SPONTANEOUS ACTIVITY IN THE INTRACARDIAC NERVOUS SYSTEM IN ZEBRAFISH (DANIO RERIO)

by

Keegan Patrick Daniel O'Shea

Submitted in partial fulfillment of the requirements for

the degree of Master of Science

at

Dalhousie University

Halifax, Nova Scotia

July 2022

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Dedication

To Bob,

I wish you could be here to see this.

I would not be where I am,

or who I am without you.

Thank you for the sandwiches.

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Abstract

The final common pathway for neural control of the vertebrate heart consists of autonomic neurons embedded in the cardiac wall, forming the intracardiac nervous system (ICNS). Parasympathetic and sympathetic efferent neurons in the ICNS project to the pacemaker, acting to reduce or increase heart rate (HR), respectively, when activated. Conventionally, ICNS neurons are considered simple relays transferring signals to the pacemaker from cardiac control centres in the brain, so should not be active without central inputs. In the isolated, spontaneously beating zebrafish heart I have shown that the muscarinic antagonist atropine, which blocks parasympathetic output, accelerates HR while the β- adrenergic antagonist timolol, blocking sympathetic output, evokes cardioinhibition. My results show that both parasympathetic and sympathetic neurons are active in the isolated heart without inputs from the brain, supporting the concept that the ICNS can operate as a local "stand-alone" system with potential for controlling HR.

List of Abbreviations Used

%	Percent
°C	Degrees Celsius
ACh	Acetylcholine
α	Alpha
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
AP	Action Potential
ATR	Atropine
AVR	Atrioventricular Region
β_2R	Beta 2 Receptor
β-AR	Beta Receptor
BLEB	Blebbistatin
bpm	Beats per minute
Ca^{2+}	Calcium (ion)
$CaCl_2$	Calcium Chloride
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyl transferase
CNS	Central Nervous System
DRG	Dorsal Root Ganglion
ECG	Electrocardiogram
G-Protein	Guanine nucleotide-binding protein
γ	Gamma

$G\beta\gamma$	g-beta-gamma subunit
G_i	G-protein-inhibitory
GP	Ganglionated Plexus
GPCR	G-protein coupled receptors
G_s	G-protein-stimulatory
GTP	guanine triphosphate
HCN	hyperpolarization-activated cyclic nucleotide–gated channels
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEX	Hexamethonium
HR	Heart rate
hr	Hour
ICNS	Intracardiac Nervous System
I_{k1}	Inward rectifying potassium channel
I_{kr}	Inward rectifying potassium channel (rapid)
I_{ks}	Inward rectifying potassium channel (slow)
I_{Na}	Inward rectifying sodium channel
ISO	Isoproterenol
K^+	Potassium (ion)
KCl	Potassium Chloride
L	Litre
LCN	Local Control Neuron
M_2R	Muscarine-2 Receptor
$MgCl_2$	Magnesium Chloride

min	Minute
mL	millilitre
mM	millimolar
MS-222	Tricaine mesylate
Musc	Muscarine
Na^+	Sodium (ion)
nAChRs	Nicotinic acetylcholine receptor
NaCl	Sodium Chloride
NE	Norepinephrine
pH	Power of Hydrogen
PKA	Protein Kinase A
PNS	Peripheral Nervous System
Q_{10}	Temperature coefficient
RAGP	Right Atrial Ganglionated Plexus
s	Seconds
SAP	Sinoatrial Plexus
SAR	Sinoatrial Region
SEM	Standard Error of the Mean
TIM	Timolol
X	Vagus Nerve
µL	microlitre
µM	micromolar
µm	micrometer

Acknowledgements

There is little research in the actual field of histology these days. This was something that was a bit of a shock to me when I found out, yet that did not stop Dr. Frank Smith from offering me a position is his laboratory when I asked after taking his undergraduate histology courses. Dr. Smith took a chance on me, someone with no research background at the time and gave me a ticket into the world of academia. Though I was not researching new techniques for creating a better tissue stain, what I have gained are techniques and skills that extend far beyond the classroom. What I have learned during my degree has been truly invaluable to me, and for that I cannot thank Dr. Smith enough.

To my supervisory committee, Dr. William Baldridge and Dr. Alex Quinn, thank you for your guidance and for testing me and pushing me to always be better. Your knowledge and support has made me a better researcher and someone who takes great care when making graphs.

To Dr. Matthew Stoyek, thank you for your insight and expertise and for always having an open door. Your knowledge of this field, your technical guidance and your advice has always had my appreciation. Thank you.

To Zachary Long, thank you for being both a lab mate and a friend. Thank you for your contributions to this project and for being there to always bounce ideas off.

To my friends, family and loved ones, thank you for your support throughout this undertaking, I truly could not have done this without you.

And finally, to the fish, your contributions to this project have been greater than anyone else. Science would not be possible without your sacrifice. Thank you.

CHAPTER 1 Introduction

1.1 An Overview of the Autonomic Nervous System

The autonomic nervous system (ANS) contributes to the peripheral nervous system, alongside the somatic and enteric nervous systems (Jänig 2006) and works to control visceral functions. These can be defined as unconscious functions necessary for survival of the organism, such as cardiac output or breathing, thus helping to maintain whole-body homeostasis, the constancy of the internal environment (Cannon, 1939). The ANS assists with maintaining homeostasis by controlling operation of visceral organs to meet the demands of the body (Wehrwein et al, 2016).

Throughout the vertebrates the ANS itself comprises two divisions, the sympathetic and parasympathetic pathways (as indicated on the left and right side of Figure 1.1), While the classic historical distinction between sympathetic and parasympathetic pathways was based purely on anatomical considerations, the modern view includes functional differences. The sympathetic and parasympathetic pathways have different patterns of innervation of their target organs and in many of these organs their respective neural effects may be opposing when activated.

The classical model of the peripheral ANS is that of a two-neuron relay from the central nervous system to the target as follows: signals originate from preganglionic neurons in the central nervous system, synapse with postganglionic neurons in peripheral autonomic ganglia, and their axons in turn project to the target organ. The cell bodies of preganglionic neurons in the sympathetic division of the ANS are located bilaterally in the intermediolateral columns of the spinal cord (Figure 1.2). Preganglionic neurons project from the spinal cord through the ventral roots and into the white rami where most synapse with postganglionic neurons in the bilateral columns of ganglia (sympathetic

chains) alongside the spinal cord (paravertebral ganglia, Fig 1.1). Referring specifically to innervation of the heart, the cell bodies of sympathetic preganglionic neurons are located in the upper thoracic/lower cervical region (C8) of the spinal cord, projecting axons to postganglionic neurons in the stellate (Fig 1.1) and middle cervical ganglia (not labelled) of the sympathetic chain. These neurons then project axons through the grey rami and sympathetic cardiac nerves to the heart.

Parasympathetic preganglionic neuron cell bodies are located in the brain stem (Figure 1.1, right side). Axons of these neurons project through the vagus (cranial nerve X) and other cranial nerves to their target organs where they synapse with postganglionic neurons in ganglia within these organs (Figure 1.3, bottom). In the case of the heart, the preganglionic neuron cell bodies are located in the nucleus ambiguus of the vagus, and project through the vagus nerve to synapse with postganglionic neurons in ganglia in the plexi within the heart.

In this classical model of a two-neuron relay, sympathetic and parasympathetic postganglionic axons synapse directly on their target effector cells, simply relaying information from the brain to the effectors. However, this model has been shown to be incomplete: a degree of "local control" of cardiac function has been surmised involving neurons within intrathoracic and intracardiac ganglia (Armour 2008). Ganglionic neurons can act as processors of information at a level intermediate between the central nervous system (CNS) and the myocardium. Within this system, there are many clusters of ganglia, organized into a hierarchy of neural control. Some ganglion cells project only within their own cluster, some to neighboring ganglia (Armour 1991), while a relatively small population project information back to the CNS (Cheng et al, 1997).

It is our understanding these local processing and interneurons make up the intracardiac nervous system (ICNS). The ICNS serves to process and modulate both intracardiac and extracardiac interactions (Campos et al, 2018). Furthermore, current understanding is that within the ICNS some elements of both sympathetic and parasympathetic divisions of the ANS converge. The ICNS is therefore the final common pathway for control of cardiac effector cells, including the pacemaker cells, that together set cardiac output.

Spontaneous output from either division of the ANS to the heart and to other viscera is referred to as tone. At the level of an individual neuron, tone can be defined as the frequency of action potentials (AP) generated by that neuron, over a set period. Sympathetic postganglionic tone is therefore the collective activity of all neurons in this system that target, for instance, the pacemaker cells, increasing heart rate when the tone increases in intensity. Conversely, parasympathetic tone, originating from postganglionic neurons in this system, drives heart rate down. The combination of both sympathetic and parasympathetic output is thus collectively designated autonomic tone; the combined output of the ICNS to the pacemaker would thus represent a combination of sympathetic and parasympathetic tone. One of the roles of the ICNS is therefore to modulate pacemaker activity by combining the "accelerator" and the "brake" to control heart rate to adjust cardiac output to match perfusion demands of the systemic vascular beds of the body.

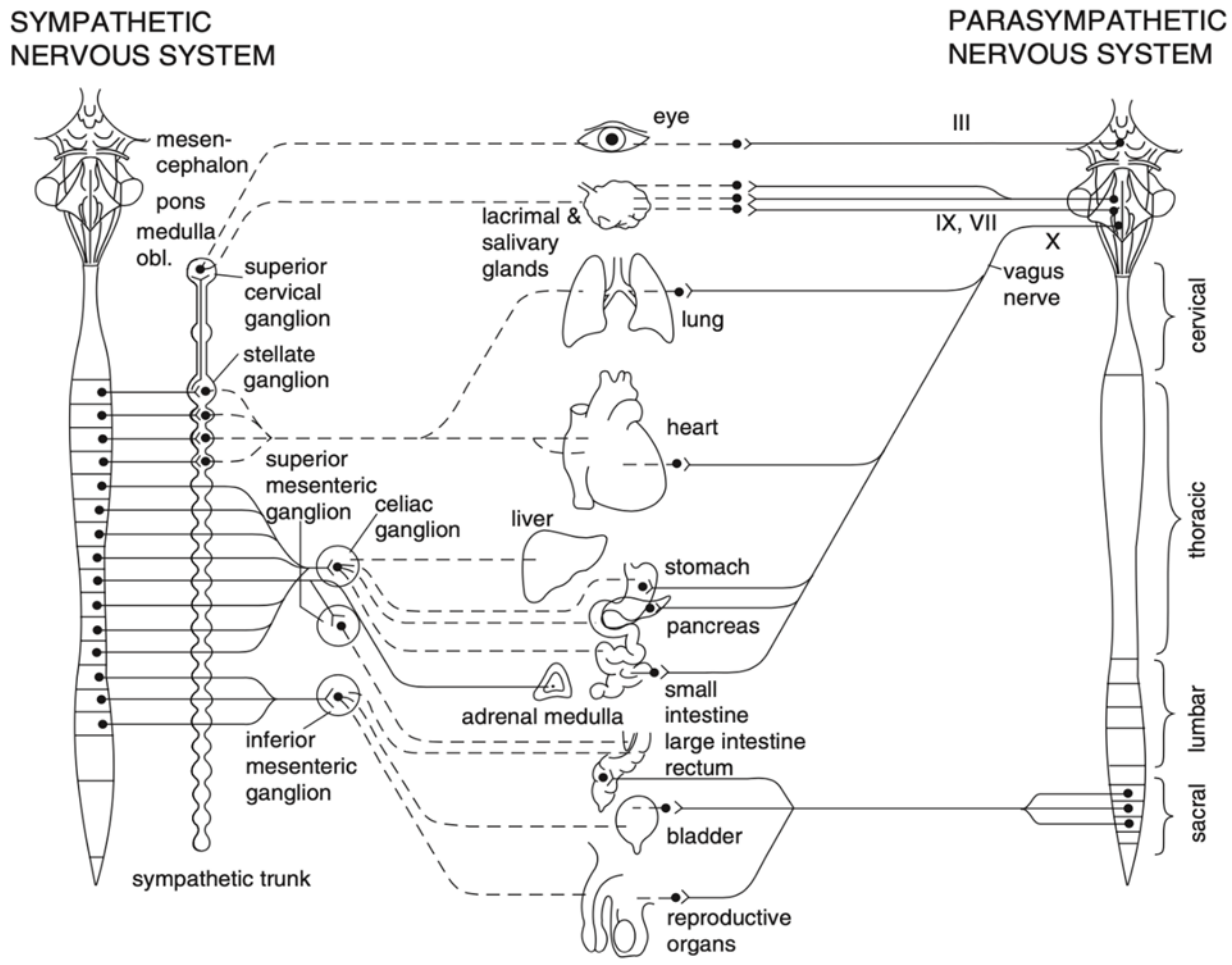

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Figure 1.1. Overview of sympathetic and parasympathetic innervation of visceral organs. Note: on sympathetic (left) side, the middle cervical ganglion (not named here) is included with the stellate ganglion. (Janig 2006; used with permission).

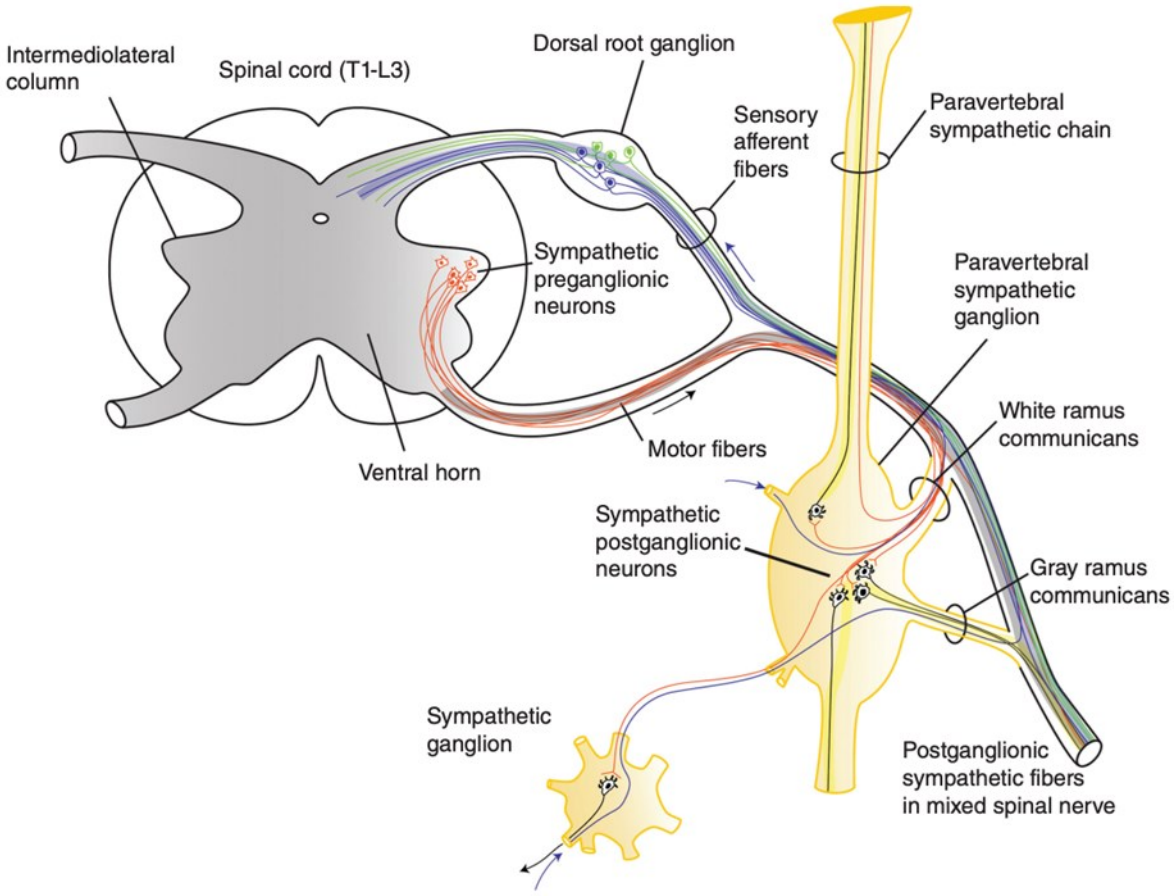


Figure 1.2. Projections of sympathetic preganglionic neurons to paravertebral or prevertebral ganglia or the adrenal medulla. (Wehrwein 2016; used with permission).

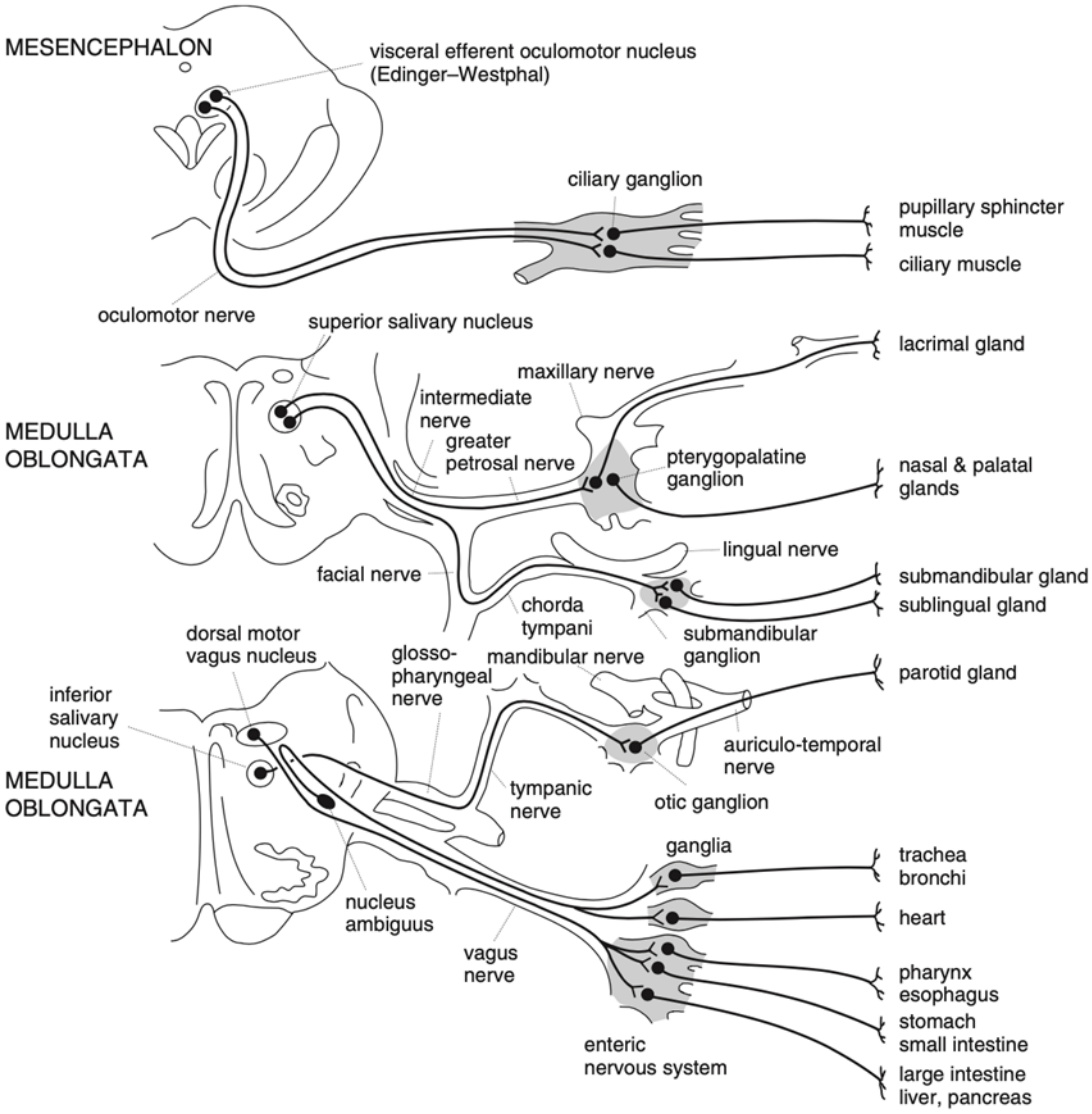


Figure 1.3. Diagram of origins and projections of parasympathetic pre-ganglionic neurons. (Janig 2006; used with permission)

1.1.1 Hierarchy of Control

Though the ICNS receives direct input from the central, preganglionic component of the ANS, it has been shown (Shivkumar et al 2016) that there are multiple feedback loops of varying latencies with potential involved with cardiac control, as shown in Figure 1.4. The existence of these control loops implies that there is a degree of local neural control residing within the heart that can be modulated by higher levels within the hierarchy. We can begin with the classical two-neuron relay model, reiterated in the hierarchy of Figure 1.4 for both the parasympathetic and sympathetic systems. Parasympathetic preganglionic neurons in the medulla are shown projecting to postganglionic efferent neuronal somata in the heart that in turn control cardiac effectors, acting via muscarinic receptors (M_2R). Sympathetic preganglionic neurons, located in cervical and upper thoracic spinal segments, project to postganglionic efferent somata in thoracic ganglia; these then project to cardiac effectors, acting via beta-adrenergic receptors (β-AR). However, as illustrated in Figure 1.4, these pathways form only part of the entire story of neuronal control loops involved in setting cardiac output.

