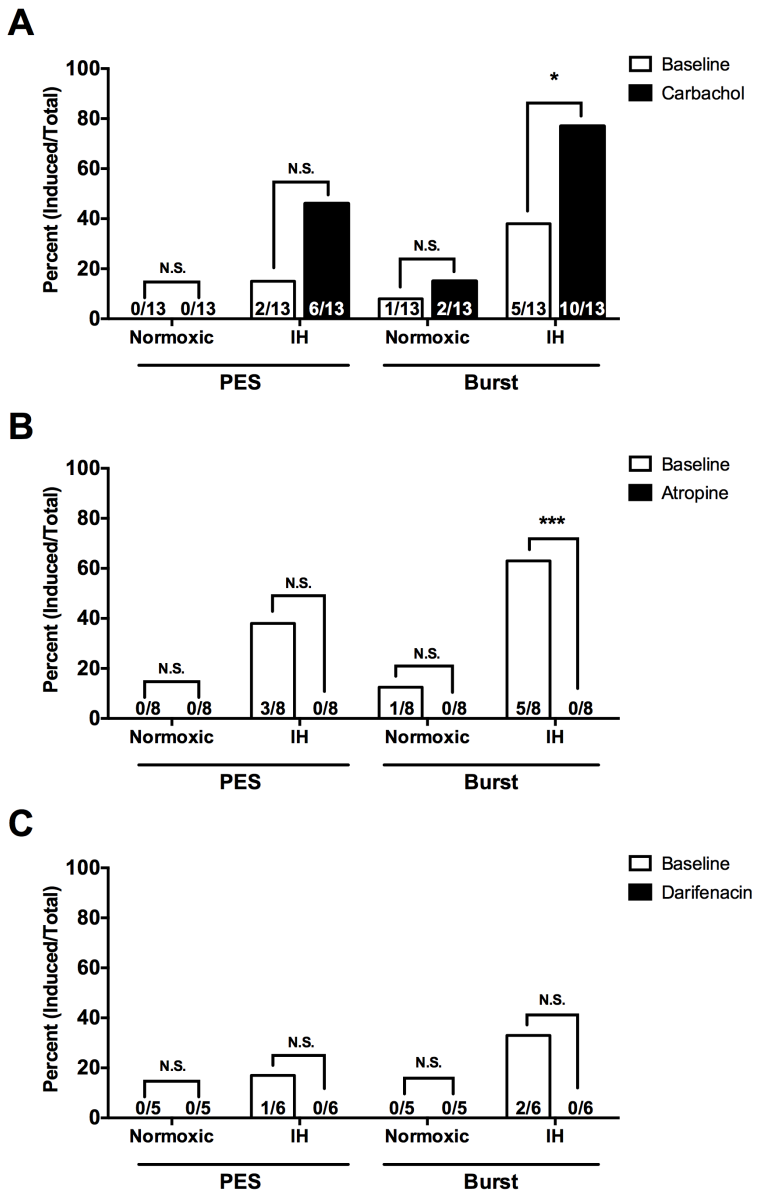

Figure 3.21 Effect of muscarinic receptor drugs on AF inducibility

Incidence of AF induced with programmed electrical stimulation (PES) or burst bacing (burst) in rats exposed to IH or normoxic conditions for 7 days in the presence and absence of carbachol (**A**), atropine (**B**) or darifenacin (**C**). In IH-exposed rats with burst pacing, carbachol enhanced susceptibility to AF induction and atropine completely prevented inducibility of AF. *, $P < 0.05$; ***, $P < 0.001$; N.S., not significant.