The long-dash line, drawn horizontally on Figure 1.4, separates the central from the peripheral components of the cardiac control hierarchy. That is, in the peripheral part of this system (below the long-dash line) there is neuronal circuitry that can function independent of the CNS and may be capable of controlling the heart. Intrathoracic ganglia, long thought to be simple relay stations, are now known to contain afferent and efferent as well as "local circuit" neuronal somata that are believed to be combined into peripheral reflex loops. Afferent neural somata were identified within intrathoracic ganglia that provided local feedback from the myocardium when the connections

between these ganglia and the central nervous system were cut (Bosnjak and Kampine 1982). This condition is referred to as "decentralization" of the ganglia. Any neuronal activity then recorded in the heart or associated intrathoracic ganglia must have originated within this part of the system, independent of input from central autonomic neurons.

According to the classic model, the stellate and middle cervical ganglia (which have inputs to and from the heart) would contain only sympathetic postganglionic efferent neurons projecting to cardiac effectors. In a chronically decentralized ganglion, stimulation of cardiopulmonary nerves at a point between the decentralized ganglion and the heart should only produce antidromic action potentials (AP) in ganglionic neurons. However, as seen in experiments performed by Armour (1983), stimulation of decentralized cardiopulmonary nerves produced synaptic responses in some of the neurons, meaning they received projections from some type of afferent neuron. Armour proposed these afferent neurons had their somata within the ICNS (as opposed to axonal branches of afferent neurons with their somata in the dorsal root ganglia or the nodose ganglia.) The implication was that these projections were part of intrathoracic reflexes which could coordinate cardiac functions without input from the brain. These intrathoracic cardio-cardiac reflexes could influence the heart on a fast, beat-to-beat basis (Armour and Ardell, 2004). These ganglia appear to receive both inhibitory and excitatory afferent input to influence cardiac function (Ardell et al, 2009). Following decentralization of the stellate ganglia, these reflexes can partially maintain control of the heart (Kember et al, 2017).

Moving further toward the heart in the schematic in Figure 1.4, there is evidence of additional, shorter-duration reflex loops within the ICNS. There are now considered to be sympathetic and parasympathetic efferent neurons, afferent neurons, and "local circuit" neurons present within the intracardiac ganglia that have similar functions to these neuronal types in the intrathoracic ganglia.

The anatomy of intracardiac ganglia thus includes connections between all of these neuron types, implying the presence of local reflexes within the heart. While the presence of sympathetic and parasympathetic efferent somata in the ICNS is well established, the afferent somata and the local control neurons are more recent findings. The existence of intracardiac afferent neurons was first reported by Ardell et al (1991). Here canine hearts were decentralized, and neuronal activity was recorded from the ICNS. Mechanical distortion of the cardiac atria and ventricles (by gently touching the surface of the heart with a finger or fine probe) caused new neuronal discharges co-incident with the distortion events to appear against a background of ongoing spontaneous neuronal activity. Furthermore, the background discharge patterns themselves could be modified by mechanical stimulation. These findings implied the presence of afferent neurons responsive to mechanical activation. Taken overall, the evidence for multiple functional neuronal types in the ICNS along with the finding that spontaneous neuronal activity could be modulated by local cardiac events, gave rise to the proposal that the ICNS is capable of operating as a “stand-alone” system.

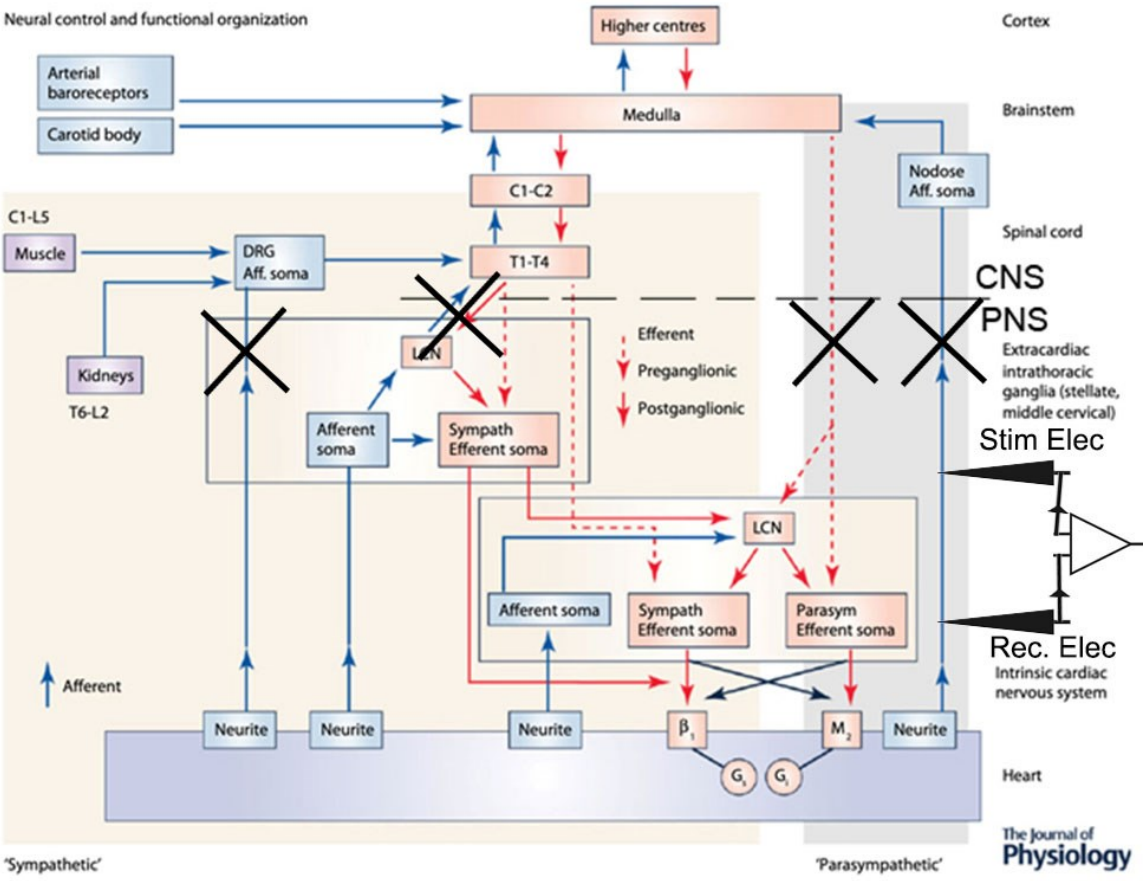


Figure 1.4. Schematic of hierarchical organization of cardiac control loops. See text for detailed explanation. X's indicate axons cut by decentralization. Electrodes indicate areas of electrode placement during experiments by Armour. (Shivkumar et al, 2016; used with permission).

1.1.2 Evidence of "Stand-Alone" ICNS

Evidence of the existence of autonomic tone of intracardiac origin has emerged sporadically in cardiac research history. One of the most notable first appearances was the findings of Gagliardi et al (1988). In this study, canine hearts were decentralized by surgically disconnecting the ICNS from the rest of the nervous system. This was achieved by an elaborate surgical procedure to section both vagosympathetic nerves (which contain sympathetic postganglionic axons as well as parasympathetic preganglionics) in the neck, and all nerves connecting the stellate and middle cervical ganglia to the heart, thus leaving this organ without any input from extracardiac neurons. Meanwhile extracellular neuronal activity, as well as ECG and right ventricular pressure were monitored. Following decentralization, spontaneous neuronal activity in the canine heart disappeared but then resumed within a short time. After decentralization the active intracardiac neurons in some cases displayed discharges phase-locked with the cardiac cycle, while others had no rhythmic activity pattern. Neuronal activity could be modified by mechanical stimulation of the heart wall with a fingertip or a probe, as described previously. The phase relationship of neuronal activity to events in the cardiac cycle could be shifted pharmacologically by administering the adrenergic agents isoproterenol (ISO) and norepinephrine (NE), and reduction in neuronal activity was seen with the application of the nicotinic antagonist hexamethonium. The major findings from this study were: 1) within the heart there was spontaneous neural activity occurring without input from higher levels in the control hierarchy; 2) intracardiac neurons appeared to receive input from cardiac mechanoreceptors; 3) neurotransmission in the ICNS was not exclusively nicotinic cholinergic but included adrenergic mechanisms, and 4) because

activity in groups of neurons in the cardiac ganglia persists after decentralization, they may be involved in local control of cardiac functions such as heart rate.

The study conducted by Butler et al. (1990) indicated that in decentralized canine hearts, regional electrical and pharmacological stimulation of ICNS neurons located in all cardiac chambers could change heart rate. For instance, electrical stimulation at the right atrial ventral ganglionated plexus (RAGP), near the pacemaker node, produced bradycardia, and following administration of atropine (ATR), an antagonist of M_2R, repeated stimulation of the same area produced tachycardia. This effect showed the presence of sympathetic postganglionic neurons as well as parasympathetic ones in the RAGP. It was postulated that the cardiac response was part of a reflex activation from afferent neurons in the RAGP. The study suggested that both afferent and efferent parasympathetic neuronal components in the left and right atrial ganglionated plexuses, as well as efferent sympathetic elements were present and were spontaneously active in intracardiac ganglionated plexuses.

Work with dissociated and cultured cardiac tissues supports the "stand-alone" concept. Cultured cardiac myocytes will beat spontaneously (Simpson, 1985). Here cellular mono-cultures were created from the hearts of day-old rats. Independent (non-coordinated) beating of early myocyte cultures occurred, and the number of actively beating cells was increased significantly by stimulation with norepinephrine. More recent studies have shown that cardiomyocyte colonies that initially beat at a low rate after one week in culture improved to 60 beats/min after one month (Belostotskaya & Golovanova 2014). The addition of cultured intracardiac neurons to myocyte cultures provided a further boost to myocyte beating performance in a study performed by Horackova et al

(1993). Myocytes that had been enzymatically dissociated from guinea pig hearts were cocultured with intracardiac neurons or stellate ganglion cells, or both. The basic electrophysiological properties of myocytes in innervated and non-innervated groups were similar, but the neuron-cocultured group showed stronger and faster spontaneous beating and had increased responses to pharmacological blockade and agonist applications. Additionally, neurons in coculture formed an extensive anatomical network that appeared to innervate all myocytes in the culture. The difference in performance of myocyte monocultures compared with that of co-cultures implies that neurons formed functional connections with the cultured myocytes that were beneficial to myocyte function (Horackova et al. 1993). Therefore, despite being enzymatically dissociated and reconstituted in culture, the myocytes and neurons were capable of reforming as a functioning tissue with local neuronal control circuitry developing *de novo* in the preparation. Thus, even within this artificial situation the presence of neurons is important to the operation of cardiac myocytes, generating what amounts to a kind of autonomic tone in culture, strengthening the potential capability for the "stand-alone" control concept of the ICNS.

1.1.3 Intrinsic Rate and Evidence of its Relationship with Tone

As discussed above, the ANS is made up of two divisions, sympathetic and parasympathetic pathways. I have defined the combination of outputs from the two pathways as autonomic tone governing all visceral organs. Blocking this tone would allow us to see what is “intrinsically” occurring during operation of the viscera. Furthermore, eliminating tone in one autonomic limb would allow an understanding of just how much tone is being exerted by the other limb. There have been attempts to do

just this in the whole organism for control of heart rate. For example, an extensive study by Altimiras (1997) sought to determine intrinsic heart rate *in vivo* in intact, unanesthetized and spontaneously breathing teleost fishes to assess intrinsic heart rate, ostensibly without autonomic neural input. The levels of standing cholinergic (representing parasympathetic) and adrenergic (representing sympathetic) tones were assessed in multiple teleost species via pharmacological administration of the receptor-appropriate antagonists while recording heart rate. It was found that cholinergic tone existed in the teleosts studied and was increased with the addition of β-blockers. In order to determine influence adrenergic tone, cholinergic tone must first be blocked using cholinergic blockers. As well, non-adrenergic-non-cholinergic tone was seen to exist in these hearts. Since these experiments were done on whole animals as opposed to isolated hearts, there may have been two confounding effects on the outcome: 1) circulating hormones may have altered cardiac status during blockade; and 2) the ICNS may remain active during both cholinergic and adrenergic blockade so may influence heart rate in the whole animal. That is, all components of spontaneous ANS tone may not have been eliminated in such *in vivo* experiments so what was considered "intrinsic" rate may not have been truly intrinsic. But what would happen if the heart containing the ICNS was isolated?

1.2 Pharmacology

The major effects of autonomic output on the heart are exerted by sympathetic and parasympathetic neuroeffector mechanisms acting to control the activity of cardiac cells. In this section I briefly review the pharmacology of these neuroeffector systems.

1.2.1 Parasympathetic Receptors and Antagonists

In the parasympathetic control of the heart, two major types of cholinergic receptors are present. The preganglionic neurons release acetylcholine (ACh) onto postganglionic neurons, acting at neuronal nicotinic acetylcholine receptors (nAChRs) located on the postsynaptic membrane of these cells. Post-ganglionic intracardiac neurons in turn release ACh which acts at M_2R on cardiac effectors.

1.2.1.1 Nicotinic Neurotransmission

Despite the proposal in 1905 by Langley that there was a nicotine-sensitive moiety involved in evoking visceral responses to ACh, it took until 1975 for the structure of nAChRs to be fully determined (Nachmansohn, 2012). Since then, receptor subtypes, their structures, and the localization of ion channels involved in nicotinic cholinergic neurotransmission have been reported (Gotti and Clementi, 2004). Neuronal AChRs are pentamers with both homomeric and heteromeric subtypes. The homomeric receptors consist of five α7 subunits while the heteromeric subtypes consist of both α and β subunits. In the parasympathetic nervous system, both the homomeric subtype (Sahibzada et al, 2002) and heteromeric subtypes are present on the membranes of postganglionic neurons (Gu et al, 2008). The activation of nAChRs is very fast, occurring in the microsecond range. The mechanism of action at the ligand-binding site is summarized in Figure 1.5. ACh or its agonists bind at the gap between an α and β subunit (or between two α subunits in the homomeric subtype), the binding causes the β subunit to rotate, opening the channel pore to allow for cations (Na^+, K^+, and Ca^{2+}) to pass through (Albuquerque et al, 2009). Sodium ions are the major charge carrier in this system,

moving inward to excite the cell, and rapidly depolarize the membrane to the action potential firing threshold.

Though several nAChR antagonists exist (mecamylamine, trimethapan) for this study I focused on hexamethonium. This is because of its wide usage, as well its ability to block sympathetic and parasympathetic transmission through the ganglia. (Ebert, 2013) Hexamethonium binds to the receptor itself in a domain separate from the ACh binding site (Gropper et al, 2019). The classical view of vagal control of the heart has only nAChRs present on postganglionic neurons within the heart. Thus, antagonizing pre- to postganglionic neurotransmission with hexamethonium should produce the same cardiac effect as blocking cholinergic muscarinic neurotransmission from postganglionic terminals on the effectors. However, this is not necessarily the case.

1.2.1.2 Muscarinic Neurotransmission

Muscarinic cholinergic receptors are a more recent discovery, having first been identified by their activation by muscarine (Musc), a derivative of the mushroom species *Amanita muscaria* (Barlow et al, 1972).) Muscarine receptors are mainly classified using one of two ways: pharmacological classification uses an upper-case M followed by a subscript number, while genetic classification uses a lowercase m with a number with no subscript. There are 5 subtypes currently recognized (M_1-M_5). The pharmacologic and genetic classifications are in the same order (meaning that m2 is the gene that codes for M_2) (Caulfield and Birdsall, 1998). For the purposes of this study, I will be using the pharmacological nomenclature, this is because I am concerned with outcomes of pharmacological agents on these receptors, and because I am not using genetically modified fish for this study. Muscarinic receptors are present on all effector tissues within

the heart, evoking responses from the ACh by postganglionic parasympathetic terminals. M_2 is the most common subtype found within the mammalian myocardium (Elgen, 2005). Muscarinic receptors have been found in zebrafish at levels that compare favourably to those of mammalian species (Williams and Messer, 2004) and M_2 receptors are known to be present in zebrafish myocardium and are genetically modifiable. (Steele et al, 2009).

Muscarinic receptors are g-protein-coupled receptors (GPCRs). The M_2 receptor is bound to inhibitory G proteins (G_i). Activation of G_i proteins inhibit the cAMP pathway by downregulating adenyl cyclase activity and decreasing the production of cAMP. As cAMP is stimulatory within the heart, downregulation of cAMP has inhibitory effects (Saternos et al, 2018). Moreover, when ACh binds with the M_2 receptor (Figure 1.6) there is a disassociation of the g-beta-gamma subunits (Gβ-γ) which stimulates inwardly rectifying potassium channels in pacemaker cells, reducing the slope of the diastolic depolarizing potential to slow heart rate. (Moss et al, 2018)

Some antimuscarinic drugs considered to be important (Rang et al, 2011) are ATR, pirenzepine and scopolamine. While scopolamine and ATR have similar effects in the periphery, scopolamine is used more often in CNS pharmacology because it can pass the blood-brain barrier. At similar doses, scopolamine is less likely to affect heart rate (HR) than ATR (Ebert, 2013). Atropine is commonly available and widely used both clinically and experimentally. It is derived from the nightshade plant *Atropa belladonna* (Brunton et al, 2010). Atropine is a competitive antagonist, reversibly binding at the receptor site of all muscarinic receptors and opposes vagal effects on the heart by blocking the binding of ACh to M_2 receptors.

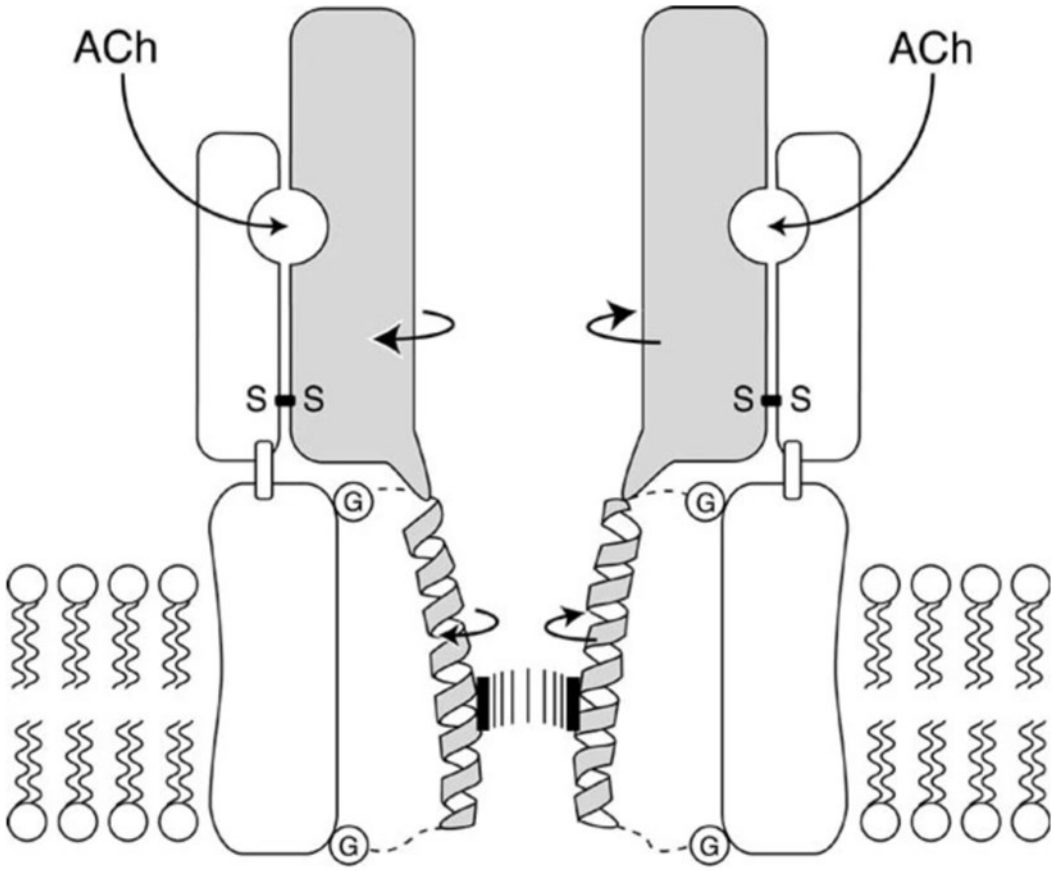


Figure 1.5. Model of nicotinic receptor binding site and mechanism of activation. Shaded shape represents β subunit while white shape represents α subunit. When ACh binds between the α and β subunit (illustrated here) the β subunit rotates, opening the channel pore for ion flow. (Miyazawa et al, 2003; used with permission)

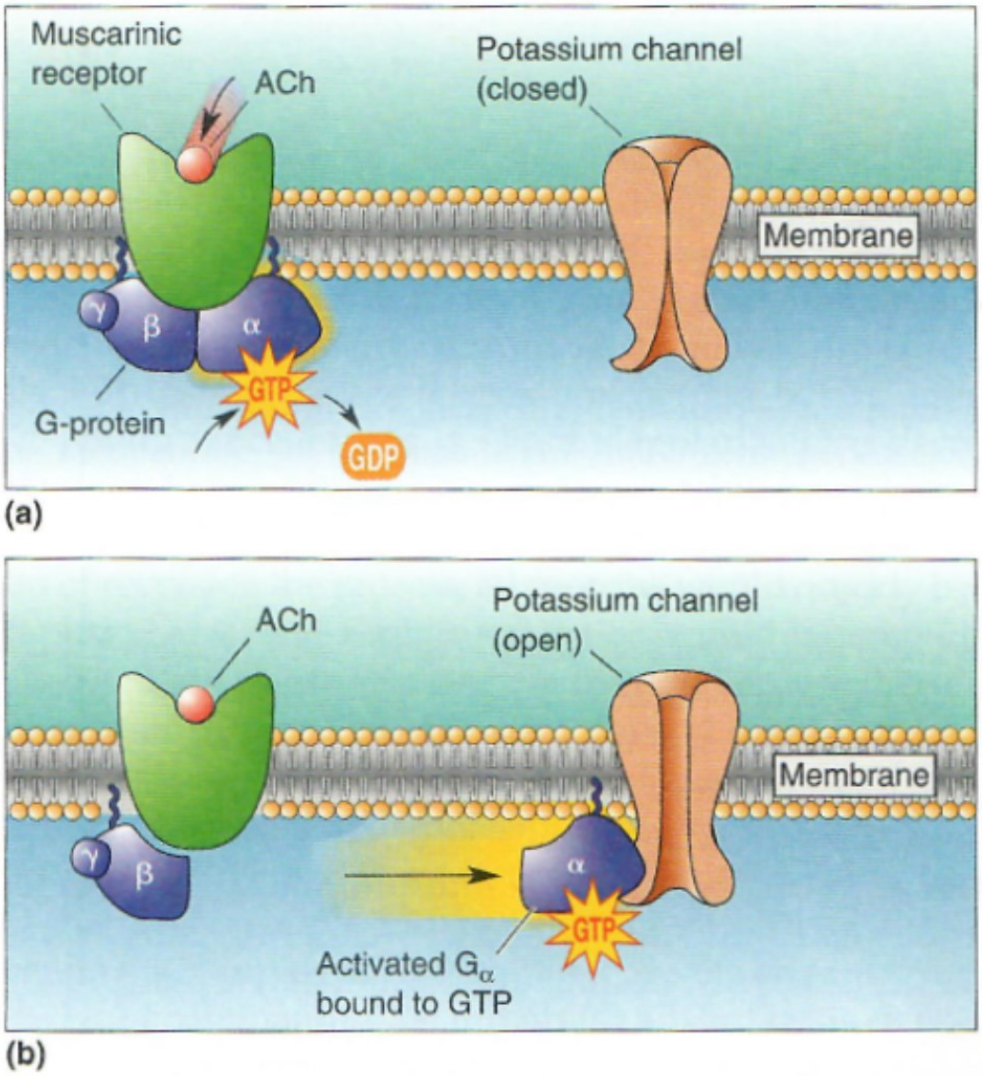


Figure 1.6. Model of muscarinic receptor binding site and mechanism of activation. When ACh binds with the muscarinic receptor (illustrated here) guanine triphosphate (GTP) binds to the Gα subunit and causes a disassociation from the Gβ-γ subunits. The Gα subunit then binds to K^+ channels, allowing their opening. (Bear et al, 1996; used with permission)

1.2.2 Sympathetic Receptors and Antagonists

The preganglionic neurons in the sympathetic system release ACh which acts at nicotinic receptors on the postsynaptic membrane of postganglionic neurons; in turn, sympathetic postganglionic neurons release the catecholamines norepinephrine and epinephrine, which act at adrenergic receptors on their target organs. In mammals there are both α and β adrenergic receptors, and these are also seen in fish. (Owen et al, 2007). In mammalian cardiac pacemaker cells, these receptors are predominantly β_1 adrenergic receptors (Greene et al, 2012); the distribution of adrenergic receptors in the fish heart is not yet well understood, but both β_1 and β_2 receptors are present in fish hearts (Joyce et al, 2022).

First described by Lefkowitz, Stadel, and Caron (1983) and fully sequenced in humans in 1987 (Kobilka et al, 1987), β_2 are transmembrane G-protein coupled receptors (Figure 1.7) with 7 α-helices, an amino-terminus, and a carboxy-terminus (Johnson, 2006). Beta-2-adrenergic receptors (β_2R) have both active and inactive forms. When associated with the α-subunit of the G_s protein and GTP, the receptor assumes the active form. Then, when activated by a β_2 agonist, the α-subunit dissociates from the G_s protein and binds with adenylate cyclase, which then causes an increase in cAMP and leading to activation of PKA; this process is summarized in Figure 1.8. This rise in cAMP leads to modulation of hyperpolarization-activated cyclic nucleotide–gated channels (HCN), causing an increase in pacemaker firing (Robinson et al, 2006). The rise in PKA also increases the phosphorylation of phospholamban, which allows for increased Ca^{2+} cycling by the sarco/endoplasmic Ca^{2+} ATPase, leading to increase pacemaker firing (Behar et al, 2016). PKA then causes the phosphorylation of the receptor which leads to

dissociation of G_s and association of G_i, which then binds to β-arrestin which allows for the activation of the mitogen-activated protein kinase pathway, leading to the inactivation of the receptor. (Johnson, 2006).

Common antagonists of β adrenergic receptors include propranolol, atenolol and timolol. In mammalian studies, atenolol may be seen more commonly as it is β_1 specific and β_1 has stronger cardiovascular effects in mammalian hearts. However, in the fish hearts it is unknown which is the dominant subtype, therefore non-selective blockers like propranolol and timolol are required. For this study I am focusing on timolol due its stronger effects at lower doses (Ebert 2013).

Application of timolol (TIM), has been shown to block the effects of β_2R activation on heart rate. β -blockers competitively bind to the receptor sites for endogenous catecholamines (Frishman, 2005). In the case of TIM, it competitively binds and blocks the effects of NE, then blocking the activation of β_2R. Preventing modulation of HCN channels, which in turn decreases pacemaker firing rate.

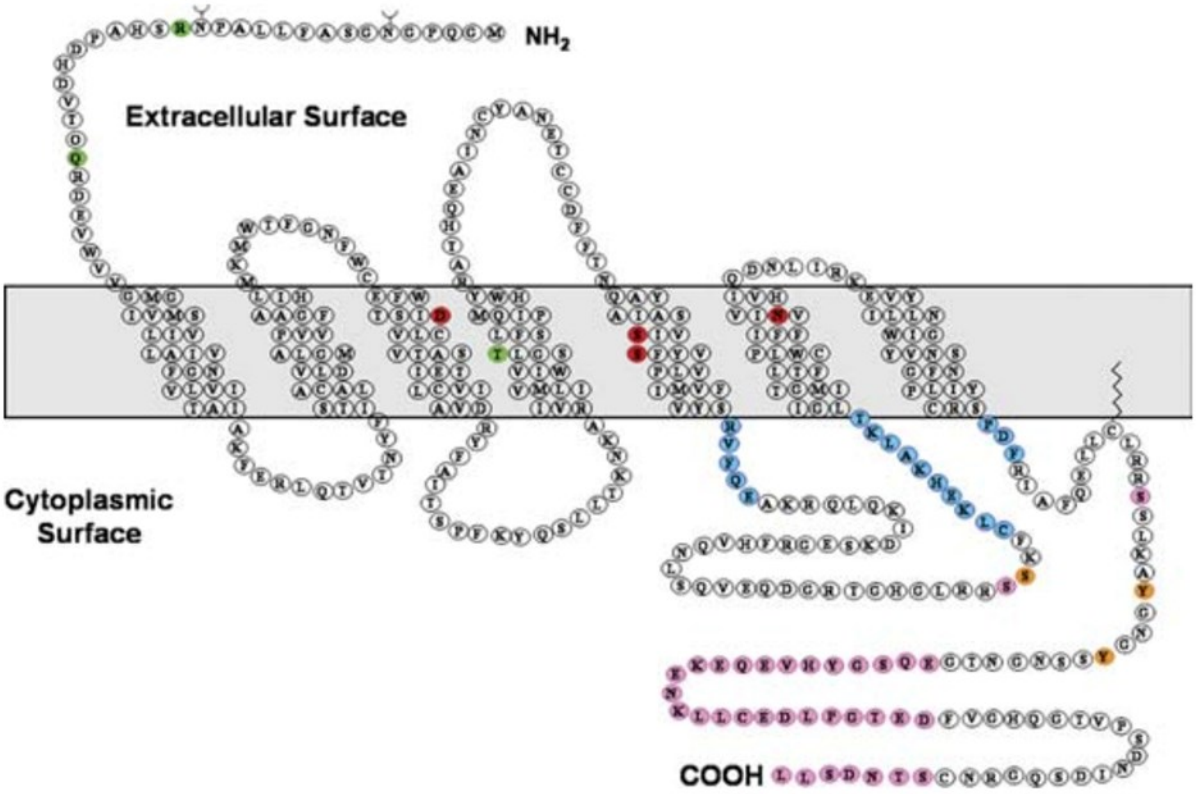


Figure 1.7. Diagram of transmembrane domain of β_2 adrenergic receptors. β_2 agonists bind to the receptor at the terminal between the 5th and 6th subunit. The Gs complex (not illustrated) is associated with the intracellular loops. (Johnson 2006; used with permission).

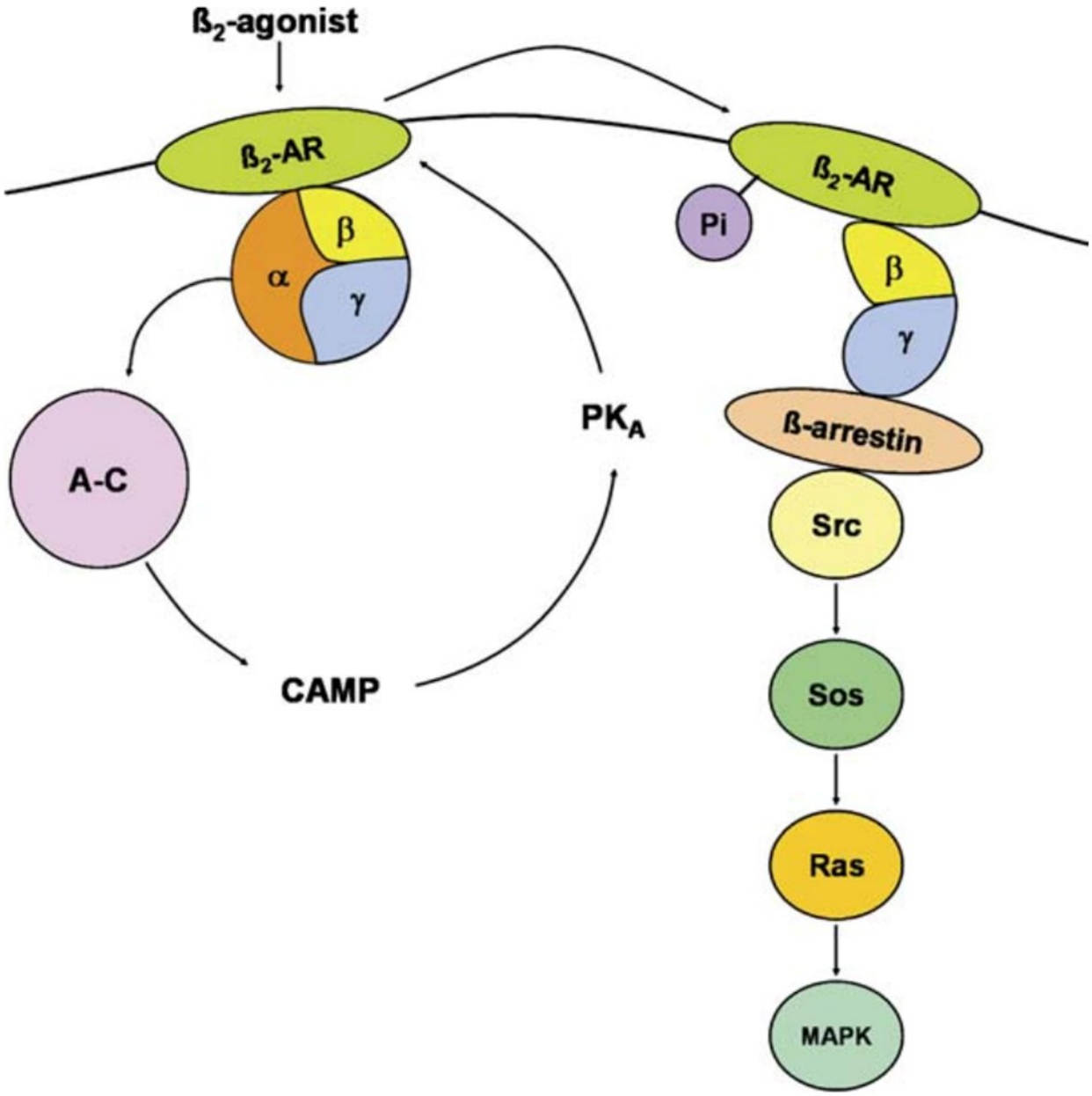


Figure 1.8. Schematic diagram of mechanisms of binding and activation of signalling of β2 receptors. Activation of β_2R causes the α subunit of Gs to disassociate and bind to adenyl cyclase, this leads to a rise in cAMP and PKA. B-arrestin then binds to the β- γ subunits and leads to inactivation of the receptor. (Johnson 2006; used with permission)

1.3 Zebrafish as a Model

Recent work in the Smith lab and others has found the zebrafish to be an ideal candidate for studying cardiovascular electrophysiology. Their small size; and accessibility of isolated heart preparations make them suitable for my work.

1.3.1 Zebrafish Model Applicability

In 2002 it was argued that the zebrafish is a model organism that should be considered fit for the study of complex integrative physiology (Briggs, 2002). In the 20 years since that landmark review, zebrafish have been used in an increasing capacity for understanding fundamental physiological questions. Their size, high fecundity, and short generation times make them ideal candidates for studying disease models. The zebrafish genome is fully sequenced (Howe et al. 2013) and 70% of human genes have one or more orthologues in zebrafish. These properties have contributed to the widespread use of this species for experimentation (Rafferty and Quinn, 2018). Most relevant to cardiovascular research, zebrafish hearts begin beating consistently 36 hours post-fertilization and at that stage of development they resemble the hearts of human embryos at three weeks of development (Dooley and Zon, 2000). In the zebrafish heart, the rapid inward rectifying potassium channel (I_{kr}) is the dominant repolarizing channel, the same channel that is dominant in human hearts (Echeazarra et al, 2021). Other advantages of zebrafish use include their optical transparency during embryogenesis, which allows for visualizing developmental cardiac physiology (Echeazarra et al, 2021). Zebrafish hearts have been used both as models for studying human electrophysiology (Vornanen & Hassinen, 2016) and cardiac disease (Leong et al., 2010).

1.3.2 Zebrafish Hearts

The mammalian heart features “double pass” circulation, deoxygenated blood moves from the heart, to the lungs where it is oxygenated. This blood then moves from the lungs, back to the heart where it is pumped out to the systemic vascular beds. However, zebrafish have a serial “single pass” circulation system. The heart pumps deoxygenated blood to the gills, it is oxygenated and then circulates though the vascular beds and moves back to the heart. Despite these differences, the zebrafish is still a useful model.

The cardiovascular system of zebrafish is the subject of numerous recent studies. Developmental cardiac mutations were studied by Chen et al (1996) and Warren et al. (2001) and the establishment of zebrafish hearts as a model of electrical conduction, similar to that of higher-order vertebrates, was reported by Sedmera et al (2003). A landmark report by Tessadori et al. (2012) described details of the pacemaker region in the zebrafish heart; this structure was long thought to be absent in fishes. Furthermore, Stoyek et al. (2022) showed that the driver of automaticity in mammals, the coupled membrane and Ca^{2+} “clock” pacemaker system, is also present in zebrafish, furthering the utility of this species as a model for studying cardiac physiology.

Recent research in the Smith lab has shown that the isolated zebrafish heart is an ideal model for studying control of cardiac function by the ANS (Stoyek et al, 2016). A major advantage of this preparation is that, unlike mammalian hearts, the ICNS is visible and accessible for manipulation in isolated, spontaneously beating cardiac preparations, which remain viable for several hours after isolation.

The ICNS in the zebrafish consists of approximately 200 neuronal cell bodies and all parts of the heart are innervated by this system. The majority of neurons in the ICNS comprise a plexus, the sinoatrial plexus (SAP), located adjacent to the valves in the sinoatrial region (SAR). Stoyek et al. (2015) showed that extracardiac input into the SAP enters the heart from the vagosympathetic nerves. These authors also mapped the distribution of neurons with specific phenotypes within the SAP: while some adrenergic and nitrergic neurons were present, the majority of neurons were shown to be choline acetyltransferase (ChAT) positive, implying that these neurons are cholinergic.

Stoyek et al. (2016) showed that isolated hearts, in addition to beating spontaneously, contained adrenergic and cholinergic receptors on both neuronal cell bodies and cardiac effectors. Putative pacemaker cells were found in the SAR and the atrioventricular region (AVR) as well. These features thus establish the isolated zebrafish heart as a viable model for the study of cardiac control by the ANS.

1.4 Temperature

Zebrafish are poikilotherms, meaning they cannot regulate their own body temperature, so this varies with changes in environmental temperature (Guschina & Harwood, 2006). Because of this, zebrafish present a unique opportunity to study possible temperature effects on the ICNS, something that would not be possible in mammals.

1.4.1 Relationship of Zebrafish Heart Rate to Environmental Temperature

Wild zebrafish occupy environments across a broad spectrum of temperatures, ranging from approximately 12 to 30°C in some cases. (Arunachalam et al, 2013). Because zebrafish are poikilotherms, many of their physiological processes are affected

by temperature change. A change in the efficiency or speed of a reaction due to a change in temperature is referred to as the temperature coefficient and is given the symbol Q_{10} (Reyes et al, 2008). Temperature coefficients exist within physiological systems, meaning that entire processes like respiration have a Q_{10}. The Q_{10} of a system represents how much faster a system would operate if the temperature was raised by10°C. A Q_{10} of 2 would therefore mean that the system would be twice as fast if raised by 10°C. Q_{10} is calculated as such ($Q_{10}= (X_2/X_1)^{(10/T_2-T_1)}$ where X represents the rates at two different temperatures and T represents the two temperatures.

The role of the heart is to supply the body with sufficient oxygen to meet the metabolic demands at a given time; with oxygen supply being directly related to cardiac output. Temperature has been shown to have a direct effect on cardiac output in fish (Farrell and Smith, 2017) which is determined by HR and stroke volume. Changes in temperature alter cardiac output primarily through effects on firing of pacemaker cells while leaving stroke volume of the heart relatively unaffected in most teleosts (Farrell and Smith, 2017). In the zebrafish heart, it has been shown that HCN channels are temperature sensitive (Marchant and Farrell, 2019). If pacemaker channels are able to be influenced by temperature, are ANS inputs to the pacemaker also temperature influenced?

1.4.2 Temperature Effects on ANS

As temperature increase, neuronal AP discharge frequency has been shown to increase (Burek et al, 2019), and it has been suggested that there is a link between the shortening of AP duration and increased temperature. This could be due in part to more

rapid gating of ion channels, the kinetics of which are highly temperature dependent (Vornanen et al, 2014)

The teleost heart receives innervation from both branches of the ANS, which contribute to setting HR via their outputs to pacemaker cells affecting pacemaker discharge rate (Sandblom and Axelsson, 2011).

If overall neuronal discharge frequency increases at higher temperatures, both sympathetic and parasympathetic tone should increase. However, there is conflicting evidence that, at higher temperatures, sympathetic tone is decreased while parasympathetic tone increases (Sandblom and Axelsson, 2011; Gilbert 2019). There is also evidence that at colder temperatures sympathetic tone is increased (Sandblom and Axelsson, 2011) but this is counter to the idea of neuronal firing being slower at lower temperatures. Again, these studies were conducted *in vivo*, therefore upstream effects from the CNS may have been affecting the results. Here I seek to understand temperature effects on the ICNS in an isolated system, allowing for greater understanding of the strength of sympathetic and parasympathetic tone at multiple temperatures and allowing for an estimation of the Q_{10} of the ICNS, if present.

1.5 Relevance and Thesis Objectives

1.5.1 Relevance

Despite the widespread use of the zebrafish heart as a model for human cardiac function (Bowley et al, 2022), there are many elements of zebrafish neurocardiology that remain unresolved. For instance, our understanding of receptor function, ANS neurotransmission and the effects of changes in these factors on autonomic tone in the isolated zebrafish heart is still incomplete. Furthermore, a 2008 review by Barros et al

highlighted the use of zebrafish for early drug discovery and the advantages of zebrafish as a model for developing and testing cardioplegic drugs. However, these authors also pointed out that, even for agents routinely used in human patients, our lack of understanding of the actions of these agents in non-mammalian models remains a severe limitation. In this thesis, I seek to explore aspects of the pharmacology of cardiotropic compounds acting via sympathetic and parasympathetic limbs of the ANS, alongside investigating tone in the isolated heart. Further understanding of the function of the ICNS will allow for more effective and more precise cardiac therapeutics in humans.