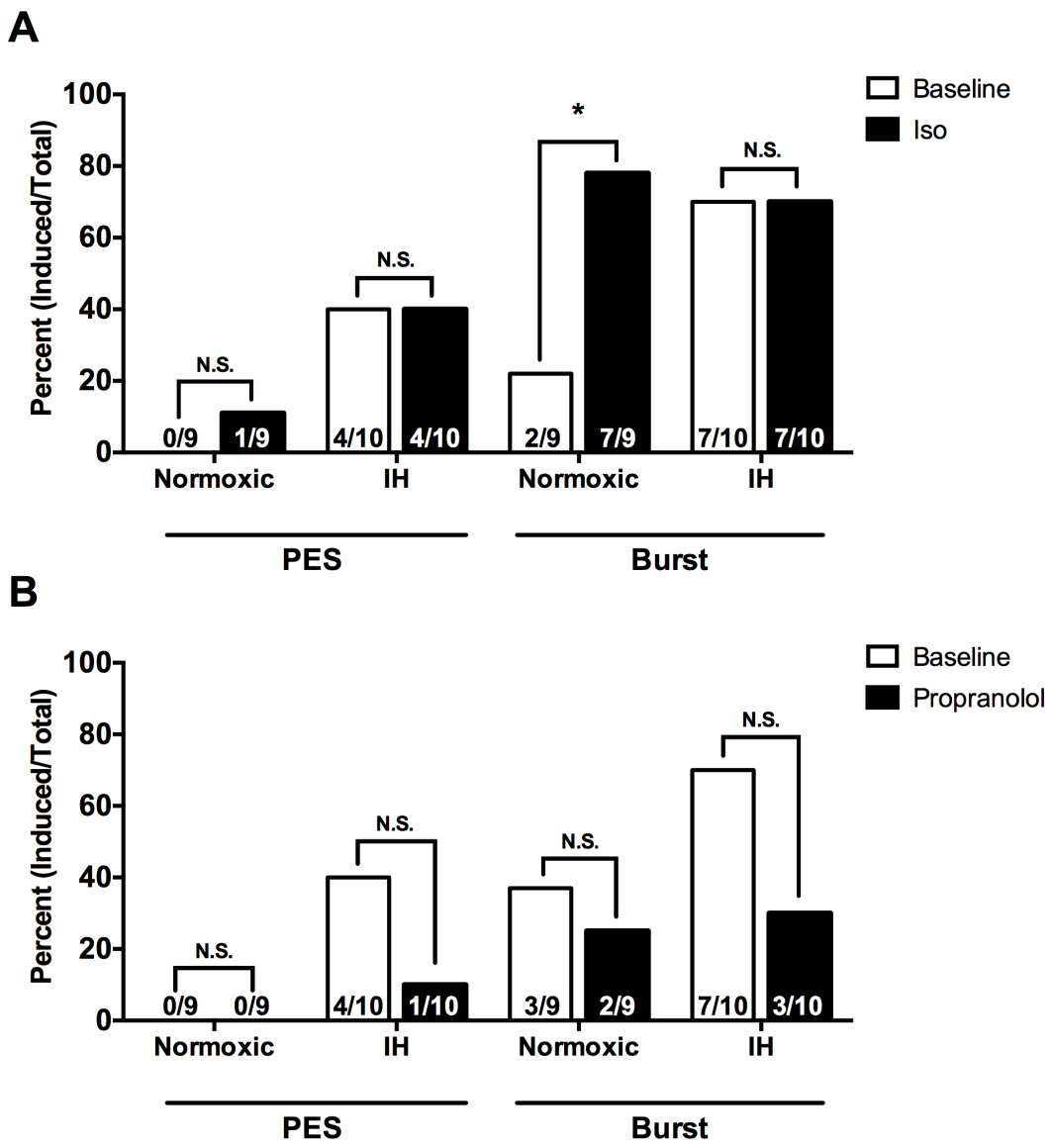

Figure 3.22 Effect of adrenergic drugs on AF inducibility

Incidence of AF induced with programmed electrical stimulation (PES) or burst bacing (burst) in rats exposed to IH or normoxic conditions for 7 days in the presence and absence of isoproterenol (**A**) or propranolol (**B**). Isoproterenol significantly increased susceptibility to AF induction with burst pacing in normoxic rats but had no effect on AF susceptibility in IH-exposed rats with either pacing method. *, $P < 0.05$; N.S., not significant.

these differences were not statistically significant. Interestingly, isoproterenol significantly increased susceptibility to AF induction with burst pacing in normoxic rats ($P < 0.05$; Figure 3.22A) but had no effect on AF susceptibility in IH-exposed rats with either pacing method (Figure 3.22A). In IH-exposed rats, β-adrenergic receptor blockade with propranolol reduced the incidence of AF induced by PES ($P = 0.121$) and burst pacing ($P = 0.074$; Figure 3.22B) but these differences were not stastically significant. Propranolol also prevented AF induction with burst pacing in one of the normoxia-exposed rats (reduced the incidence of AF from 3/9 to 2/9; $P = 0.510$; Figure 3.22B).

4.0 DISCUSSION

4.1 Overview

This study, to our knowledge, is the first to demonstrate AF promotion in an animal model of OSA, using IH alone to mimic obstructive apneas. This matches the enhanced AF susceptibility seen in OSA patients and, for the first time, demonstrates a role for IH in atrial arrhythmogenesis. We found substantially enhanced AF vulnerability in IH-exposed rats compared to control animals exposed to normoxic conditions using both PES and burst pacing to induce AF. Susceptibility to AF induction with PES indicates that IH rats have a highly vulnerable atrial substrate; on the other hand, control animals were insensitive to PES-induced AF. We investigated the underlying pathological determinants of the AF-promoting substrate and identified a number of atrial substrate changes that have not been reported previously in an IH model of OSA, including (1) lowered atrial Cx 43 content, (2) heightened cholinergic sensitivity with increased muscarinic receptor protein expression and (3) alterations in adrenergic function characterized by enhanced responses to propranolol and blunted responses to isoproterenol. Atropine completely prevented AF inducibility, and the sensitivity to carbachol-induced AF was enhanced, in IH-exposed rats, indicating that parasympathetics were critical for AF inducibility. Adrenergic activation also played a role, since propranolol prevented burst-induced arrhythmias in a third of IH-exposed rats that were inducible at baseline. These findings highlight a causal role for chronic IH in OSA-related AF susceptibility and in the formation of AF-promoting vulnerable substrates.

4.2 Considerations of the model

The first overarching aim of this thesis was to establish that an IH model of OSA had enhanced AF inducibility to facilitate investigation of the underlying vulnerable substrates. We used an IH regimen composed of repeated 80-second cycles of hypoxia alternated with 120-second cycles of normoxia, resulting in exposure of rats to 18 events per hour, thereby emulating a moderate form of OSA based on the AHI (AASM Task force, 1999). The IH stimulus was applied for 8 hours per day, during the diurnal sleep period of the rat, and based on behavioural assessment, our rats slept normally during this time, with the polyphasic pattern characteristic of rats (Simasko and Mukherjee, 2009). Specifically, rats appeared to be sleeping for most of the exposure duration, with the exception of microarousals typically observed in laboratory conditions (for example, 396 wake-ups on average in a 12 hour diurnal period; Clancy et al., 1978). In IH rats, respiration rate increased during phases of hypoxia, which was not due to hypercapnia since eucapnic conditions were maintained within the chambers. This observation is an indication that the fractional inspired O_2 within the chambers was producing intermittent hypoxemia, since hypoxia in the blood induces hyperventilation (Bisgard and Neubauer, 1995). Overall, this suggests that our results were due to IH.

The usefulness of our IH model of OSA can be understood from previously reported characteristics (Moreau and Ciriello, 2013, 2015) and an appreciation of the critical pathophysiological role of IH, enabled by over two decades of OSA research using numerous variants of the basic paradigm. Earlier studies have demonstrated that our IH model displays many features of human OSA, including significant

alterations in body energy balance (Moreau and Ciriello, 2013) as well as blood pressure elevation and reduced baroreflex gain with 95-day exposures, indicating substantial cardiovascular dysfunction (Moreau and Ciriello, 2015). Studies of laboratory rodents subjected to diurnal IH are the most frequently used OSA paradigm, even though rodents do not experience the intrathoracic pressure swings or hypercapnea (Fletcher et al., 1992a) that occur in OSA patients during apnea (for review, see Dempsey et al., 2010). The focus on IH is based on the hypothesis (Dematteis, 2009) that among the three pathophysiological components of OSA, IH is the most important in the development of cardiovascular complications. Indeed, IH by itself generates sympathetic hyperactivity (Gonzalez-Martin et al., 2009; Zoccal et al., 2008) and hypertension (Allahdadi et al., 2008; Zoccal et al., 2008; Zoccal et al., 2007) in experimental animals. Thus, although our model does not include all attributes of human OSA, our findings provide important lessons for understanding the human counterpart and address previously unanswered questions about the role of IH in atrial arrhythmogenesis.