1.5.2 Hypothesis and Objectives

It is clear from the discussion above that an index for evaluating the existence and degree of intracardiac autonomic tone, arising from the operation of local neural circuitry within the heart, is needed to understand its role in cardiac control. My overall hypothesis is that this tone exists in the isolated zebrafish heart, and my work in this thesis explores the development and testing of an index to evaluate its properties using pharmacological methods.

I hypothesize that both sympathetic and parasympathetic tone are present in isolated zebrafish hearts. Blocking sympathetic and parasympathetic receptors on the pacemaker cells using TIM and ATR, respectively, should alter heart rate, thus demonstrating the presence of this tone. This work is the main focus of Chapter 2.

Following the experimentation and findings in Chapter 2, several secondary questions arose, listed below and the details of investigating these are discussed in Chapter 3.

1. Given that cholinergic neurons constitute the most common neurotransmitter phenotype in the zebrafish ICNS (Stoyek et al, 2016), it is reasonable to suppose that nicotinic neurotransmission is involved in interactions between neurons in this system that result in the generation of tonic outputs to the pacemaker cells. Therefore, does blockade of such activity with the nicotinic antagonist hexamethonium modify rate in the isolated heart?

2. Given that the trials of sympathetic and parasympathetic antagonists provided indirect evidence that there is spontaneous neural output from the ICNS to the pacemaker cells in isolated hearts, is it possible to directly record ongoing spontaneous neural activity from within the ICNS itself?

3. What is the source of this tone? Does it arise from the ongoing activity of afferent neurons with their somata located within the ICNS that are driven by mechanical movements of the heart during contraction? Alternatively, could spontaneous activity be generated by intrinsically discharging neuronal populations within the ICNS?

Chapter 4 consists of a summary of my findings and an overview and discussion of the implications of these results for understanding neural control of the heart as well as considering the challenges and limitations of this project and my hopes for future directions in this study.

CHAPTER 2 Presence of Autonomic Tone

2.1 Introduction

The main hypothesis in this thesis is that there exists a degree of spontaneous neuronal activity within the isolated zebrafish heart which can influence heart rate. This hypothesis is based largely on lines of circumstantial evidence from a variety of previous studies across the vertebrates; some of these are cited in Chapter 1, Section 1.1.2. I sought to test the hypothesis through a series of experiments employing pharmacological blockade of sympathetic and parasympathetic efferent influences on pacemaker cells, since autonomic tone could originate from elements of either pathway within the ICNS.

2.1.1 Parasympathetic Blockade

As I alluded to in Chapter 1, Section 1.2.1.2, ATR is commonly used both clinically and experimentally as an antimuscarinic. I am focusing on ATR because this agent has a greater potency at a given concentration than do other agents of this type used in blocking cholinergic drive to the pacemaker (Ebert, 2013)

In mammalian studies of isolated hearts, ATR has been effective at a concentration of ~5 μM (Alpin et al, 1995; Langley et al, 1977). In teleosts ATR has been used over a range of concentrations. In whole-animal experiments by Axelsson et al. (1992), ATR was injected into the circulation via a ventral aortic cannula at 1.2 mg/kg. When injected into the coelomic cavity in work done by Bastos-Ramos (1998), ATR was used at a concentration of 25 mg/kg. In work done by Perry and Desforges (2006), rainbow trout were pre-treated at a dose of 1 μmol/kg injected into the dorsal aorta, and ATR has been used at 100 nmol/kg by injection into the circulation in experiments done by McKendry et al (2001). The issue with all of these studies is that at the actual receptor sites, the concentration of ATR is unknown. Herein lies one of the advantages of the

isolated heart preparation: a known concentration of a pharmacological agent, applied in the perfusate, can be delivered directly to the tissue containing the receptors.

Recent work in this lab by Stoyek et al (2016) reported that 10 μM is sufficient concentration of ATR for cessation of bradycardia induced by vagal stimulation in the isolated zebrafish heart. However further work needs to be done on the issue of effective ATR concentration in this preparation. Is 10 μM the optimum concentration? Or is the blockade greater at higher concentrations? Here I set out to uncover possible concentration-dependent effects of ATR in the isolated heart. Therefore, I explored cardiac effects of this agent across a range of concentrations bracketing (3 μM, 10 μM, 30μM).

In order to ensure that ATR was targeting and blocking muscarinic receptors I applied the agonist muscarine to the isolated heart before and during exposure to ATR; in a few trials, I also used the alternate muscarinic agonists bethanechol (broad-spectrum agonist) and methoctramine, (M_2R-specific subtype agonist).

2.1.2 Sympathetic Blockade

While β_1-AR is the predominant subtype on mammalian pacemaker cells mediating adrenergic increases in HR (Rodefeld et al, 1996; Wehrwein et al, 2016), the situation in other vertebrate groups is not so straightforward. In fish there is apparently a mix of β_1- and β_2-AR involved in HR control. In zebrafish, Joyce et al. (2022) have shown that in larval zebrafish both β_1- and β_2-AR may be involved in adrenergic tachycardia, and that if β_1- AR are genetically eliminated, β_2-AR take over. In isolated adult zebrafish hearts β_2-AR have been shown immunohistochemically to be present in the sinoatrial region (containing the pacemaker), the atrium, and ventricle; furthermore,

ISO acts on these hearts to increase rate (Stoyek et al, 2016). Maciag et al (2022) has confirmed in zebrafish that ISO is a potent β-agonist in the heart, likely acting at both β_1- and β_2-AR. Because both subtypes of adrenergic receptor may be involved in rate control in the zebrafish heart, TIM was chosen for use in this study because it is a non-selective β-antagonist and may be more potent at lower doses compared with other common non-selective blockers like propranolol (Achong et al, 1975). However, as with ATR (section 2.1.1 above), little is known about cardiac effects of TIM in any teleost, let alone zebrafish. In the isolated zebrafish heart, Stoyek et al. (2016) showed that TIM at a concentration of 10 μM blocked the tachycardia induced by ISO, so I have used a concentration range of 3-30 μM TIM to evaluate the effect of this agent on HR. The efficacy of TIM to antagonize β-AR activation was evaluated by its ability to eliminate ISO-induced tachycardia.

2.1.3 Temperature

As I have pointed out in Chapter 1, Section 1.4.1, fish hearts display an increase in heart rate with rising temperature, expressed by a Q_{10} in the range of 1.5-2 (Farrell and Smith, 2017). Given that HR in zebrafish increases with temperature (Marchant and Farrell, 2019) I seek here to determine if there is a similar relationship between temperature and the efficacy of the neural control of the heart. That is to say, does the activity of the ICNS itself have a Q_{10}? To test this, I developed protocols for sympathetic and parasympathetic blockade trials at two temperatures, 22°C and at 27°C, the latter being the acclimation temperature of the zebrafish bred and held in the Faculty of Medicine CORES Zebrafish Facility at Dalhousie University. An additional higher temperature was not selected because above 30°C hearts began to fail. Any differences in

cardiac rate responses to pharmacological blockade between the two temperatures could be due to a change in the efficacy of the ICNS in modulating HR.

2.2 Materials and Methods

2.2.1 Ethics

Animal use procedures followed the "Guidelines on the Care and Use of Fish in Research, Teaching and Testing" document issued by the Canadian Council on Animal Care (CCAC 2005 ed.). I qualified for the Fish Usage Certificate through training and testing provided by the Dalhousie Animal Training Program. Ethical approval for the work in this thesis was covered by Protocol #21-080, issued to F. Smith by the Dalhousie University Committee on Laboratory Animals.

2.2.2 Animals

Wild-type zebrafish (AB line) were mainly acquired from the Zebrafish CORES facility in the Faculty of Medicine. Fish were raised and maintained in 3-10 L tanks (Aquatic Habitats, nif-0000-31933, Apopka, FL, USA) at 28.1°C, pH 7.48. Water conditions were monitored and adjusted with Pentair Aquatic Eco-Systems Monitor (Pentair Aquatic Eco-Systems, Inc. Apopka, FL, USA). Fish were fed GEMMA Micro 300 nutrient flakes (Skretting USA, Westbrook, ME, USA) and were subjected to a 14:10 hr light: dark cycle. During parts of this study, zebrafish were also acquired from the Aquatron Laboratory Facility at Dalhousie University, the National Research Council of Canada (NRC) Laboratory in Halifax, Franz-Odendaal Laboratory in the Biology Department at Mount Saint Vincent University and were raised from eggs obtained from Zebrafish International Resource Center (ZIRC, University of Oregon, Eugene OR, USA). For further details of zebrafish sourcing, see Limitations section, Ch. 4.

2.2.3 Pharmacology

Atropine and TIM were made up in stock solutions of 1 mL aliquots of 1 mM in distilled water and frozen. Aliquots of these stocks were dissolved in zebrafish saline (Stoyek et al; (2016) with the composition: 142 mM NaCl, 4.7 mM KCl, 10 mM HEPES, 10 mM Glucose, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 7.2p H, 22°C) at one of 3 concentrations (3 μM, 10 μM, or 30 μM of either antagonist in 200 ml saline). Antagonist dilutions were made up on the day of experiments. All antagonists were delivered in saline supplied to the preparation by gravity feed from reservoirs hung above the bath, which was perfused at a constant flow rate (generally 5 ml/min) during the experimental protocols.

For trials to test the efficacy of the autonomic antagonists, hearts were first exposed to 5 μL boluses (1 mM) of agonists. Boluses of Musc, or ISO were delivered directly to the heart in the vicinity of the SAR and nearby atrium via focal pipettes placed within 0.5 mm of the tissue surface. The pipettes were connected to either a Hamilton microsyringe (0.1 ml total volume) coupled to a microliter injector (Hamilton Company, Reno, NV, USA) or to a Picospritzer II pressure ejection valve (Parker Hannifin, Cleveland, OH, USA). After recording HR responses to bolus agonist application alone, agonist doses were repeated in the presence of the appropriate antagonist in the perfusate. Lack of agonist responses during antagonist exposure was taken to indicate that the antagonists were blocking their respective receptors in this preparation.

All pharmacological agents used in this study were obtained from Sigma-Aldrich (Sigma-Aldrich Canada Co., 2149 Winston Park Drive, Oakville, ON, L6H 6J8, Canada).

2.2.4 Heart Isolation and Preparation

Zebrafish were placed in a room temperature vial containing buffered tricaine anesthetic (MS-222, 1.5 mM, 7.2 pH, Sigma Aldrich) dissolved in tank water for euthanasia. Dissection was begun when swimming and breathing movements ceased and the fish lacked a response to a tail pinch by forceps (approximately 5 min of MS222 exposure). Fish were transferred to a dissecting dish with a silicone rubber bottom (Sylgard, Dow Corning, Midland, MI, USA) and were pinned ventral side up with 100 μm diameter stainless steel pins inserted through the jaw, anal fin region, and both sides of the gill arches to provide stability for dissection. The pectoral fins and operculae were removed and a ventral incision was made from the isthmus between the gill openings caudally to the anal fin to expose the heart and open the coelomic cavity for removal of the viscera. Once exposed, the heart was removed from the body by transecting the rostral end of the bulbus arteriosus and caudal end of the sinus venosus. Hearts were transferred to a 22°C, 5 mL recording chamber with a Sylgard bottom, pinned through the bulbus arteriosus, ventricle and sinus venosus and were perfused with saline for 15 minutes to for acclimation before beginning experimentation.

2.2.5 Whole Heart ECG Recording

In all experiments ECG signals were recorded from the surface of the atria using a bipolar wire electrode and were differentially amplified (AM Systems Model 3000 AC/DC Differential Amplifier, AM Systems, Sequim, WA, USA; total system signal gain 500). Signals then underwent analog-digital conversion (Digidata 1322A, Axon Instruments, Foster City, CA, USA) and were stored as digital files on a personal computer.

2.2.5.1 Antagonist Exposure at 22°C

Following acclimation at 22°C, heart rate before and during drug application was recorded according to the following protocol: two HR control samples (20 sec duration each) were taken at 5 min intervals to ensure that heart rate was stable before drug application. The second control sample was designated as the "pre" value. Antagonists were then applied in the perfusate for 10 minutes at a constant flow rate of 5 mL/min. ECG samples of 20 s duration were recorded each minute during antagonist exposure.

2.2.5.2 Antagonist exposure at 27°C

Experiments at elevated temperature were done on separate hearts from those at 22°C. In the experiments at raised temperature, hearts were first held at 22°C while two 20 s samples of ECG were recorded 5 min apart to ensure HR stability. Then the incoming perfusate and the bath were heated to 27°C using a temperature control unit (model TC324B, Warner Instruments, Holliston, MA, USA) coupled to an inline heater for the bath substrate. Once HR had stabilized at 27°C, two further control recordings were taken. Then antagonists were applied to the preparation following the same protocol as in Section 2.2.5.1 above.

2.2.5.3 Evaluation of Antagonist Effectiveness.

Experiments to assess targeting of receptors by antagonists were done on groups of hearts separate from the main antagonist experiments outlined above. To first evaluate the effect of agonist exposure, two 60 s samples of ECG were taken 5 min apart; the first was taken as control for comparison with the agonist response. At 10 s after starting the second recording, a bolus of agonist was applied to the atrium. Recording continued for

the remainder of the 60 s sample period and continued for a further 60 s to capture the full agonist response. After 5 min of saline perfusion to "wash out" the agonist and allow HR to return to the control value, the heart was perfused with saline containing the antagonist, recording began, and the bolus dose of agonist was repeated at 10 seconds into the recording.

2.2.6 Data Collection and Analysis

For analysis of HR responses, data files were visualized offline using Axoscope 10 (Axon Instruments, Foster City, CA, USA). HR during 20 s ECG sample recordings was determined by counting beats during the sample period, then multiplying by 3 to determine beats per minute (BPM).

Statistical analysis was performed in accordance with Zar (2014). Statistical analysis and graphing were performed using PRISM (Version 9.4.0) (GraphPad Software, San Diego, CA, USA). Data were segregated into groups according to antagonist type, concentration, and temperature. Testing for normalcy or skewness (Kurtosis) of data was done with the D'Agostino-Pearson normality test. Comparisons within-group between Pre and antagonist mean HR were done using Students' paired t-test. This was done independently for all concentrations of both ATR and TIM at 22°C and 27°C. Comparisons of the differences in mean control HR between 22°C and 27°C trials were done with t-tests to establish the effects of change in temperature on basic HR for calculating Q_{10} values. One way analysis of variance (ANOVA) analyses were run to test for differences in control HR among the three groups at a given temperature. ANOVA was also used to analyze responses during antagonist effectiveness trials for comparing pre vs agonist, agonist vs agonist and antagonist, and pre vs agonist and

antagonist. In the case of significant F-values, Tukey's *post-hoc* multiple-means comparison tests were used to determine significant differences among specific means. All values are presented as mean ± SEM; *p*-values of < 0.05 were considered significant.

2.3 Results

2.3.1 Temperature

When the temperature of the perfused saline and experiment chamber was increased from 22°C to 27°C, baseline heart rate increased significantly. The mean "Pre" HR for each group was higher than the corresponding values recorded in hearts at 22°C, demonstrating that an increase in temperature caused a general increase in HR. Q_{10} was calculated as described in Chapter 1. (Pre-3 µM 110 ± 5 to 156 ± 7, p=0.0003, t test, Q_{10}=1.79 ± 0.23; Pre-10 µM 90 ± 3 to 149 ± 6, p<0.0001, t-test, Q_{10}=1.58 ± 0.12; Pre-30 µM 114 ± 4 to 168± 6, p=0.04, t-test, Q_{10}=1.59 ± 0.11) Average "pre" rate increased from 123±4 to 156±4 bpm (n=49, p<0.0001, t-test). Average calculated Q_{10} of all hearts in both studies was 1.65.

2.3.2 Parasympathetic Blockade

2.3.3.1 Effects of ATR Blockade at 22°C.

Experiments to test the effect of ATR exposure on HR at 22°C were done on hearts isolated from a total of 36 zebrafish. Before application of ATR, values for mean HR were not significantly different among the Pre value in the three groups (one-way ANOVA; F =2.33, p =0.11). Kurtosis of Pre HR values from individual hearts in each group was determined to be minimal; these values were thus taken to be normally distributed.

The effects of exposure to ATR are presented in Figures 2.1 and 2.2. Experimental protocol and sample ECG records taken before and during ATR exposure are shown in Figure 2. In these experiments each heart was evaluated for response to ATR at only one drug concentration. The responses of all hearts tested are summarized in Figure 2.2. Panel A of this figure shows that the trend in ATR response was cardioacceleration in all hearts in all ATR groups. The mean change in HR for each concentration of ATR reflects this, as shown in Figure 2.2 panel B. Application of 3 μM ATR induced an increase in HR from 109 ± 8 to 119 ± 10 bpm (approximately 10% rise from control); 10 μM resulted in an increase from 93 ± 5 to 111 ± 5 bpm (19% rise); and 30 μM evoked an increase from 119 ± 9 to 128 ± 8 bpm (approximately 8% rise). Paired t-tests showed that ATR-related HR changes from Pre values were significant in all groups (see Figure 2.2 caption for test details). Therefore, ATR in this range of concentration and at this temperature was effective in modulating HR.

2.3.2.2 Effects of ATR Blockade at 27°C.

Experiments on the effects of ATR at an elevated temperature were done at 27°C because this temperature was close to the acclimation temperature of the fish. In these experiments hearts from 19 zebrafish were exposed to the same ATR doses and with the same procedure as for experiments at the lower temperature. Values for mean HR were not significantly different among the pre groups (one-way ANOVA; $F = 0.95$ $p = 0.41$), Kurtosis of Pre HR values within each group was minimal. Rate responses of hearts to ATR at 27°C are shown in Figure 2.3. Panel A of this figure indicates that in all hearts tested the trend was for cardioacceleration during ATR exposure in all 3 groups.

The mean values for effects of ATR at 27°C are summarized in Figure 2.3B. At a concentration of 3 μM, ATR increased HR from 148 ± 11 to 160 ± 15 bpm (8% rise). 10 μM ATR resulted in an HR increase from 163 ± 8 to 182 ± 8 bpm (approximately 12% rise). ATR at 30 μM produced an increase in HR from 168 ± 5 to 173 ± 5bpm (3% rise). The degree of cardioacceleration evoked by ATR was significant within each group, as indicated by the asterisks in Figure 2.3B (t-test details in figure caption). As for the results of the experiments at 22°C outlined above, ATR across this range of concentrations was also consistently effective at 27°C in evoking cardioacceleration.

I attempted to consider the proportional effects of ATR at the three drug concentrations used by normalizing the data presented above in a plot showing percent change in HR from control values evoked within each group; this plot was done for both temperatures and is shown in Figure 2.4. There are clear trends in these proportional responses: for the data recorded at either temperature, the proportional response for the 10 μM ATR group was qualitatively stronger than those recorded at lower and higher ATR concentrations, suggesting perhaps that this was close to the optimum dose for this response, and was consistent over the temperature range used. However, because the 22°C and 27°C data sets were obtained at different times and from different sample populations no statistical comparisons were made between them.

2.3.2.3 Evaluation of Effectiveness of ATR Blockade.

In order to ensure that ATR used in these experiments was adequate to actually antagonize muscarinic receptors on the cardiac pacemaker cells, I tested the capability for ATR to eliminate responses to bolus doses of muscarine applied directly to the heart. A sample recording of the rapid-onset bradycardia evoked by muscarine, and its elimination

by 10 μM ATR, is demonstrated in panel B of Figure 2.1. In a total of 9 hearts, boluses of muscarine evoked a decrease in mean HR from 89 ± 6 to 69 ± 5bpm, as presented in Figure 2.5 (ANOVA details in figure caption). Panel A shows that the muscarine responses of the individual hearts all followed the same trend, while panel B indicates that mean HR during muscarine application was significantly less than the pre-application value. ATR, delivered in the perfusate, eliminated the effect of a repeat dose of muscarine (ATR + Musc bar in Figure 2.5B); mean HR during combined ATR and muscarine application was not significantly different from the mean Pre HR value (89 ± 6 to 88 ± 6bpm respectively). No agonist-antagonist trials were done for ATR at either 3 or 30 μM.