4.3 Principal findings

Although IH has been used extensively to study numerous OSA-related comorbidities, this study is the first to examine the effects of an IH model on atrial arrhythmia susceptibility. We found that repeated bouts of IH, as occur due to OSA, are sufficient to significantly enhance AF susceptibility after just 7 days. Two different pacing protocols were used to identify AF susceptibility: PES with a single extrastimulus and atrial burst pacing. PES is thought to initiate AF as it occurs

physiologically, since AF episodes are often preceeded by premature atrial beats (Hoffman et al., 2006). On the other hand, atrial burst pacing more reliably induces AF by promoting cardiac electrical instability (Jones et al., 2008). Susceptibility to AF induced by PES was previously not reported in the rat, but in other small animals such as wild-type mice, AF is rarely induced by PES with a single extrastimulus (Tuomi et al., 2010). Thus, it was expected to be similarly non-inducing in the rat. Indeed, we found that AF could not be induced with PES in normoxia-exposed control rats (0%, 0/27), but IH exposure significantly increased AF inducibility with PES (26%, 7/27, $P < 0.01$). Susceptibility to AF induction with PES indicates that IH-exposed rats possess a highly vulnerable atrial substrate, demonstrating the profound effect and early latency of chronic IH during the sleep period, as it occurs in OSA of moderate severity.

Since PES is thought to more readily induce re-entry than triggered activity (Jalife et al., 2009), PES-induced AF susceptibility suggests a re-entrant mechanism underlying the enhanced atrial arrhythmias in IH rats. This is supported by the fact that AF was more readily induced in the MRA, where AERP was significantly shorter than in the HRA since myocardial regions with shorter refractory periods (Jalife et al., 2009) and enhanced spatial dispersion of refractoriness (Allessie et al., 1976) are more vulnerable to reentry. These observations are important because they are consistent with recent studies demonstrating significantly diseased reentry substrates in the atria of OSA patients (Dimitri et al., 2012). Compared to patients with AF only, those with both OSA and AF demonstrate site-specific conduction abnormalities, areas of low voltage and regions of electrical silence, suggesting underlying conduction dissociation, with no difference in refractory period at rest

(Dimitri et al., 2012). In this study, highly variable activation patterns were recorded simultaneously from high and mid-right atrial sites during AF, indicating areas of conduction block and underlying structural and/or electrical heterogeneities, consistent with OSA patients. This observation also provides further support for reentry, since activation heterogeneities and areas of conduction block are key determinants underlying the initiation of reentry in the right atrium in particular (Aslanidi et al., 2009). Moreover, substrate vulnerability in our IH rats was not due to lowering of AERP in the resting state, similar to OSA patients (Dimitri et al., 2012). Taken together, this suggests that the electrophysiological substrate in our IH rats resembles that found in OSA patients.

Having successfully established an IH model of OSA-related AF promotion, the remaining objectives of this thesis were to investigate the responsible underlying vulnerable substrates. We noted a number of atrial substrate changes that may serve as pathological determinants of AF susceptibility; however, parasympathetic activation was found to be particularly important for AF promotion in our model. Atropine consistently suppressed AF inducibility in IH-exposed rats. Moreover, IH-exposed rats had significantly enhanced sensitivity to AF induction in the presence of carbachol with both PES and burst pacing, similar to models of AF associated with enhanced cardiac parasympathetic function (Guasch et al., 2013; Tuomi et al., 2010). Very little is known of the effects of chronic IH on atrial parasympathetic function, so we used muscarinic receptor agonist and antagonist drugs as tools to identify potential changes in the atrial cholinergic responses to stimulation and antagonism. Cholinergic enhancement may be due to (1) an increase in parasympathetic activation of the myocardium, either from elevated vagal tone or as

a result of changes in local circuit neurons of the ICANS, or (2) increased cholinergic sensitivity at the end-organ level. From the findings of this thesis, IH-exposed rats had significantly enhanced responses to cholinergic stimulation with carbachol compared to normoxic controls with no difference in sensitivity to muscarinic receptor blockade with atropine between the two groups. These results can be interpreted to mean that, while not affecting the resting level of cholinergic activation, IH exposure augments cholinergic sensitivity. This was probably at least partially due to an increase in muscarinic receptor number, since we found significantly higher atrial M2 muscarinic receptor protein in 7 day IH-exposed rats. Given the ability of atropine to prevent AF induction and the higher incidence of carbachol-induced arrhythmias in the IH group, this enhanced cholinergic sensitivity likely contributed significantly to the enhanced AF susceptibility we observed in these animals. Studies of transgenic mice have shown that both enhanced M2 (Posokova et al., 2013; Guasch et al., 2013) and M3 (Tuomi et al., 2010) receptor function in the atria are associated with enhanced susceptibility to electrically-induced AF, which is consistent with the current data. In these models, cholinergic enhancement was due to a lack of RGS proteins, which normally limit muscarinic receptor signalling. In addition to the increase in atrial muscarinic receptor protein content that we observed, it is possible that reductions in RGS proteins may also play a role in the enhanced cholinergic responses and carbachol-induced AF susceptibility. Characterizing changes in RGS protein expression in our model would be an interesting experiment for future study.

The enhanced cholinergic sensitivity we observed is a very interesting and novel finding, and may have implications for earlier reports that chronic IH

attenuates baroreflex control of the heart rate but enhances heart rate responses to vagal stimulation (Lin et al., 2007; Gu et al., 2007). These observations could be explained by our findings, although relatively long exposure lengths in these studies and differences in methodology make extrapolation of our findings to theirs difficult.