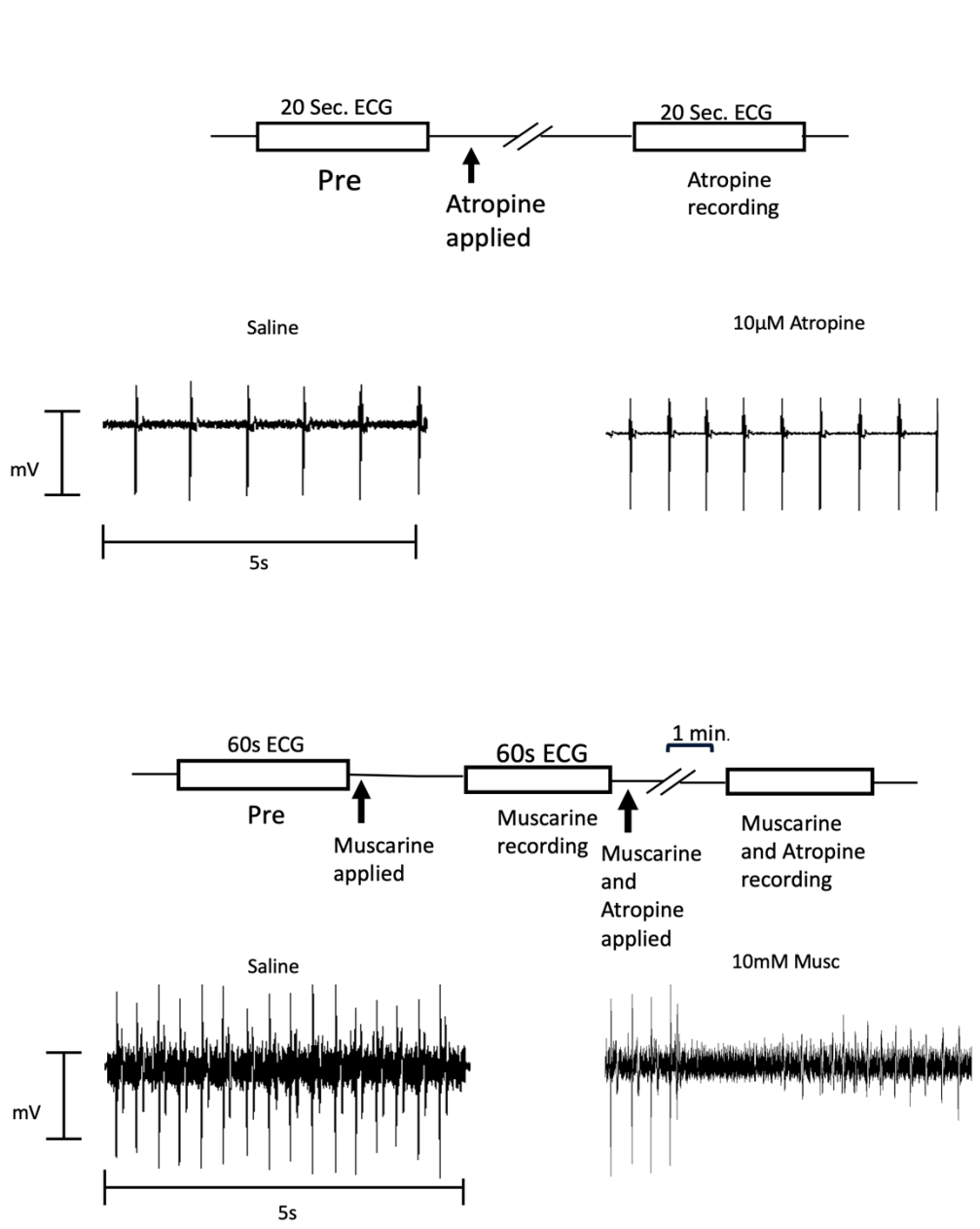


Figure. 2.1. Experimental protocol and ECG traces of parasympathetic blockade at 22°C A) Experimental protocol and representative sample before (left side of panels) and during (right side) exposure to ATR (concentration given above response trace). B) Experimental protocol and ECG trace for heart exposure to muscarine. Bottom: inhibitory effect of muscarine on HR (5 µl bolus, 1mM, via pipette close to atrial surface.) Amplitude indicated by scale bar on right side of figure: A: 0.2mV; B: 1mV

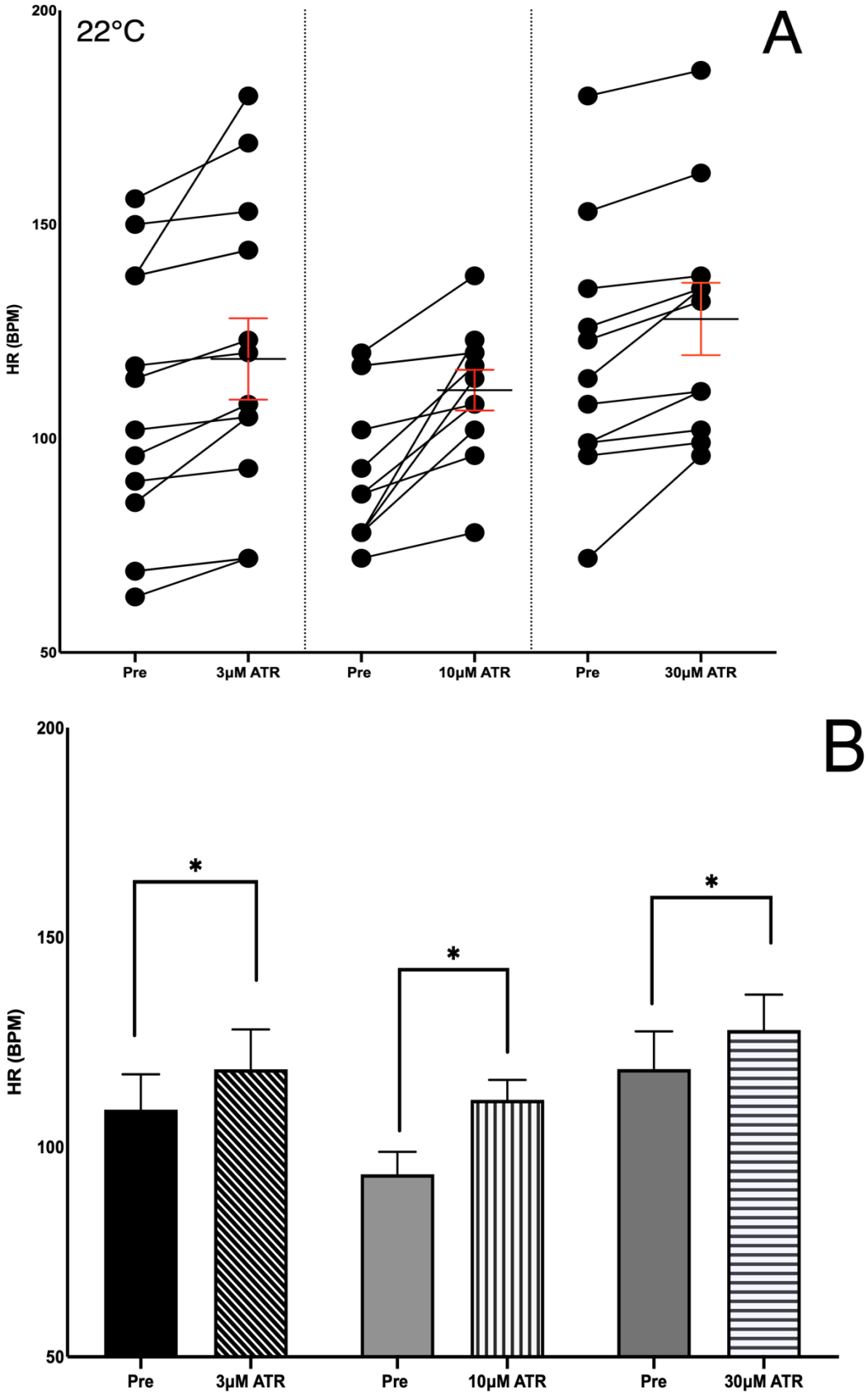
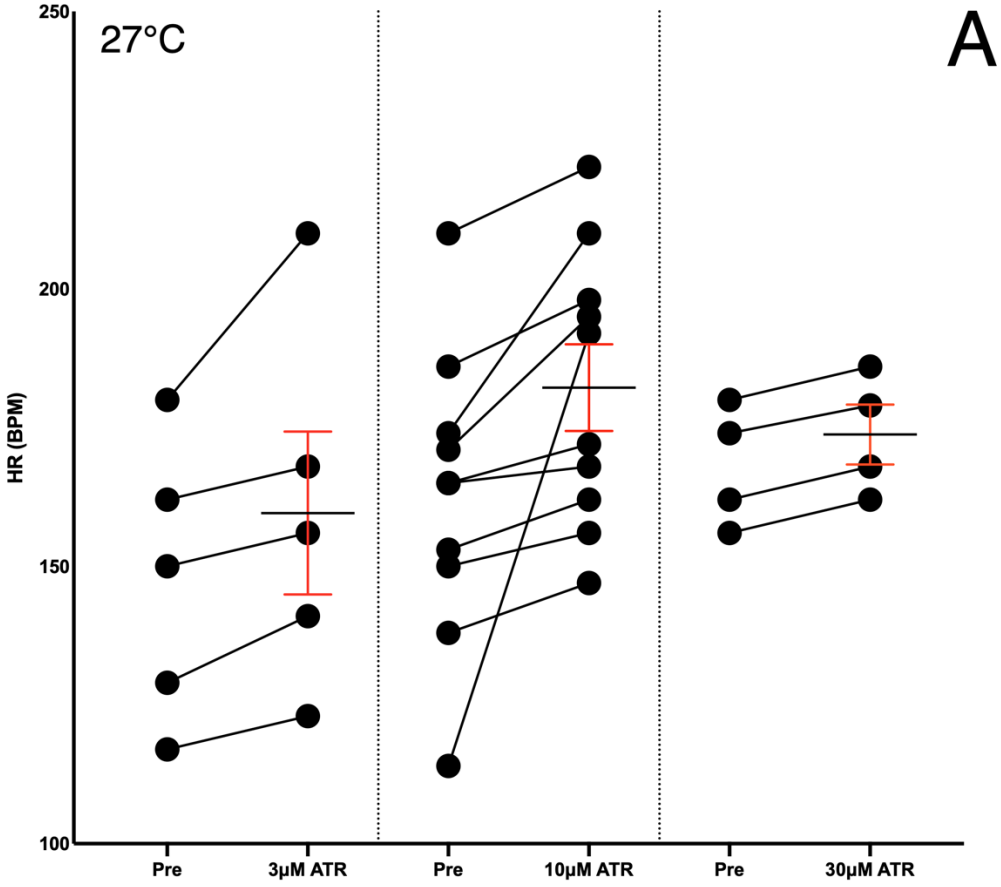

22°C
A
200
150
100
50
HR (BPM)
Pre
3μM ATR
Pre
10μM ATR
Pre
30μM ATR
B
200
150
100
50
HR (BPM)
*
*
*
Pre
3μM ATR
Pre
10μM ATR
Pre
30μM ATR

Figure 2.2 Summary of HR responses to ATR application at 22°C. A) Responses of individual hearts to ATR, grouped by concentration. Each heart within a concentration group is represented by a pair of data points connected with a line, from pre-ATR value to its respective value during ATR exposure. Only one dose of ATR was applied to any heart. Within each ATR group, mean HR values before and during exposure are illustrated by long horizontal lines with accompanying shorter horizontal lines indicating ± 1 standard error (SE). B) Summary of data in panel A replotted with vertical bars showing mean HR ± 1 SE before and during ATR exposure within each group. Asterisks represent significant differences within group between mean values, compared by t-test (3 μM: n=14, p=0.0047; 10 μM: n=11, p=0.0018; 30 μM: n=11, p=0.0018).


27°C
A
250
200
150
100
HR (BPM)
Pre
3μM ATR
Pre
10μM ATR
Pre
30μM ATR

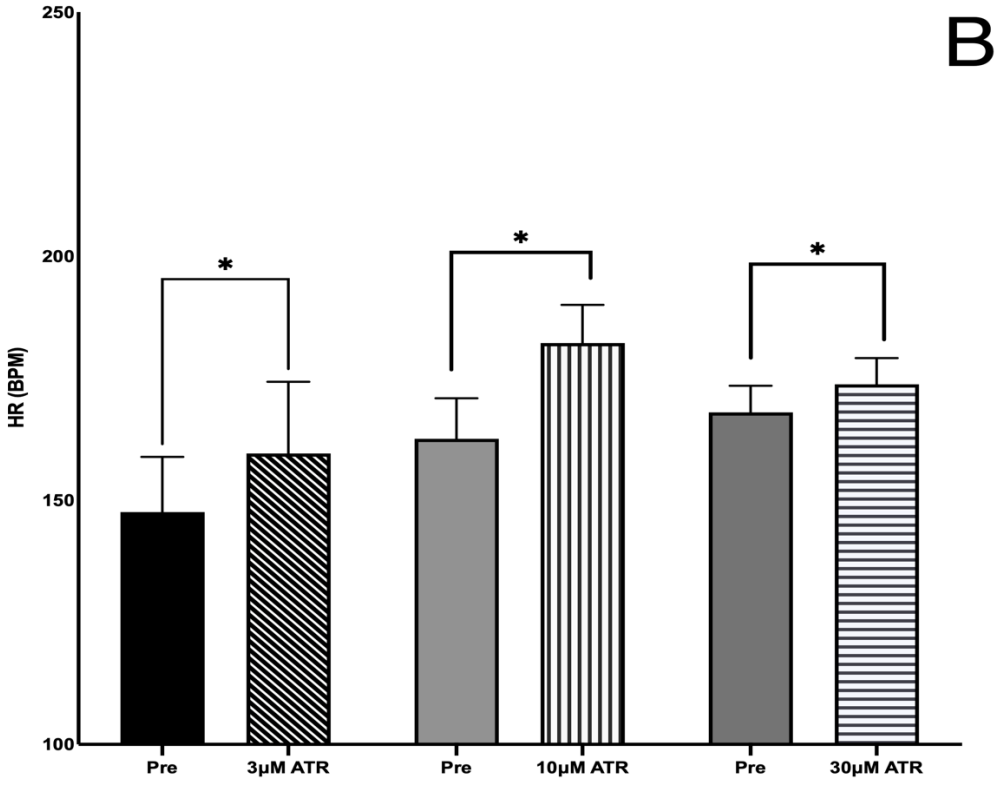

B
250
200
150
100
HR (BPM)
*
*
*
Pre
3μM ATR
Pre
10μM ATR
Pre
30μM ATR

Figure. 2.3. Summary of HR responses to ATR application at 27°C. Data presentation in this figure follows that of Figure 2.2. A) Responses of individual hearts to ATR, grouped into one of 3 concentrations. B) Summary of data in panel A replotted with vertical bars showing mean HR ± 1 SE. Asterisks indicate significant differences within-group between mean control HR and values during antagonist exposure (t-tests: 3 μM: n=15, p=0.0306; 10 μM: n=10, p=0.0234; 30 μM: n=14, p=0.0002).

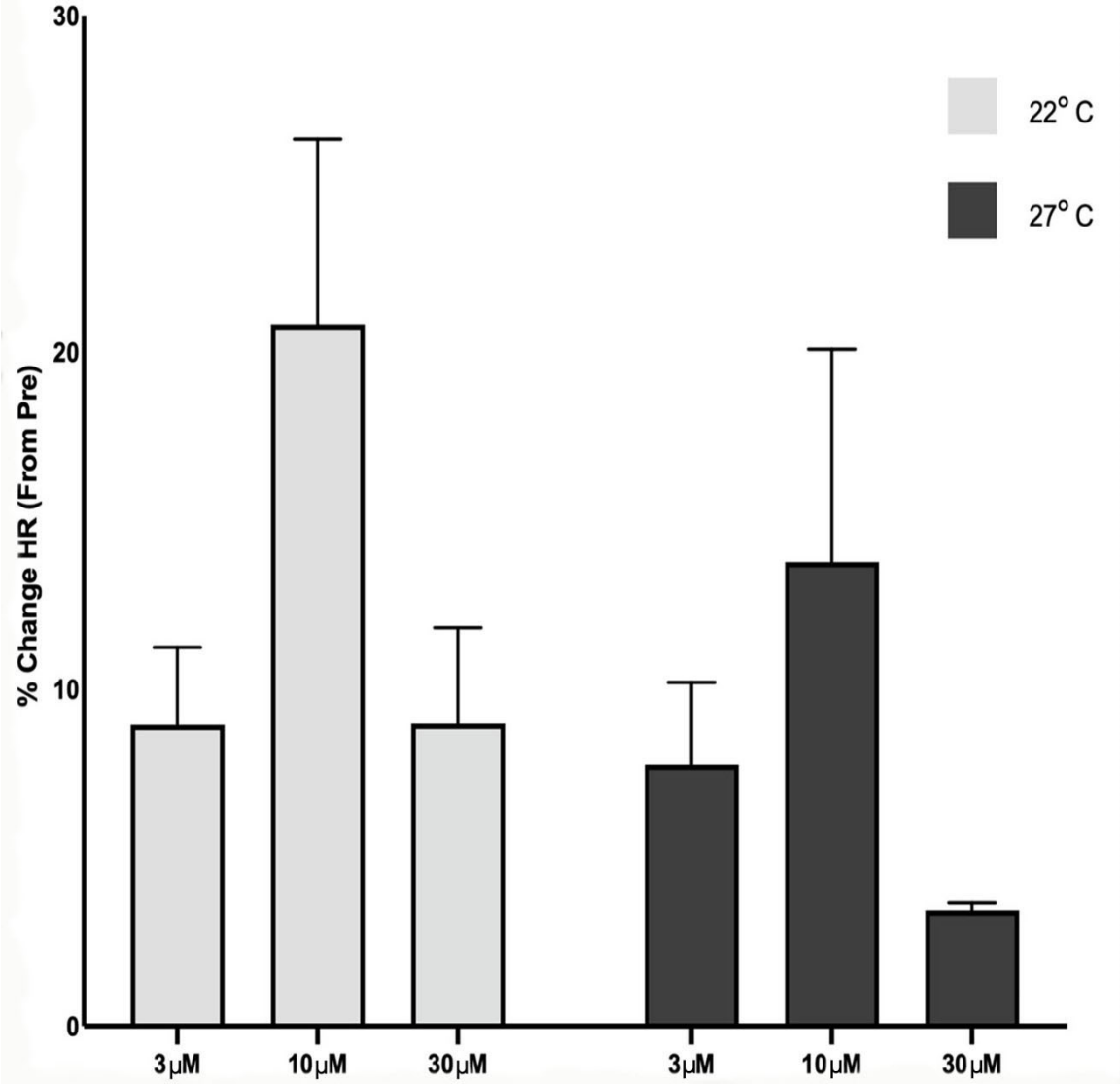
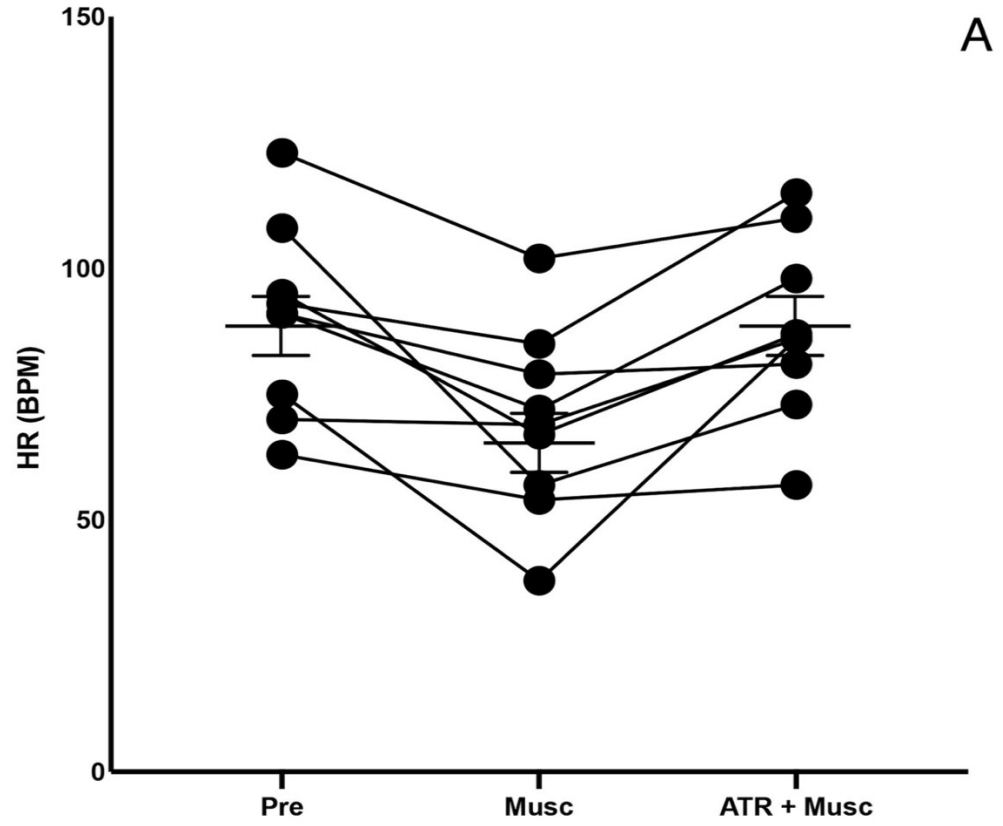


Figure 2.4. Proportional changes in mean HR induced by ATR. HR responses from Figures 2.2 and 2.3 were normalized and plotted as per cent change from the pre-exposure values for 22 and 27°C, for all concentrations of ATR (X-axis). Error bars presented as mean HR ± 1 SE.