In addition to cholinergic enhancement, we used pharmacological tools to identify novel changes in atrial adrenergic function in the IH-exposed rats, which agree with and extend observations from previous models of IH. After 7 days, we observed enhanced responses to adrenergic receptor blockade with propranolol in IH-exposed rats compared to normoxic controls. This effect is likely a reflection of an increase in baseline adrenergic receptor activation due to IH-mediated potentiation of sympathetic outflow (Dick et al., 2007; Xing and Pilowsky, 2010). Although we did not record nerve activity, previous studies have shown that IH augments tonic sympathetic nerve activity beginning during the first day of IH exposure (Dick et al., 2007; Xing and Pilowsky, 2010). With increasingly chronic exposures, the manifestations of IH-induced sympathetic potentiation mirror those seen in OSA patients (Narkiewicz et al., 1999; Carlson et al., 1996; Narkiewicz et al., 1998; Sajkov et al., 1994), including elevated plasma catecholamines (Gonzalez-Martin et al., 2009), increased tonic sympathetic nerve firing (Zoccal et al., 2007, 2008), increased chemoreflex control of sympathetic activity (Braga et al., 2006; Huang et al., 2009) and the loss of baroreflex control of sympathetic activity (Yamamoto et al., 2013), all of which result in hypertension. Elevated plasma catecholamines (Gonzalez-Martin et al., 2009) and increased sympathetic nerve activity (Zoccal et al., 2007, 2008) have been reported as early as day 8 and 15 of IH exposure, respectively, and blood pressure elevation can occur as early as day 7 or 8 (Fletcher

et al., 1999; Sica et al., 2000). More recently, chronic radiotelemetry studies indicate that MAP is significantly increased during both day and night by the third day of IH exposure and continues to rise until day 7, with concomitant loss of baroreflex control of sympathetic activity (Yamamoto et al., 2013). These studies suggest that the enhanced responses to propranolol we observed at day 7 were due to early sympathetic potentiation.

Early potentiation of sympathetic activity may also explain the blunted responses to adrenergic receptor stimulation with isoproterenol observed in IH-exposed rats. Although speculative, IH-mediated sympathetic enhancement may cause functional agonist-dependent adrenergic receptor desensitization that underlies the isoproterenol insensitivity. As sympathetic activation becomes more severe with increasingly chronic exposures, desensitization may also occur in the form of reduced adrenergic receptor levels, such as that which occurs due to sympathetic overactivity caused by heart failure (Bristow et al., 1982, Ihl-Vahl et al., 1996) or aging (White et al., 1994). This would also explain the reduction in β-adrenergic receptor content we observed in 95-day IH-exposed rats. We recognize that further studies, including evaluation of sympathetic tone and radioligand assessment of receptor function, are required to explore these possibilities.

Activation of sympathetic activity appeared to be less important for AF promotion than parasympathetic influences in our model. Adrenergic blockade with propranolol was modestly effective at reducing the incidence of AF in IH-exposed rats compared to baseline levels, but these differences did not reach statistical significance. In contrast, atropine abolished AF inducibility in all IH-exposed rats,

lowering the incidence of AF from 38% to 0% with PES (P = 0.055) and from 63% to 0% with burst pacing (P = 0.007). It is recognized that differences between the ability of atropine and propranolol to prevent AF are dose-dependent, and that a relatively high dose of atropine or a low dose of propranolol, compared to ED_{50} values, could explain this effect. Dose-response curves were ascertained in pilot studies (see appendices), and doses of 1.0 mg/kg (i.p.) atropine and 10 mg/kg (i.p.) propranolol were selected, as they similarly increased heart rate by approximately 25%. In addition, the 1.0 mg/kg dose we used for atropine is farther from the LD_{50} reported in the literature for the rat (280 mg/kg; Cahen and Tvede, 1952) than the chosen 10 mg/kg dose of propranolol is from the corresponding LD_{50} of propranolol in the rat (76 mg/kg i.p.; RTECS, 2015). Overall, this suggests that parasympathetic activation was more important than sympathetic activation for AF inducibility in IH-exposed animals. Similar findings have been reported in a study that investigated the mechanisms of enhanced AF inducibility during individual episodes of simulated apnea in pigs (Linz et al., 2012). In this study, AF inducibility was significantly attenuated by atropine while renal sympathetic denervation had a more modest effect and atenolol did not significantly reduce AF susceptibility.

IH-exposed rats exhibited significant reductions in Cx 43 content in the atria, which may contribute to the electrophysiological substrate underlying enhanced arrhythmias in our model. Although we didn't use pharmacological tests to demonstrate the importance of Cx 43 for AF inducibility as we did with the autonomic receptors, there is considerable evidence that reductions in Cx 43 can promote AF. In AF patients, loss of function Cx 43 mutations associated with reduced gap junctional coupling have been reported (Thibodeau et al., 2010) and reduced Cx 43

levels are found in chronic AF patients (Kostin et al., 2002) and animal models of AF, while restoring Cx 43 can prevent AF inducibility (Igarashi et al., 2012). In an earlier study, our lab used the same pacing protocols employed in this thesis to show that reduced Cx 43 content was associated with enhanced AF susceptibility in a genetic mouse model of Oculodentodigital dysplasia (ODDD) (Tuomi et al., 2011). Although the ODDD model is associated with a more significant reduction of total atrial Cx 43 protein content (60%; Manias et al., 2008) than what we observed in our IH rats (25% reduction in IH versus normoxic rats at 7 days), it is still likely that IH-induced Cx 43 lowering contributed to AF susceptibility if one considers a threshold model of arrhythmogenesis, which recognizes that multiple physiological factors can produce the same electrophysiological outcome. The complex, heterogeneous pathophysiology of AF is well described by a threshold model as it involves multiple factors that contribute to substrate vulnerability. In this case, it can be concluded that reduced atrial Cx 43 contributed to substrate vulnerability to some extent, since it was present together with enhanced AF inducibility and its role is well established.

Together with Cx 40, Cx 43 proteins comprise atrial gap junction channels, the subcellular structures that determine cardiac conduction velocity. As discussed in chapter 1, slow conduction velocity lowers the wavelength of re-entry circuits and facilitates AF maintenance, so a reduction in connexin expression would be expected to promote re-entry mechanisms that maintain AF. However, in strands of atrial myocytes from Cx 40-/- and Cx 43-/- mice, loss of Cx 43 reduces conduction velocity while loss of Cx 40 accelerates it (Beauchamp et al., 2006). Genetic models with reduced Cx 43 levels have enhanced AF susceptibility (Tuomi et al., 2011), while studies of Cx 40-/- mice have reported that AF inducibility was not different

from that of wild type mice (Schrickel et al., 2002) and that Cx40-/- were more resistant to AF induction in the presence of carbachol (Tuomi et al., 2011). Here, we found that Cx 43 protein and mRNA were significantly lower following 7 days of IH compared to normoxic controls and at day 95 of IH, Cx 43 mRNA was also lower in IH-exposed rats compared to normoxic controls, demonstrating sustained Cx 43 reductions induced by IH. Corresponding protein levels were not measured at the 95 day point. On the other hand, levels of Cx 40 mRNA and protein were not different in IH and normoxia-exposed rats at any of exposure lengths examined. Given the roles of the dominant atrial connexin isoforms in AF promotion, the lowering of Cx 43 and lack of change in Cx 40 we observed at day 7 in IH-exposed rats is consistent with the increased AF vulnerability in these animals.