A
150
100
50
0
HR (BPM)
Pre
Musc
ATR + Musc

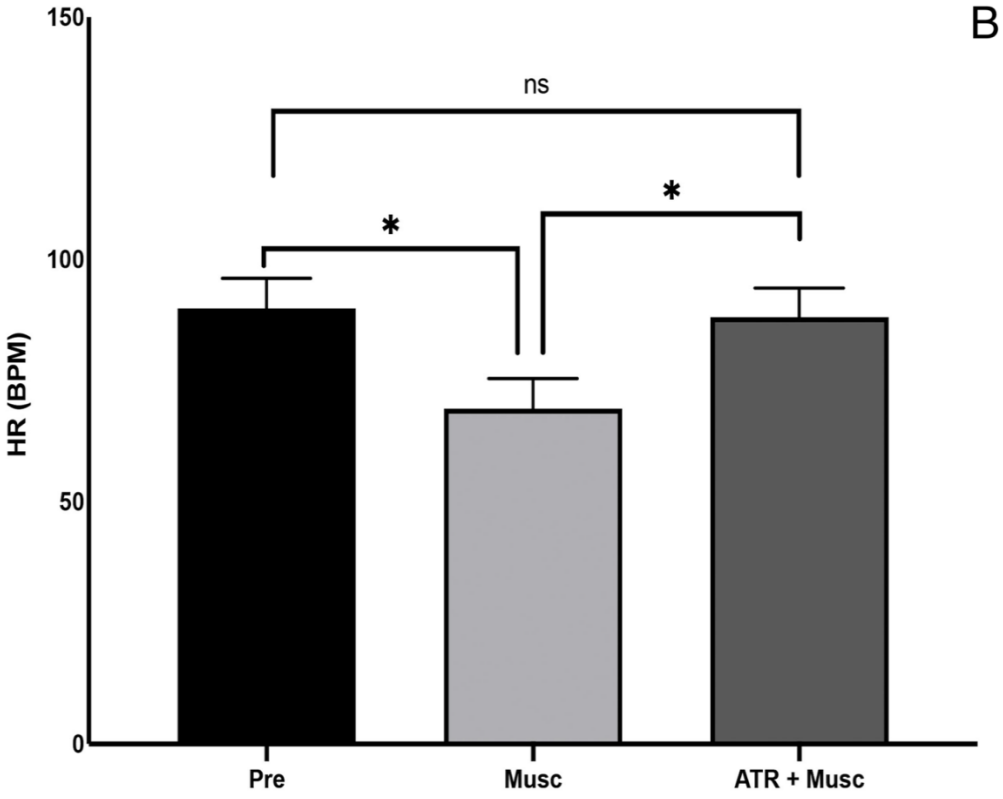

B
150
100
50
0
HR (BPM)
ns
*
*
Pre
Musc
ATR + Musc

Figure 2.5. Effectiveness of ATR blockade on muscarinic receptors on cardiac pacemaker. A) Responses of 9 individual hearts to application of bolus doses of muscarine, delivered to the atrium by pressure ejection from a local pipette (Musc); and effect of 10 μM ATR in perfusate, in combination with the bolus of muscarine (ATR + Musc). HR recorded from each heart is represented by data points connected by lines; mean values of HR in each data point column are represented by long horizontal lines with accompanying SE indicators. B) Summary of mean HR for data shown in panel A. Asterisks represent significant differences: between mean HR values taken before (pre) and during muscarine application ($p=0.0109$, ANOVA); and between HR values during exposure to muscarine alone and ATR in combination with muscarine (ATR + Musc) ($p=0.0113$, ANOVA). There was no significant difference between mean control and that recorded during ATR in combination with muscarine ($p=0.9509$, ANOVA).

2.3.3 Sympathetic Blockade

2.3.2.1 Effects of TIM Blockade at 22°C.

The effects of TIM exposure on HR at 22°C were tested in hearts isolated from a total of 53 zebrafish. In these experiments each heart was evaluated for response to TIM at only one drug concentration, so the data were separated into three independent groups reflecting the concentration of TIM applied to each group (3, 10 or 30 μM). In order to determine whether mean HR before TIM application (designated "pre") differed among the groups, a one-way ANOVA was performed ($F = 10.29$, $p = 0.0001$). This test produced a significant F-value so Tukey's *post-hoc* multiple-means comparison test was used to determine significant differences among these means. Mean Pre HR in the 10 μM TIM group was significantly lower than the values for the 3 and 30 μM groups but there was no significant difference between the latter two group means. Independent analyses of kurtosis patterns of Pre HR data in the three groups showed that within each group the HR values were normally distributed.

Cardiac chronotropic responses to three concentrations of TIM are summarized in Figure 2.6. Panel A shows that the overall trend in TIM response was consistent cardioinhibition at all antagonist concentrations. The change in mean HR evoked by TIM within each group was significant, as shown in Figure 2.6 panel B (asterisks; see Figure 2.6 caption for details of within-group t-tests). Application of 3 μM TIM caused a decrease in HR from 112 ± 5 to 101 ± 6 bpm (fall of 10% from pre); 10μM evoked a decrease from 90 ± 3 to 78 ± 3 bpm (13% fall). Application of 30 μM TIM decreased mean HR from 112 ± 5 to 95 ± 5 bpm (15% fall). Therefore, antagonism of β-AR over

this range of concentration and at this temperature was effective in evoking cardioinhibition in isolated hearts.

2.3.2.2 Effects of TIM Blockade at 27°C.

The Pre HR value for each group was significantly greater (3 µM, 112 ± 5 to 161 ± 11 , $p<0.0001$, t test; 10 µM, 90 ± 3 to 135 ± 8, $p<0.0001$, t-test; 30 µM 111 ± 5 to 169 ± 9, $p<0.0001$, t-test) than the corresponding group values for hearts at 22°C, demonstrating the temperature effect on HR expressed by the calculation of the corresponding Q_{10} values for this variable (3 µM, Q_{10}=1.86, 10 µM Q_{10}=1.81, 30 µM Q_{10}=1.96). One-way ANOVA analysis to determine whether pre-TIM mean HR was different among the three groups produced a significant F-value ($F =3.52$; $p =0.043$). Tukeys *post-hoc* multiple-means comparison tests showed that the Pre -TIM mean HR at 10 µM was significantly lower than the values in the other concentration groups. Independent analyses of kurtosis within-groups showed that distribution of pre-TIM HR values in the 30 µM group was skewed (kurtosis = 2.28). Despite the skewness of this value, I determined that a t-test was still appropriate to assess the difference between Pre and post values for 30 µM TIM, since this is robust and moderate kurtosis values do not obviate its use (Garren and Osborne, 2021).

To evaluate responses to TIM, hearts from 30 zebrafish were exposed to the same antagonist concentrations and with the same procedure at 27°C as for experiments at the lower temperature. Heart rate responses to TIM are shown in Figure 2.7. Panel A indicates that the trend of TIM responses showed consistent cardioinhibition in all 3 groups. The mean values for effects of TIM are summarized in Figure 2.7B; TIM evoked significant HR changes from Pre values at all concentrations tested (asterisks; see caption

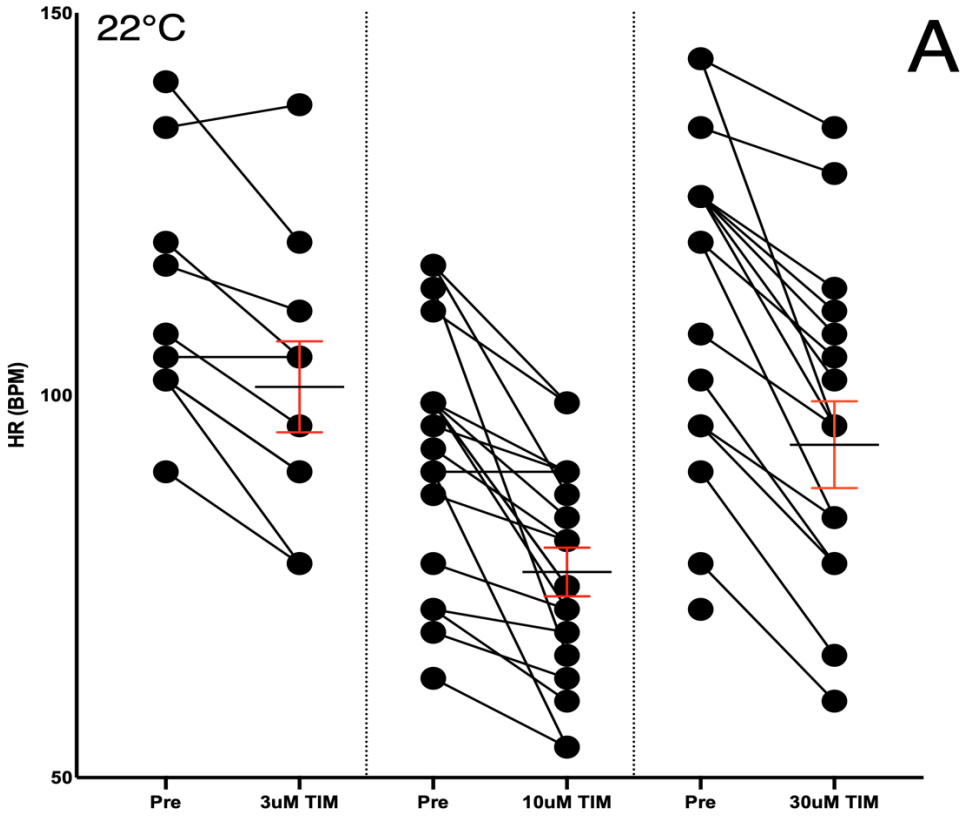
for Figure 2.7 for details of t-tests). TIM at 3 μM resulted in a decrease in HR from 161 ± 11 to 130 ± 10 bpm (19% decrease). TIM at 10 μM resulted in a fall in HR from 136 ± 8 to 106 ± 7 bpm (22% drop). Administration of 30 μM TIM produced a decline in HR from 169 ± 9 to 140 ± 9 bpm (22% decline). In summary, as for the results of the experiments at 22°C outlined in Section 2.3.3.1, TIM across the range of concentrations tested was consistently effective at 27°C in evoking cardioinhibition.

I attempted to consider the proportional effects of TIM on HR at the three drug concentrations used by normalizing the data for each group presented above and plotting these data to show percent change in HR from their respective control values. This plot was done for responses at both temperatures and is shown in Figure 2.8. Trends in HR responses to TIM are apparent qualitatively: proportional changes in HR at all concentrations at 27°C were all perceptibly greater than those evoked by the same concentration at 22°C. However, because the 22°C and 27°C data sets were obtained at different times and from different sample populations no statistical comparisons were made between them.

2.3.2.3 Evaluation of Effectiveness of TIM Blockade.

To ensure that the application of TIM was adequate to actually antagonize activation of β-AR on pacemaker cells, I tested the capability for TIM to eliminate responses to bolus doses of ISO applied directly to the heart by focal ejection from a pipette with its tip close to the atrium. In a total of 10 hearts, boluses of ISO evoked increases in HR (mean change in rate was from 81±6 to 96±5bpm), as presented in Figure 2.9. Panel A shows that ISO responses of individual hearts all followed the same trend (Pre to ISO). Panel B indicates that the mean HR during isoproterenol application was

significantly greater than the pre-application value (asterisk, Pre-ISO comparison; see details of ANOVA analysis in caption). TIM, delivered in the perfusate (10 μM), eliminated the effect of a repeat dose of isoproterenol on each heart (Figure 2.9A) and collectively (Figure 2.9B; asterisk, ISO to TIM+ISO comparison). There was no significant difference between mean Pre HR and the value during combined exposure to ISO and TIM (81 ± 5 to 82 ± 5bpm respectively). No agonist-antagonist trials were done for TIM at either 3 or 30 μM.


22°C
A
150
100
50
HR (BPM)
Pre
3uM TIM
Pre
10uM TIM
Pre
30uM TIM

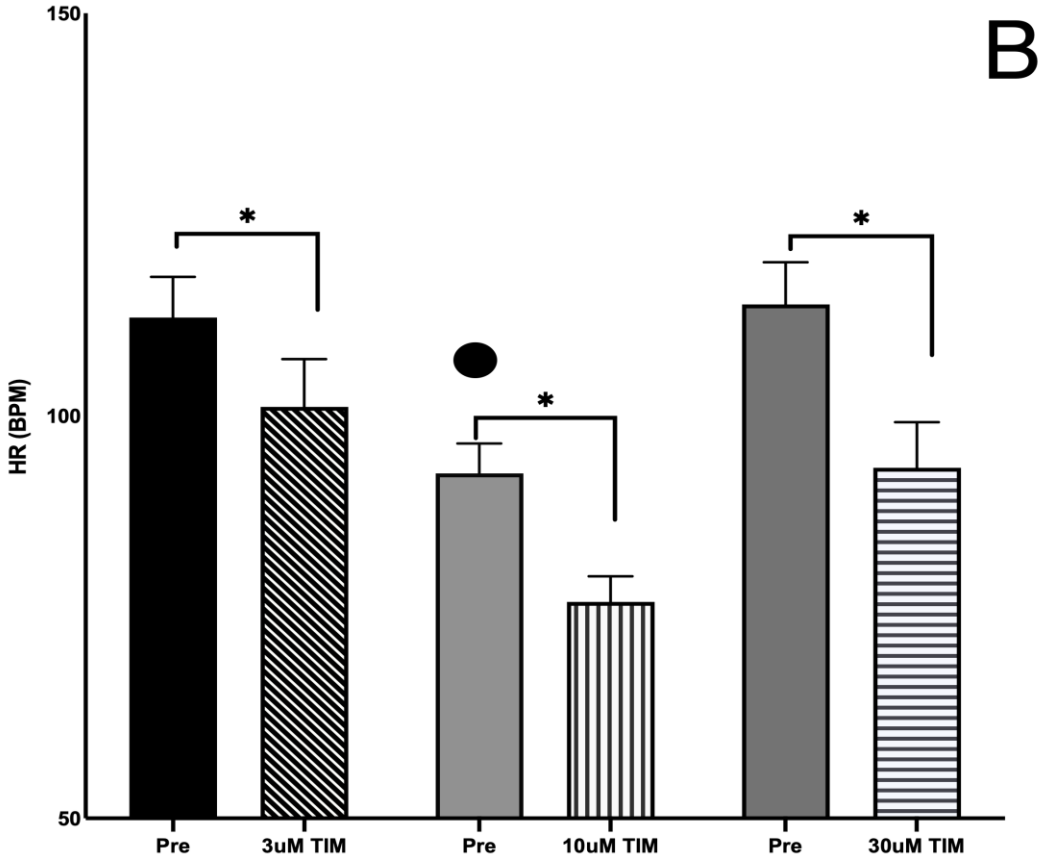

B
150
100
50
HR (BPM)
*
*
*
Pre
3uM TIM
Pre
10uM TIM
Pre
30uM TIM

Figure 2.6. Summary of HR responses to TIM application at 22°C. Data presentation in this figure follows that of Figure 2.2. A) Responses of individual hearts to TIM, grouped by concentration. B) Summary of data in panel A replotted with vertical bars showing mean HR ± 1 SE before and during TIM exposure. Circle indicates that the f-value of Pre group of 10μM was significantly lower than other pre groups, compared by ANOVA. Asterisks represent significant differences between within-group mean values, compared by t-test (3 μM: n=10, p=0.0023; 10 μM: n=23, p=0.0014; 30 μM: n=20, p<0.0001).

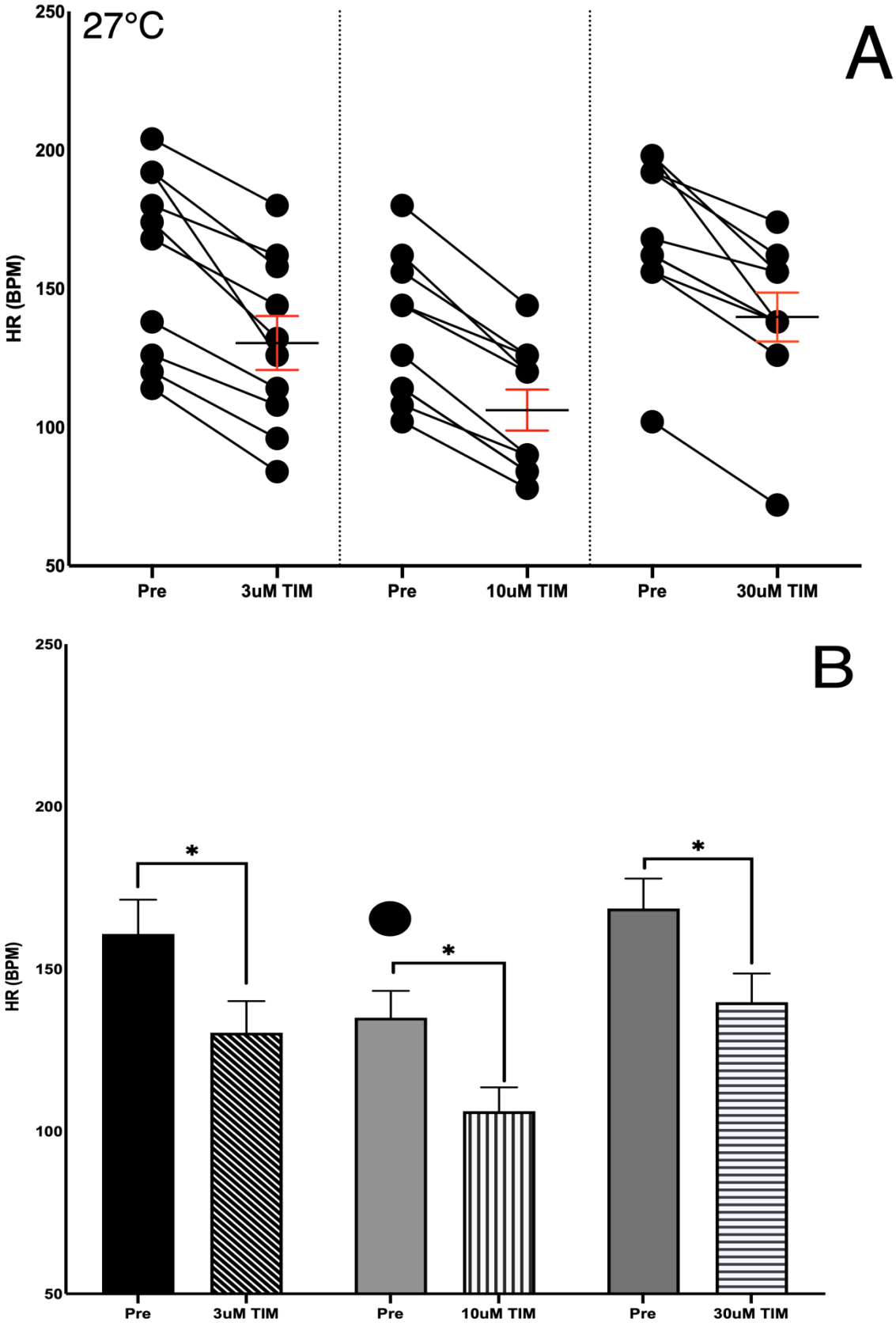

27°C
A
250
200
150
100
50
HR (BPM)
Pre
3uM TIM
Pre
10uM TIM
Pre
30uM TIM
B
250
200
150
100
50
HR (BPM)
*
*
*
Pre
3uM TIM
Pre
10uM TIM
Pre
30uM TIM

Figure 2.7. Summary of HR responses to TIM application at 27°C. Data presentation in this figure follows that of Figure 2.6. A) Responses of individual hearts to TIM, grouped by concentration. B) Summary of data in panel A replotted with vertical bars showing mean HR ± 1 SE. Circle represents value of Pre group of 10μM was significantly lower than other pre groups, compared by ANOVA. Asterisks represent significant differences between within-group mean values (t-test: 3 μM: n=10, $p<0.0001$; 10 μM: n=10 $p<0.0001$; 30 μM: n=10, $p<0.0001$).