When studies began, there were no published papers demonstrating enhanced AF susceptibility in an animal model of OSA. Recently, Iwasaki *et al.* (2014) were the first to publish findings of enhanced atrial arrhythmia inducibility in a long-term OSA paradigm, which mimicked apneas by intermittently closing the airways of intubated rats. Our findings of connexin remodelling agree with, and shed light on, the results of Iwasaki's study. Consistent with our data, enhanced AF vulnerability was accompanied by reductions in atrial Cx 43 protein, along with significant atrial conduction slowing, as has been recently observed in OSA patients. Our data suggest that IH is at least partially responsible for these observations since we observed similar lowering of Cx 43 using IH alone rather than repetitive airway obstructions to mimic OSA.

4.4 Impact of research and implications

Despite its clinical importance, the pathogenesis of OSA-related AF is poorly understood. Although IH has been used extensively to study numerous OSA-related comorbidities, this study is the first to examine the effects of a chronic model of IH alone on atrial arrhythmia susceptibility. One issue addressed by this thesis was to determine the role of the autonomic nervous system in AF susceptibility associated with chronic OSA, which was previously unknown. Researchers have primarily focused on role of autonomics in mediating the arrhythmogenic effects of individual episodes of simulated apnea or anoxia (Ghias et al., 2009; Linz et al., 2011; Linz et al., 2012; Iwasaki et al., 2012; Linz et al., 2013). Ghias *et al.* (2009) found that AF inducibility was increased during 2 minutes of anoxia in dogs, which was preventable with GP ablation or combined pharmacological blockade. In a pig model, Linz and colleagues demonstrated that enhanced AF inducibility during 2 minutes of simulated apnea could be abolished by atropine or vagotomy and reduced by renal sympathetic denervation (Linz et al., 2011; Linz et al., 2012). While useful for demonstrating the arrhythmogenic properties of acute apneic episodes and the prominent role of the autonomic nervous system, none of these models emulate chronic OSA, for which the enhanced risk of AF has been characterized.

This work is the first to demonstrate the role of the autonomic nervous system in AF susceptibility in a chronic OSA model. Interestingly, in our chronic IH model of OSA, the parasympathetic nervous system was critical for AF promotion while the sympathetic nervous system appeared to be less important, paralleling that which occurs during simulated apneas (Linz et al., 2011; Linz et al., 2012). However, the

underlying mechanisms of chronic autonomic dysfunction with IH are very different from the acute surges of cardiac autonomic activation that occur with apneic episodes. For example, during acute apnea, enhanced cardiac vagal activation due to IH-mediated chemoreflex activation (Franchini and Krieger, 1993) likely explains the dependence of AF inducibility during apnea on parasympathetic activation (Linz et al., 2011; Linz et al., 2012), while our findings suggest that enhanced cholinergic sensitivity rather than enhanced vagal activation underlies parasympathetic enhancement with chronic IH exposure. At the same time, the role of sympathetic activity in contributing to AF susceptibility during acute apneas (Linz et al., 2012) is probably related to chemoreflex-mediated sympathetic activation (Franchini and Krieger, 1993), while chronic potentiation of sympathetic activity due to IH (Zoccal et al., 2007; Gonzalez-Martin et al., 2009) most likely contributes to modulating AF promotion in our model. Taken together with findings from acute apnea studies, our results point to a complex role for autonomic alterations in OSA-related AF pathophysiology, for the first time demonstrating long-term effects on atrial arrhythmogenesis.

This thesis has also been the first to demonstrate a role for IH in OSA-related electrical remodelling. OSA patients exhibit slower atrial conduction velocities and prolonged atrial conduction times (Dimitri et al., 2012; Cagirci et al., 2011; Maeno et al., 2013a, 2015b), but it was previously unclear whether these alterations were due to OSA itself or a comorbid condition. The recent Iwasaki study described in section 4.3 of this chapter represents the first published experimental evidence in support of the hypothesis that OSA itself causes AF-promoting electrical remodelling. This thesis, with the findings of reduced Cx 43 content associated with enhanced AF

vulnerability, sensitivity to PES induction and activation heterogeneities in the right atrium, has provided additional experimental support for that hypothesis, as well as additional insights into the underlying mechanisms, highlighting IH as a critical factor. Further studies are needed to characterize the conduction characteristics of our IH model of OSA.

Based on our results and those of previous studies, I propose the following model for OSA-related AF pathophysiology. During apnea, the atria become vulnerable to AF primarily as a result of cardiac vagal outflow associated with peripheral chemoreceptor stimulation by hypoxia (Franchini and Krieger, 1993; Boscan et al., 2001). Activation of the chemoreceptors also augments sympathetic activity (Franchini and Krieger, 1993; Boscan et al., 2001), which contributes to AF vulnerability to a lesser extent (Linz et al., 2011), while the resultant blood pressure surges activate the baroreflex to further augment cardiac vagal tone and put the breaks on sympathetic flow (Braga et al., 2007). Over time, chronic exposure to IH during the sleep period leads to formation of a highly vulnerable atrial substrate, as indicated by susceptibility to PES-induced AF in IH-exposed rats. This substrate persists after the sleep period in the absence of apneas, explaining the enhanced risk of AF in awake OSA patients. Parasympathetic enhancement, characterized by increased cholinergic sensitivity, is the principal mediator of chronically enhanced AF vulnerability. However, through enhancement of chemoreceptor sensitivity (Braga et al., 2006; Huang et al., 2009) and the attenuation of baroreflex control of sympathetic activity (Yamamato et al., 2013) chronic IH leads to sympathoadrenal activation (Gonzalez-Martin et al., 2009; Zoccal et al., 2007, 2008; Peng et al., 2014), chronic IH augments sympathetic activity, which also contributes to AF

susceptibility. Substrate vulnerability is further enhanced by IH-induced lowering of Cx 43 levels, potentially due to reductions in atrial conduction velocity, as has been found in association with reduced connexin 43 levels in a model of OSA induced by repetitive trachel occlusions (Iwasaki et al., 2014). By reducing the wavelength, slow conduction velocity favours re-entrant mechanisms of AF maintenance, consistent with the present data.

In addition to providing novel pathophysiological insights, our findings have clinically significant implications. Despite the wealth of epidemiological data in support of a causal relationship between OSA and AF (Kanagala et al., 2003; Fein et al., 2013), animal models are important in order to determine causality and explore underlying mechanisms in the absence of confounding factors present in clinical databases. We demonstrated that IH alone substantially enhances AF susceptibility and induces a highly vulnerable atrial substrate that includes right atrial activation heterogeneities, connexin remodelling and both cholinergic and adrenergic dysfunction. Thus, exposure to chronic IH and its sequalae may contribute to the greater risk of procedural failure seen in OSA patients (Ng et al., 2011). The dependence of enhanced AF susceptibility on autonomic influences in our IH rats highlights the potential importance of combining standard ablation approaches with adjunctive procedures that target the ANS, such as GP ablation (Katritsis et al., 2013), for successful treatment of AF in OSA patients.

Importantly, we found that enhanced AF susceptibility and vulnerable substrate formation were evident after just 7 days of a moderate IH stimulus. This highlights the fact that the latency of the onset of arrhythmogenesis in OSA may be

shorter than previous studies have suggested (Iwasaki et al., 2014). The feasibility of therapies targeted at OSA for reducing the arrhythmia burden has been demonstrated (Fein et al., 2013; Kanagala et al., 2003). Given the mechanistic link and potentially short latency of AF onset demonstrated by this thesis, early identification and treatment of OSA may be critical to prevent the progressive worsening of AF due to remodelling. OSA is extremely common, found in 17-26% of men and 9-28% of women (Young et al., 1993) and yet, the true burden of OSA is likely underestimated since over 85% of patients remain undiagnosed (Young et al., 1997; Kapur et al., 2002). We have shown that AF-promoting mechanisms are initiated within days by IH alone, which could mean that in many undiagnosed OSA patients, the process of arrhythmogenesis has already begun. Clearly, the need for improved screening and/or diagnostic methods in order to identify OSA in its early stages is a significant clinical problem.

4.5 Limitations and future studies

Like all animal models of human disease, the one we used here has limitations. As discussed in section 4.2 of this chapter, OSA models with IH alone have become a gold standard in the field of OSA-related cardiovascular disease research even though they do not mimic the alterations in intrathoracic pressure and hypercapnea (Fletcher et al., 1992a) experienced by OSA patients during each episode of apnea (Dempsey et al., 2010). This is because IH by itself produces the autonomic and cardiovascular alterations caused by OSA, which has led to the conclusion that IH is the most important pathophysiological component (For review,

see Dematteis, 2009). Thus, although our IH stimulus does not capture all aspects of the apneic insults as they occur in OSA patients, our findings provide important lessons for understanding the human counterpart. Clinical investigation is needed to validate the applicability of our findings to humans.

In OSA, hypoxia in the lungs during apnea results in lowering of the partial pressure of oxygen in the blood, which in turn causes chemoreflex activation. During phases of hypoxia, we exposed rats to fractional inspired oxygen concentrations of 6.5-7%, but did not measure the resultant arterial hypoxemia. However, based on visual assessment, respiration rate appeared to increase rapidly during phases of hypoxia, which was not due to hypercapnia since eucapnic conditions were maintained within the chambers. This observation is an indication that the 6.5-7% FiO_2 was producing hypoxemia, since hypoxia in the blood induces ventilation (Bisgard and Neubauer, 1995). In an earlier study that used an IH regime and apparatus similar to ours to model IH in the rat, a more modest lowering of the inhaled oxygen fraction to 10% during the hypoxic phase produced significant reductions in arterial blood oxygen levels (from 97 mmHg during normoxia to 57 mmHg during hypoxia; nadir HbO_2 86.3%) and hyperventilation (Gonzalez-Martin et al., 2009), as was observed in our rats. Overall, this suggests that our IH stimulus induces hypoxemia, but further studies should be performed in our lab in order to validate and quantify the cyclic changes in oxygen saturation in our model.

Sympathetic activity should also be assessed in future studies. The enhanced responses to adrenergic receptor blockade we observed in IH-exposed rats is an indication of increased activation of adrenergic receptors but measurements of

plasma catecholamines or sympathetic nerve activity are required to determine whether 7 days of exposure to our IH model increases overall sympathetic activity. Although previous studies employing similar IH models have demonstrated blood pressure elevation following 7 days of IH exposure (Fletcher et al., 1999; Sica et al., 2000) and significantly elevated plasma catecholamines as early as day 8 (Gonzalez-Martin et al., 2009), subtle differences in methodology introduce problems for extrapolating these findings to our model.

We attempted to quantify connexins and autonomic receptor protein levels with western blotting in 95 day IH- and normoxia-exposed rats, but were unsuccessful because of sample degradation as a result of freezer break-down. Therefore, our findings of lower β-adrenergic receptors and Cx 43 in 95 day IH-exposed rats are based solely on mRNA expression, which does not always correlate with protein expression. In addition, we used overall mRNA and protein content as an index for connexin dysfunction, but remodelling can also occur at the level of connexin phosphorylation status or changes in distribution. Connexin function also depends on intracellular pH, Ca^{2+} and protein-protein interactions, none of which were assessed in this study. For adrenergic and muscarinic receptors, we examined mRNA and protein expression and used atrial responses to receptor agonist and antagonist drugs as physiologically relevant indices of receptor function. However, we did not explore potential changes in components of the associated intracellular signalling cascades, which would be an interesting issue for future study.

The novel findings in this thesis provide rationale for further experiments. With the new knowledge that our IH model leads to reductions in atrial Cx 43 content and right atrial activation heterogeneities associated with AF vulnerability, characterizing the conduction characteristics in our model is an important experiment for future study. Since pharmacological blockade prevented AF inducibility, a logical next step would be to demonstrate whether an analogous clinically relevant procedure, such as GP ablation, produces the same effects. It was recently demonstrated that carotid body ablation prevents the sympathoadrenal activation and hypertension induced by chronic IH In rats (Peng et al., 2014). Given the central importance of peripheral chemoreceptors in mediating the autonomic effects of IH, it would be worthwhile to investigate whether chemoreceptor destruction with carotid body ablation could ameliorate the enhanced AF vulnerability and autonomic alterations demonstrated in this study. Finally, to determine how the AF-promoting substrate advances with time, our studies should be repeated in rats subjected to longer IH exposures; perhaps two or three weeks at first, and eventually, 95 days to observe extremely chronic effects.