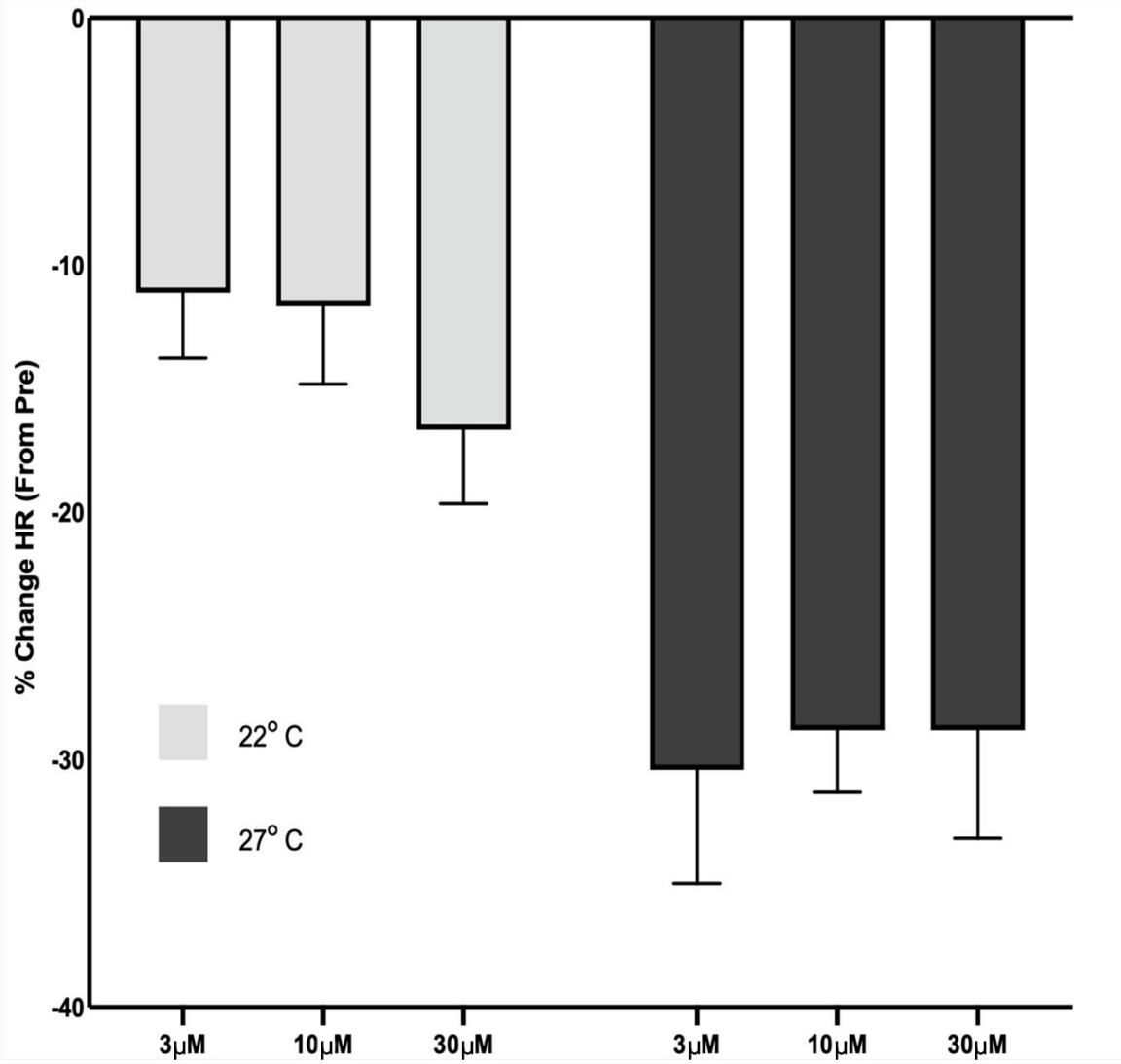
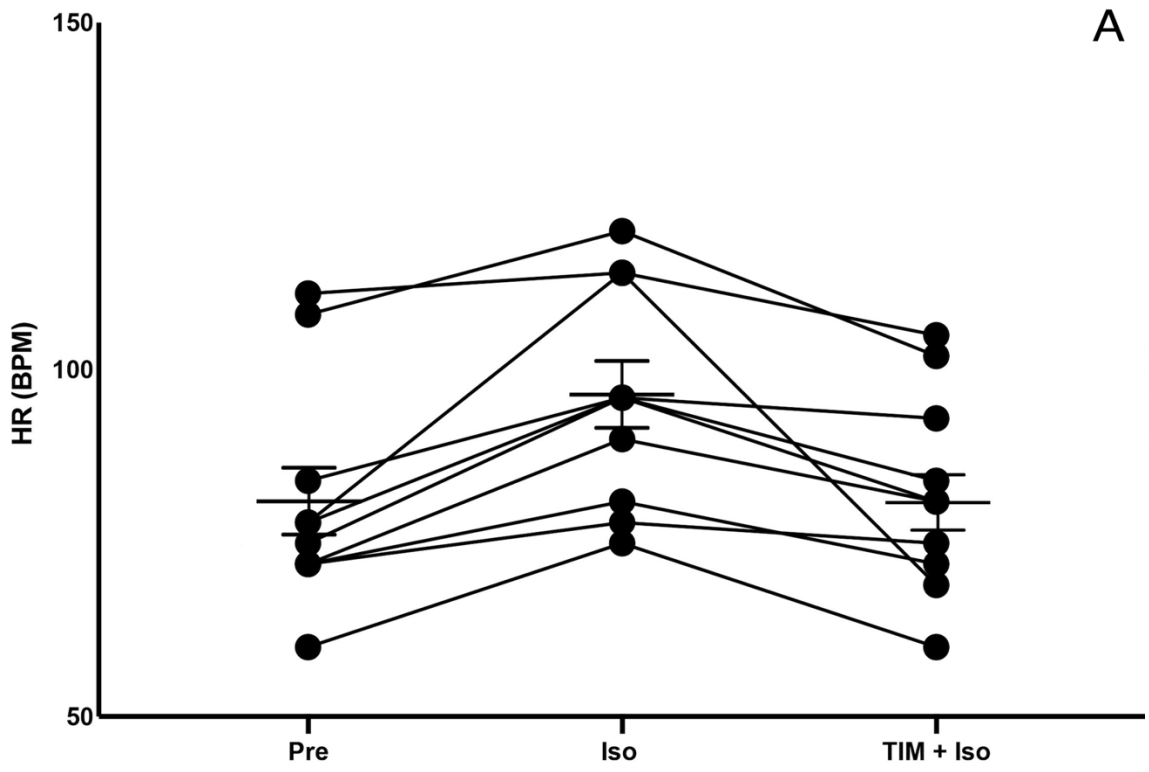


Figure 2.8. Proportional changes in mean HR induced by TIM. HR responses from Figures 2.6 and 2.7 were normalized and plotted as per cent change from the pre-exposure HR values for 22 and 27°C, for all concentrations of TIM (X-axis). Error bars represent ± 1 SE.


A
150
100
50
HR (BPM)
Pre
Iso
TIM + Iso

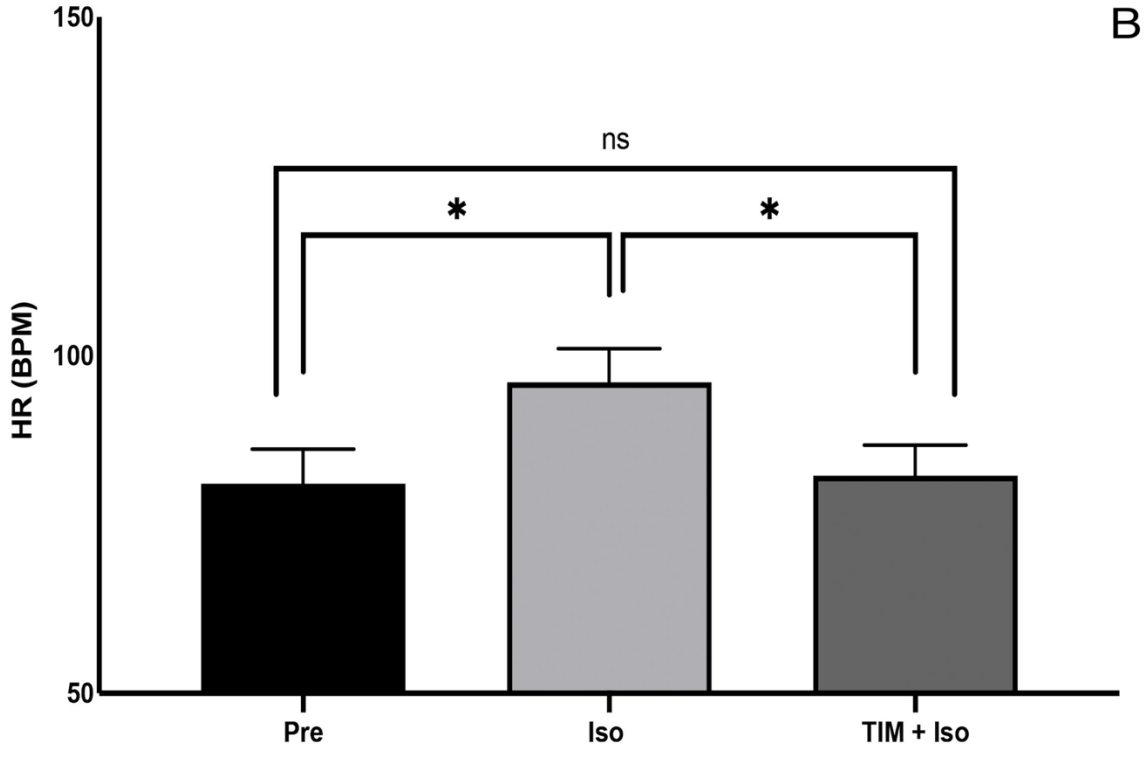

B
150
100
50
HR (BPM)
ns
*
*
Pre
Iso
TIM + Iso

Figure 2.9. Effectiveness of TIM blockade on β-adrenergic receptors on cardiac pacemaker. A) Responses of 10 individual hearts to application of bolus doses of Iso, delivered to the atrium by pressure ejection from a local pipette (Iso); and effect of 10 μM TIM in perfusate, in combination with Iso (TIM+Iso). HR recorded from each heart is represented by data points connected by lines; mean values of HR at each data point column are represented by long horizontal lines with accompanying SE indicators. B) Summary of mean values of HR ± SE for data shown in panel A, analyzed by one-way ANOVA ($f=14.34$; $p=0.0007$). Asterisks represent significant differences: between mean HR values taken before (Pre) and during Iso application (Iso) ($p=0.0016$, ANOVA); and between HR values during Iso exposure and TIM in combination with Isoproterenol (TIM + ISO) ($p=0.0137$, ANOVA). There was no significant difference between mean Pre HR and that recorded during TIM in combination with Iso. ($p=0.8785$, ANOVA)

2.4 Discussion

In this study, the presence of spontaneous autonomic tone in isolated zebrafish hearts has been established. As well, the effects on temperature on this tone, and the concentration effects of pharmacological antagonists have been explored. While the effects of variations in drug concentrations have been explored previously in mammalian and even human studies (Horiguchi and Nishikawa, 2002), the present study was novel in its approach to investigating this aspect of characterizing autonomic tone.

2.4.1 Hierarchy of Control and Stand-Alone ICNS

While the classical view of autonomic outflow has been via a two-neuron relay with all processing power being held within the CNS, here I have further illustrated the multiple levels in the hierarchy of control as in Figure 1.4 and provided further evidence to reinforce the concept of a "stand-alone" system in the ICNS. Application of pharmacological antagonists produced chronotropic changes, illustrating the presence of tone, both sympathetic and parasympathetic in origin. This tone exists independent of extracardiac input from the central nervous system and is able to modulate rate.

2.4.1.1 Parasympathetic Tone

In the isolated zebrafish heart, application of ATR invokes tachycardia. On the parasympathetic branch of ANS, pre-ganglionic projections begin in the medulla and synapses with post ganglionic neurons at or near the target organ. In the case of the heart, the post ganglionic neurons lie within the heart. In the isolated heart, parasympathetic efferent neurons exist, however because of the isolation there is no extracardiac signalling to them to carry an upstream influence on pacemaker discharge. Considering that M_2R on the pacemaker cells are the furthest down-stream signalling receptors in this

system, blocking input to them should have no effect on cardiac output. However, the change in HR due to ATR application in my experiments implies that there is parasympathetic tone generated up-stream from the effectors within the isolated heart that is sending signals to pacemaker M_2R, to drive HR down.

2.4.1.2 Sympathetic Tone

On the sympathetic side of the ANS, preganglionic neurons synapse with post-ganglionic neurons in the sympathetic chain. These post ganglionic neurons then project to their target organs and in the heart, extend directly to activate β-adrenergic receptors influencing pacemaker firing. In the classical view of this system in which there are no sympathetic postganglionic neurons in the ICNS, signalling to β_2R should not be possible. Sympathetic efferent neurons should not exist. However, as I have shown, blocking of β_2R with TIM invokes bradycardia. The evoked decrease due to TIM implies that there is drive from sympathetic efferent soma in the ICNS to the pacemaker that is elevating HR above its intrinsic value, thus validating the proposal of such neurons embodied in the model of Figure 1.4. The presence of both sympathetic and parasympathetic efferent neurons in the heart, as well as the generation of tone by both these populations further legitimizes the theory of the ICNS being able to act as a stand-alone system to modulate HR bidirectionally.

2.4.2 Direct Receptor Antagonism

In chapter 1, section 1.5.1, I cited a review by Barros et al (2008) that stated that our lack of understanding of the actions and mechanisms of common cardiotropic drugs in zebrafish remains a severe limitation in the field. Here I explored that issue by directly applying these agents to the isolated zebrafish heart.

2.4.2.1 Parasympathetic Antagonism

In the literature in this field, the range of ATR concentrations used in teleosts has been vast. 100 nM/kg to 25 mg/kg. However, the various method of exposing cardiac tissue to ATR in these studies cofounds the estimation of the effect of ATR due to unreliable measures of the actual concentration at the tissue. Application of agents in tank-water does not consider problems with transmission (or potential lack there-of) across the gills of the fish, and even exposure by injecting into the blood does not account for any upstream effects (such as ATRs effect on the gills) that may occur as the drug continues to pass through the system. Simply put, level of drug at the receptors and responses to said drug has remained problematic. By applying ATR directly to the isolated heart, I have avoided the issue of effects due to concentration at receptors being unknown, (because agents are not being diluted/removed by the system) and have established a concentration range for ATR that is sufficient to block M_2R.

In studies of isolated mammalian hearts, 5 μM ATR was found to be effective (Alpin et al, 1995; Langley et al, 1977), while in isolated zebrafish hearts, 10 μM ATR was found to effectively block the effects of vagal stimulation (Stoyek et al, 2016). Here I have found that a concentration as low as 3 μM ATR can provide significant cardioacceleration in an isolated zebrafish heart. While the lack of paired data has prevented further analysis of comparisons across concentrations to evaluate the optimal ATR dose, I do believe that with further experimentation and the use of multiple doses on the same heart, an optimal dose for maximal cardiac response could be found.

2.4.2.2 Sympathetic Antagonism

There have been fewer studies regarding the effects of adrenergic antagonists such as TIM than of cholinergic antagonists in teleosts. Stoyek et al (2016), showed that 10 μM TIM was sufficient to eliminate ISO induced tachycardia in zebrafish, little recent literature exists on TIM usage, let alone usage in zebrafish models. In isolated rat hearts, TIM was shown to reduced HR at a concentration of 50 μM (Nasa et al, 1992).

By applying TIM directly to isolated hearts, I have addressed the issue of concentration being unknown at the level of receptors in the heart, and I have shown a range concentration of TIM from 3 to 30 μM is sufficient to effectively antagonize β-receptors. In my experiments as little as 3 μM TIM can induce significant cardioinhibition. Interestingly, Vornannen has stated (2017) that resting adrenergic tone may be nonexistent in teleost hearts. But here I have shown that resting adrenergic tone is present, contributing proportionally between 7 to 19% of spontaneous heart rate.

2.4.3 Temperature and ICNS Q_{10}

At 22°C the average percentage change due to ATR application was an increase of 12%. The average change due to TIM was a decrease of 7%. Combined, this could imply that there is more parasympathetic tone driving HR down, and that the intrinsic rate of these hearts is slightly faster than their spontaneous rates when isolated from the body.

Raising of the temperature of the system evoked a significant overall increase in HR. The calculated Q_{10} of 1.65 is similar to that found by Perrichon et al. (2017) in the maki-maki. While this value is less than that found in some other species of teleosts (2.37, thunnus albcares (Korsemeyer et al, 1997); 2.21, Ptychocheilus oregonen(Kolok

and Farrel, 1994); 2.65 Catostomus macrocheilus (Kolok et al, 1993)) It is worth noting, that thermal acclimation (chronic change in temperature) has been shown to have an effect on the intrinsic HR of teleosts and that fish raised at a higher temperature have a lower Q_{10} than those at a lower temperature (Farrell and Smith, 2017).

Raising the temperature of the system results in the modulation and change of expression of cardiac ion channels in myocytes which can influence excitability. In brown trout, myocyte I_{K1} does not deteriorate below 35°C while expression of I_{KR} increases up to 28°C, but I_{Na} decreases below 20.9°C (Vornanen, 2017). As long as the influence of I_{K1} does not outweigh I_{NA}, myocyte APs will continue to fire at an increased rate at higher temperatures. However, temperature effects on ion channels in cardiac pacemaker cells in fish are not well understood. Furthermore, in the context of the nervous system, higher temperatures have been found increase excitability in mammalian motor neurons (Kiernan et al, 2001) due in part to the increase in inactivation rate of Na^+ channels in an axon. Increasing inactivation rate of these channels allows for a shorter AP by reducing the overlap between competing inward Na^+ and outward K^+ channels (Yu et al, 2012) and also likely an overall increase in AP firing rate. However, temperature effects on the ANS are not well understood in any vertebrate group.

At 27°C the average percentage change due to ATR application was an increase of 9%. At the increased temperature TIM induced a 19% decrease. Such a temperature increase would have raised conduction velocity of neural axons within the heart and therefore partially increased its capacity to respond to change. Additionally, an increase in AP firing rate in the ICNS is likely. The temperature related difference in effectiveness of ATR and TIM application on HR would imply that at 27°C there is more sympathetic

drive increasing HR and that intrinsic HR is lower than starting rate. In the context of recent findings in fish, acute warming of bluefin tuna was seen to increase the efficacy of adrenergic stimulation (Shiels, 2017). However, Vornannen (2017) suggests that adrenergic tone in fish is low, and that modulation of rate cannot occur through I_{Ks} as in mammalian heart, but only through I_{KR} or I_{K1}. In my experiments I saw an increase in adrenergic tone with an increase in temperature, but the mechanism for this effect is unclear.

The trend toward a decrease in the effect of ATR at 27°C may be partially attributed to a physiological ceiling on the capacity of fish hearts to maintain cardiac output at higher temperatures. A study by Barrioneuvo and Burggren (1999) in zebrafish found that resting HR of fish reared at 28°C peaked at ~190 bpm at 20 days old and settled to ~140 bpm by 3 months of age. The average HR of zebrafish at 27°C in my experiments was 157 bpm which is already above the resting rate found by Barrioneuvo and Burggren so application of ATR at the higher temperatures may result in diminishing returns for HR responses due to this potential physiological cap on rate.

CHAPTER 3 Investigating Autonomic Tone

3.1 Introduction

Given the evidence outlined in Chapter 2 that the pacemaker is influenced by outputs from the ICNS in the isolated heart, a number of questions arise about how this system may work. I propose here three specific questions about the properties of this system, to focus the studies in this chapter.

1) What neurotransmitter mechanisms might be involved in generating autonomic tone in the ICNS?
2) What are the patterns of neuronal discharge in the ICNS that together make up the spontaneous activity that is ultimately funnelled into sympathetic and parasympathetic outputs to the pacemaker cells?
3) What is the origin of the drive to the ICNS that results in outputs to the pacemaker?

In the following sections I examine these questions in more detail, generate hypotheses and briefly outline approaches to test these hypotheses. The model illustrated in Figure 3.1 acts as a guide to frame these studies.

3.1.1 Role of Nicotinic Neurotransmission in Autonomic Tone

In the mammalian heart neurons expressing a variety of neurotransmitters have been described, including cholinergic, adrenergic, peptidergic, purinergic and nitrergic (summarized in Ardell and Armour (2016) and others). In the fish heart similar neuronal phenotypes have been identified (for example, Stoyek et al, 2015; 2016). However, in hearts from all vertebrate groups so far examined, the majority of intracardiac neurons appear to express ACh. It is also apparent from a number of functional studies of neurotransmission in this system that the most common mechanism for synaptic

operation is that of nicotinic cholinergic excitation of postsynaptic membrane receptors (Ardell and Armour, 2016; Stoyek et al, 2016; Rajendran et al, 2019). This synaptic mechanism therefore presumably underlies ongoing spontaneous neural activity within the ICNS that is responsible for local autonomic drive to the pacemaker in both intact hearts in situ and isolated hearts *in vitro*. However, the details of the synaptic interactions responsible for this activity are not clear in the hearts of any vertebrate group so far examined. On this basis **I hypothesized that nicotinic cholinergic neurotransmission is the major mechanism underlying the neuronal activity within the ICNS that results in modulating pacemaker discharge rate in the isolated zebrafish heart.**

In order to investigate the role of nicotinic cholinergic synapses in generating autonomic tone in the zebrafish heart, I used a known ganglionic synaptic blocker, hexamethonium. This agent antagonizes cholinergic nicotinic neurotransmission by blocking the ion channel associated with the nicotinic postsynaptic receptor. This should therefore eliminate any neural effects on the pacemaker cells that originate within the ICNS. Both sympathetic and parasympathetic neurons efferent to the pacemaker are believed to be present in the heart and drive via both of these output pathways would presumably be affected by interrupting nicotinic synapses (indicated at locus "**1**" in Figure 3.1). However, the possibility that "upstream" nicotinic blockade with HEX could alter both sympathetic and parasympathetic effects on HR may lead to ambiguity in the outcome of such experiments. Thus, if application of hexamethonium alters HR, differentiation of the effects of such blockade on sympathetic versus parasympathetic efferent neuronal populations is required. This is illustrated by two entries for locus "**1**"

in Figure 3.1. Treating each case independently, if excitatory inputs via nicotinic synapses on parasympathetic efferent neurons are driving bradycardia, then application of hexamethonium should increase HR much as would the application of ATR acting at the neuroeffector junctions on pacemaker cells. However, if inputs via nicotinic synapses on sympathetic efferent neurons are driving tachycardia, hexamethonium should decrease heart rate by removing presynaptic drive to these neurons. Therefore, potential confounding effects of hexamethonium application on adrenergic drive at the pacemaker should be eliminated with application of timolol concurrently with hexamethonium application, uncovering the role played by muscarinic postjunctional receptors. Conversely, cofounding effects of hexamethonium on muscarinic drive at the pacemaker should be resolved by application of ATR concurrent with hexamethonium, thus differentiating the role played by adrenergic postjunctional receptors. If, however, only one autonomic limb is active at a given time, application of hexamethonium should generate the same effect as application of the antagonist for that limb. Overall, these experiments would confirm the role of nicotinic cholinergic synapses within the ICNS in generating autonomic tone to the pacemaker.