4.6 Summary

For the first time, we demonstrated AF promotion in an IH model of OSA. After just 7 days of diurnal exposure to chronic IH, a highly vulnerable atrial substrate forms, evidenced by susceptibility to PES-induced AF in IH-exposed rats. Enhanced arrhythmia susceptibility was accompanied by atrial substrate changes including cholinergic enhancement, autonomic imbalance, and connexin remodelling. Cholinergic enhancement was particularly important. These findings

provide novel insights into the mechanisms underlying OSA-related AF and the formation of vulnerable substrates, highlighting a causal role for IH, and a novel model for further exploration of the underlying mechanisms. Addressing these mechanisms may provide novel therapeutic targets for AF in OSA patients, the need for which will become increasingly important as the prevalence of OSA increases with the aging of the population (Duran et al., 2001; Young et al., 2004).

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APPENDICES

Appendix 1 – Dose-response curves of autonomic receptor drugs

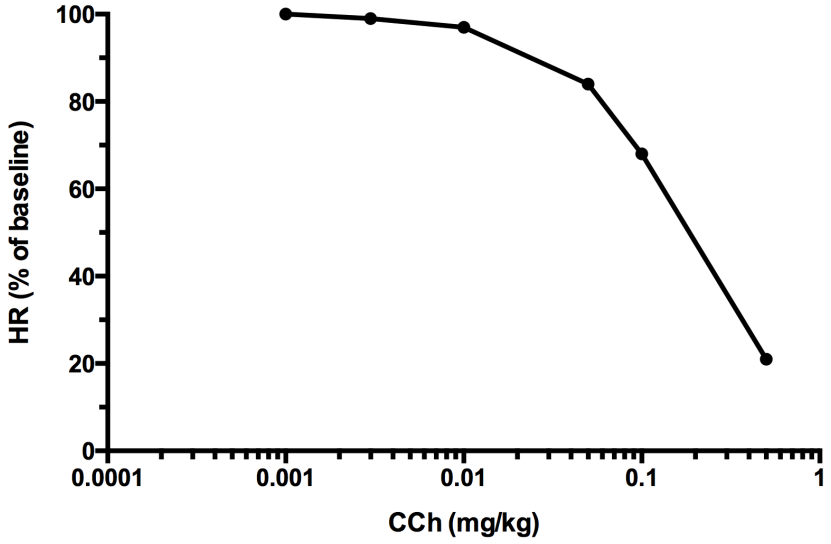

Figure 1 Negative chronotropic effects of cumulative doses of carbachol (CCh) administered intraperitoneally in an adult, male Sprague-Dawley rat. Heart rate (HR) at each dose is given as the percent of the steady-state baseline value immediately before that dose. Values represent maximal responses.

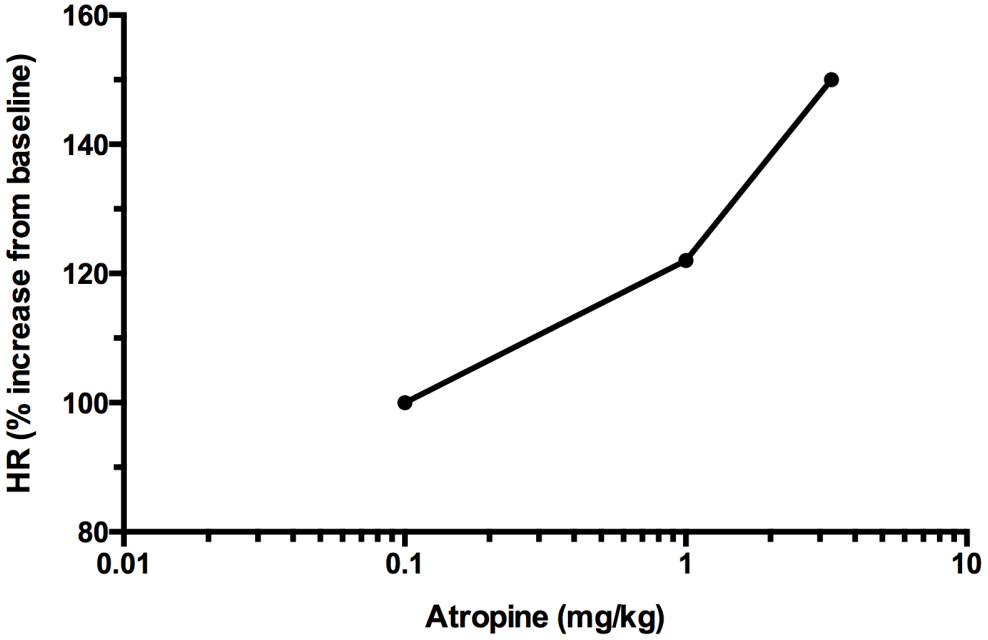

Figure 2 Positive chronotropic effects of cumulative doses of atropine administered intraperitoneally in an adult, male Sprague-Dawley rat. Heart rate (HR) at each dose is given as the percent increase from the steady-state baseline value immediately before that dose. Values represent maximal responses.

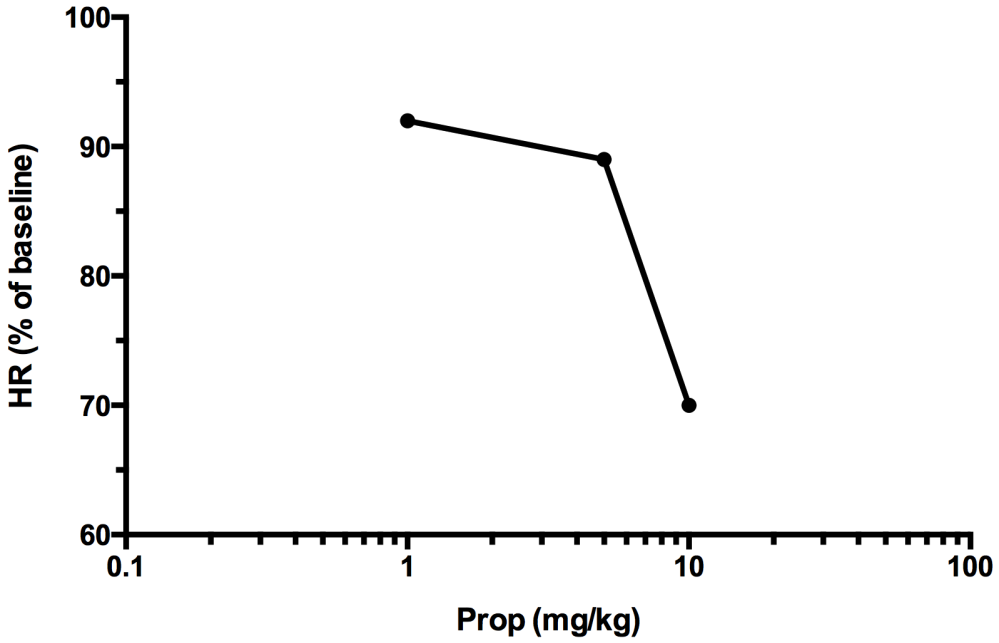

Figure 3 Negative chronotropic effects of cumulative doses of propranolol (prop) administered intraperitoneally in an adult, male Sprague-Dawley rat. Heart rate (HR) at each dose is given as a percent of the steady-state value immediately before that dose. Values represent maximal responses.

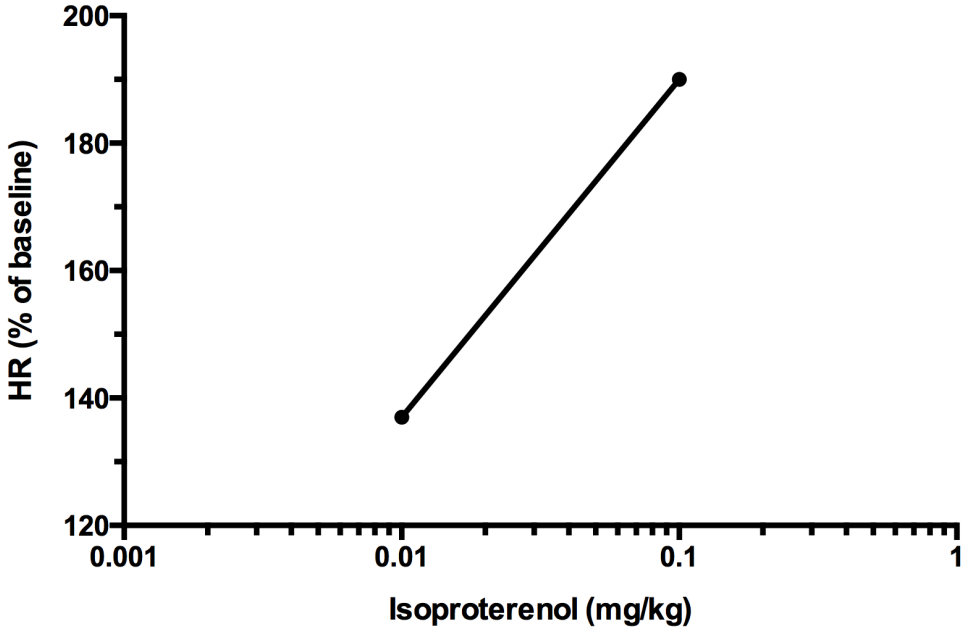

Figure 4 Positive chronotropic effects of cumulative doses of isoproterenol administered intraperitoneally in an adult, male Sprague-Dawley rat. Heart rate (HR) at each dose is given as a percent of the steady-state value immediately before that dose. Values represent maximal responses.

Appendix 2 – Literature used for initial test dose selection

Table 1. Rationale and references for chosen autonomic receptor drug dosages.

Drug	Dose, route	Rationale and references
Isoproterenol	0.1 mg/kg, i.p.	In rats, 0.17 mg/kg isoproterenol was administered intraperitoneally, but the resultant change in heart rate was not reported (Nomura et al., 1982). In mice, 1.0 mg/kg (i.p.) isoproterenol increased heart rates by 50-60% (Petric et al., 2012), which is too high for the purposes of this study. Doses of 0.01 and 0.1 mg/kg i.p. were tested in pilot studies in a single rat (Figure 4); 0.1 mg/kg most reliably produced a heart rate increase of 20-40% within 3 minutes of injection that lasted at least 20 minutes. The 0.1 mg/kg dose resulted in heart rates as high as 550 bpm (which is too rapid to permit cardiac pacing at drive train cycle lengths of both 150 and 100 ms).
Propranolol	10 mg/kg, i.p.	10 mg/kg propranolol administered intraperitoneally was an effective β-blocker in mice (Argawai and Bose, 1967) while doses as high as 50 mg/kg (i.p.) have been used in similar studies of rats (Lima and Sourkes, 1986). In another study, 10 mg/kg propranolol (i.p.) was administered to rats but the resultant change in heart rate was not reported (Nomura et al., 1982). In pilot studies, cumulative propranolol doses of 1.0, 5.0 and 10 mg/kg (i.p.) were administered to a single rat (Figure 3).
Carbachol	0.05 mg/kg, i.p.	The carbachol dose of 0.05 mg/kg (i.p.) was used for intracardiac EP studies in mice (Tuomi et al., 2011, Wakimoto et al., 2001). Wakimoto et al. ascertained pharmacokinetics and dose-response relationships of i.v. and i.p. carbachol (Wakimoto et al., 2001). They found that i.v. carbachol had a low therapeutic index with unfavourable effective/lethal dose ratio while i.p. carbachol was safe and clearly able to induce a change in heart rate with stable hemodynamic conditions. An ascending dose-response curve (Figure 1) was generated with doses ranging from 0.001-0.5 mg/kg i.p. in a single rat; 0.05 mg/kg produced heart rate reductions of 16%.
Atropine	1.0 mg/kg, i.p.	The atropine dose of 1.0 mg/kg i.p. has been used to increase AERPs by roughly 30% in mice (Tuomi et al., 2010) and similar LD_{50} values have been reported for rats (280 mg/kg i.p.; Cahen and Tvede, 1952) and mice (250 mg/kg i.p.; Cahen and Tvede, 1952). In pilot studies, 1.0 mg/kg (i.p.) atropine increased the HR by 20% (Figure 2).
Darifenacin	1.0 mg/kg, i.v.	1.0 mg/kg darifenacin was administered intravenously to reduce AERP by about 20% in mice (Tuomi et al., 2010).

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