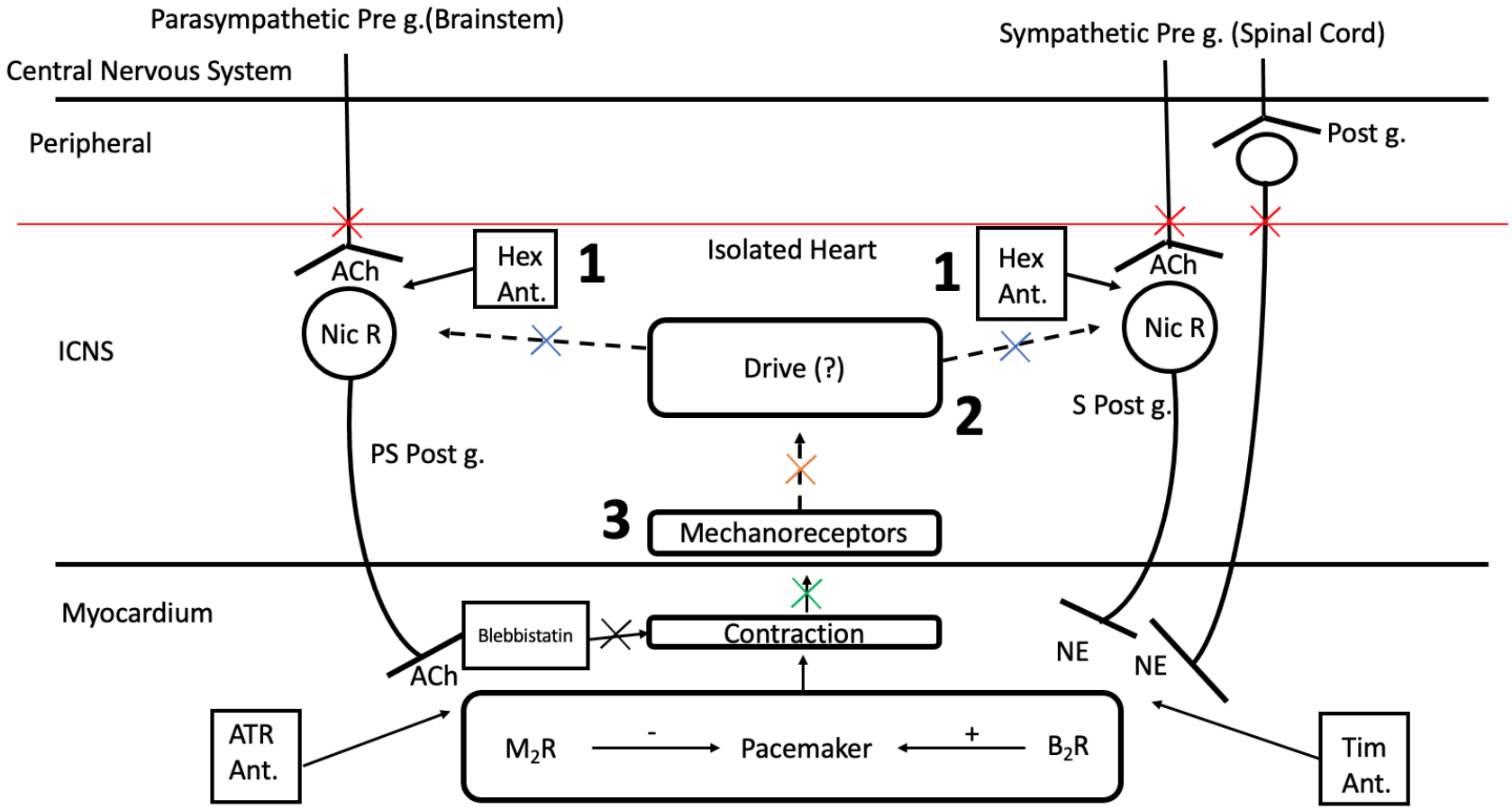

Parasympathetic Pre g.(Brainstem)
Sympathetic Pre g. (Spinal Cord)
Central Nervous System
Peripheral
Post g.
ACh
Hex Ant.
1
Isolated Heart
1
Hex Ant.
ACh
ICNS
Nic R
Drive (?)
Nic R
2
S Post g.
PS Post g.
3
Mechanoreceptors
Myocardium
Blebbistatin
Contraction
ACh
NE
NE
ATR Ant.
M2R
-
Pacemaker
+
B2R
Tim Ant.

Figure 3.1. Diagram of sites to target autonomic tone in the heart. Removal of the heart from the body eliminates extracardiac inputs from the brain to the heart, via preganglionic axons (parasympathetic and sympathetic) and sympathetic postganglionic axons, as indicated by red Xs at the "Isolation" line. The portions of the neural pathways remaining after isolation of the heart from the body are located below the "isolation" line. Antagonists are shown within square boxes and their sites of blocking are indicated by associated arrows. **1**) HEX application antagonizes NIC receptors on neurons within both parasympathetic and sympathetic circuits (blockade represented by blue Xs). The dotted line between the box labelled "drive" and neurons expressing nicotinic receptors indicated that this drive is hypothesized but not confirmed. **2**) Neural APs responsible for driving autonomic tone should be detectable by focal extracellular electrodes placed near active clusters of somata. **3**) BLEB application uncouples contraction of myocytes from the electrical signal to contract (black X). Contractions related to the mechanical cycle of the myocardium are proposed to excite mechanoreceptors so BLEB application should offload the mechanoreceptors (green X), thus eliminating inputs to the neuronal circuitry (orange X).

3.1.2 Neuronal Discharge Patterns in the ICNS

The experiments reported in Chapter 2 of this thesis have shown the presence of autonomic outputs directed to the pacemaker, but these results provide only indirect evidence of electrical activity of the intracardiac neurons themselves. Given that at least a subset of efferent intracardiac neurons must be spontaneously discharging APs in order to influence pacemaker cells, **I hypothesized that evidence of such activity should be directly detectable by extracellular electrical recording using electrodes placed near active intracardiac neurons** (Figure 3.1, locus "**2**"). This technique has been widely used in the mammalian ICNS to record ongoing, spontaneous APs (Ardell et al, 1991; Butler et al, 1990), I thus attempted to record activity from small clusters of spontaneously active ICN at localized sites in the SAR.

In addition to recording spontaneously active neurons, this technique should be able to detect stimulation-evoked activity in the ICNS. Stoyek et al (2016) provided evidence that electrical stimulation of vagosympathetic trunks attached to isolated zebrafish hearts invoked bradycardia. This occurs through a 2-stage process in the ICNS; First terminals of incoming preganglionic axons in the extracardiac nerves targeting parasympathetic postganglionic efferent neurons activate these neurons by nicotinic postsynaptic depolarization. Then the efferent neurons that project to the pacemaker cells cause bradycardia by muscarinic inhibition of these cells. In the present study, I propose to use stimulation of the vagus nerves to evoke APs in intracardiac neurons to characterize their discharge. Further confirmation of the role of nicotinic synaptic neurotransmission in the ICNS should also be provided by the application of

hexamethonium during stimulation, as proposed in Section 3.1.1, which would act to block vagally-evoked neuronal activity.

3.1.3 Origin of Autonomic Tone

Two major possibilities for the origin of spontaneous activity in the ICNS of isolated hearts are: 1) afferent neurons stimulated by mechanical movement of the heart, as alluded to by Gagliardi et al (1988); or 2) a spontaneous neuronal rhythm-generating circuit within the heart as proposed by Pokrovksii (2005). Indirect evidence supporting the former proposition has been provided by a study of cardiac mechanoreceptor input patterns to the canine ICNS (Rajendran et al, 2016), so **I hypothesized that ongoing neuronal activity within the ICNS results from activation of afferent intracardiac neurons by mechanical forces from cardiac contraction**. To determine if mechanical stimulation is the drive, I pharmacologically decoupled the electrical and mechanical functions of the myocytes with the compound blebbistatin (Figure 3.1, locus "**3**"). Blebbistatin is an excitation-contraction uncoupling agent that acts to inhibit myosin ATPase activity by preferentially binding to the ATPase intermediate, thus stopping myosin motility (Kovács et al 2005). Blebbistatin has been shown to stop motion within the heart without affecting electrophysiological parameters (Marchant, 2021; Stoyek et al, 2022; Swift et al 2012).

Should blebbistatin affect HR, this would imply that removal of afferent activity driven by mechanical events in the cardiac cycle plays a role in generating autonomic tone. This role would be detected by recording changes in HR after blebbistatin application. Alternatively, if HR, and thus autonomic tone, are unaffected by removal of afferent activity it may be that there is a group of neurons within the ICNS that is capable

of generating a regular discharge pattern to modulate the efferent output to the pacemaker cells. This could be a population of neurons similar to the P cells seen in the study by Edwards et al. (1995) in the guinea pig heart.

3.2 Materials and Methods

For all experiments outlined in these studies, the same processes and protocols were used as described in Chapter 2 for heart preparation, application of pharmacological agents dissolved in the perfusate, ECG recording and data acquisition during drug responses.

3.2.1 Hexamethonium Exposure

In order to antagonize nicotinic neurotransmission in the ICNS, hexamethonium (as hexamethonium chloride, Sigma Aldrich) was applied to the heart in the perfusate. This agent was dissolved in saline on the day of the experiment at a concentration of 10 μM. The heart was first perfused with saline, and 20 s control ECG samples recorded, then perfusion was switched to the hexamethonium-containing solution and ECG samples were recorded at 1 min intervals during 10 min exposure periods.

3.2.2 Electrical Activity in ICNS

In order to facilitate making extracellular electrical recordings of AP activity, the isolated heart was pinned to the chamber bottom to expose the SAR where most of the neurons are located, and the ventricle was removed to reduce whole-heart motion artefact. Two types of unipolar recording electrodes were used in trials to record spontaneous, ongoing APs: custom-made carbon fibre electrodes (Millar, 1992; see Appendix A for details of construction) or commercially made tungsten microelectrodes with 1 μ tip diameter (Tungsten Electrodes Fine; FHC, Brunswick ME, USA). The

electrode was held in a micromanipulator and its tip was visually guided to a position close to various small clusters of neuronal somata that were apparent at the junctions of the vagosympathetic nerves with the intracardiac nerve plexus in the region of the SAR. Electrodes of either type were connected to a high-gain AC amplifier (gain 10,000x) and the amplifier output, along with an ECG signal, was saved to the computer hard drive using Axoscope (see Chapter 2 for details).

In trials to record evoked neuronal activity in the ICNS the vagal stimulation protocol developed by Stoyek et al. (2016) was followed to attempt to evoke APs in neurons in the ICNS. Briefly, bipolar wire electrodes attached to a Grass Instruments Model S88 stimulator via a constant-current stimulus isolation unit (Grass PSIU6) were used to stimulate a vagosympathetic trunk. Rectangular, monopolar stimulus pulses of 0.5 ms duration and varying intensity were applied to the nerve, either singly or over a frequency range of 1-20 Hz. Stoyek et al. (2016) reported that a stimulus intensity of 300 μA was optimal for evoking changes in HR, so this intensity was used as a starting point for trials in my experiments. Extracellular recordings as described above were used to detect neuronal discharge responses to single stimulus pulses or pulse trains.

3.2.3 Blebbistatin Exposure

Blebbistatin (Toronto Research Chemicals, Toronto, ON, Canada) was dissolved in DMSO and frozen. On the day of experiments stock was thawed and mixed in a saline solution to give a final concentration of 30 μM. During all of these experiments the preparation was shielded from room light and illuminated by a fibre-optic cable with red light (long-pass filter, 700 nm). Blebbistatin is an expensive compound, therefore constant perfusion of the drug, over the tissue and into a waste container is not

sustainable. Therefore, using a stop-bath is required; this procedure involved filling the recording chamber with perfusate containing blebbistatin, then stopping flow and leaving the tissue exposed to the compound for 1 hr. Experiments involving blebbistatin exposure had a modified protocol to account for the time required to allow blebbistatin to penetrate the tissue and cease mechanical movement. During experimentation I discovered that perfusion of the tissue during "pre" recordings caused an increase HR, so the protocol was adjusted to account for this. 20s recordings were taken during a "pre" phase with saline perfusion, then during a second" pre" phase during which saline perfusion was stopped. Blebbistatin was then perfused into the chamber and left for 1 hour to allow for tissue penetration. A 20s ECG recording was taken at the 1-hour mark, and then perfusion of blebbistatin was turned on again while another 20 s recording was taken. In order to determine whether the passage of time could affect HR, control experiments were run with the same protocols using saline instead of blebbistatin.

3.3 Results and Discussion

3.3.1 Role of Nicotinic Neurotransmission in Autonomic Tone

Stoyek et al (2016), in reporting the only study to date on effects of the nicotinic channel blocker hexamethonium in the fish heart, showed in isolated zebrafish that a concentration of 10 μM in the perfusate effectively blocked nicotine-induced bradycardia as well as that induced by stimulation of the vagosympathetic trunks attached to the heart. However, this study did not report whether application of hexamethonium alone affected HR. In my pilot experiments in 3 rhythmically beating hearts, application of hexamethonium at the same concentration used by Stoyek et al (2016) had no discernable effect on HR (data not shown). These trials were done in the first year of my thesis work.

Several further hexamethonium trials done later on also demonstrated no HR change, but these results were not conclusive because at least some of these hearts developed arrhythmias during the experiments. I was therefore not able, on the basis of these studies, to either determine whether hexamethonium was in fact antagonizing nicotinic neurotransmission in the ICNS, or to show whether such blockade (if it was in fact effective) had any effect on spontaneous HR in the isolated zebrafish heart.

3.3.2 Electrical Activity in the ICNS

In the mammalian heart, extracellular recordings of cardiac neural APs were found typically in the range of 0.1~0.3mV (Ardell et al, 1991; Rajendran et al, 2016). The small size of these signals may lead to problems in detection, including that the heart is inherently electrically noisy, and that expected AP signal magnitude may be close to the intrinsic noise level of the recording electrodes. An additional problem is that the amplitude of the ECG (around 1 mV or greater in the isolated zebrafish heart) may be more than a decade larger than that of the largest extracellularly recorded neuronal APs, so the ECG could mask the much smaller AP signals. Furthermore, in mammalian hearts the intracardiac neurons are embedded in adipose deposits which act to electrically insulate neuronal APs from the ECG signal (Butler et al, 1990). The zebrafish heart, in which there is no adipose tissue associated with the ICNS, does not have this advantage.

As indicated in section 3.2.2, I first developed electrodes made from fine carbon fibres, insulated to their tips with glass in an attempt to maximize the ability to pick up highly localized neuronal discharges near small clusters of intracardiac neurons (see details of electrode construction in Appendix A). When inserted into the perfusate bath these electrodes displayed a high level of inherent noise which was greater than the

expected amplitude of the extracellular APs (and also exceed the ECG signal amplitude, which I attempted to record via direct contact of these electrodes with the atrium). Thus, over multiple trials in 6 hearts I was not able to make any successful recordings of neuronal signals with this type of electrode in the beating heart. I then attempted to use commercially manufactured tungsten microelectrodes with fine tips, as used for extracellular recording of the activity of mammalian intracardiac neurons. These had a much lower inherent noise level, and in trials on 5 hearts I could clearly detect large-amplitude ECG signals from atrial tissue. However, even with the tip of the electrode in contact with tissue directly adjacent to groups of neuronal cell bodies I was not able to record any spontaneous APs that might have been between the ECG signals.

Given the lack of success in detecting spontaneous intracardiac neuron activity with tungsten electrodes, I attempted to evoke such activity by electrically stimulating the vagosympathetic trunks attached to the heart, as described in Section 3.2.2. Stimulation of preganglionic axons in these extrinsic cardiac nerves should produce postsynaptic excitation in postganglionic intracardiac neurons, some of which project to the pacemaker to inhibit heart rate. I reasoned that activation of intracardiac neurons should produce APs that were synchronized to the nerve stimulus, thus providing a means of reliably and predictably evoking APs with short latencies following stimulus pulses. In experimental trials in 17 hearts, I initially used stimulus parameters (pulse duration, intensity and frequency) reported by Stoyek et al (2016), but I was unable to obtain the expected bradycardia. I then varied these parameters but could still not evoke bradycardia.

This in itself is noteworthy (in regard to health and development of the zebrafish at the time) as vagal stimulation to induce bradycardia has a relatively long history and a

large body of literature behind it, including use in isolated mammalian hearts (McEwen, 1956) and more recently, being used in clinical studies for anti-arrhythmic activity in heart failure candidates (DeFerrari, 2014). Lack of vagal response was seen when stimulating some individual thoracic branches in a study by Randall et al (1985), but their procedure did not stimulate the entire vagal trunk. Lack of response to vagal stimulation could imply underdevelopment of neural pathways in the heart, or possible down-regulation of M_2R channels in the hearts I used. This is supported by the findings of Fisher et al (2004) who saw a lack of bradycardia following vagal stimulation in mice who had M_2R knockout.

In all of these trials noise from the nerve stimulation artifact overrode all electrical signals in the system. In a final attempt to solve this problem I constructed a modified type of suction electrode for recording that was made with an extra artifact-suppressing lead as designed by Stys (1992). In a further 6 trial hearts these electrodes were also unsuccessful in detecting either stimulus-evoked or spontaneous APs.

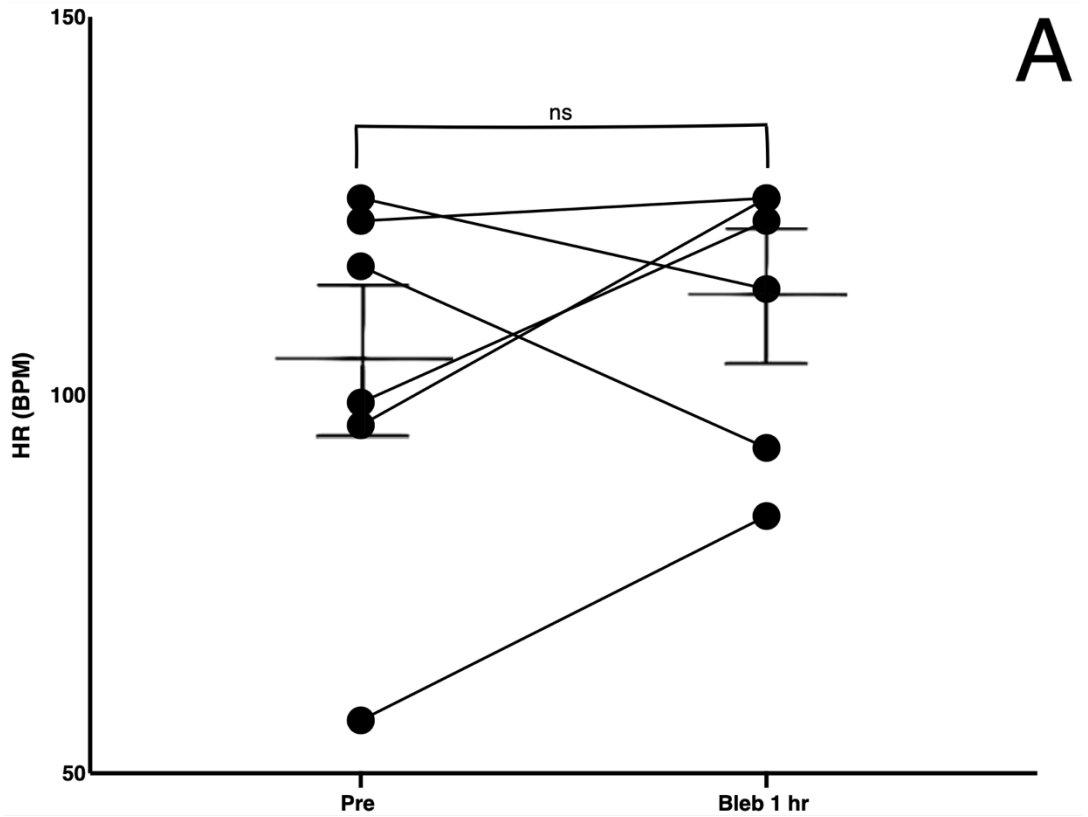
In theory any or all of these approaches should provide useful information on ongoing neuronal activity in the ICNS in isolated hearts. It is possible that, had I been able to continue my experiments and solve the technical difficulties with these AP recording experiments, I may have been able to test my hypothesis, but I did not have this opportunity so was not able to conclude this study.

3.3.3 Effects of Blebbistatin Exposure

Experiments to test the effect of blebbistatin exposure on HR were done on hearts isolated from 6 zebrafish. The HR effects of exposure to 30μM Blebbistatin are shown for individual hearts in Figure 3.2A. These responses were variable in different hearts:

rate increased in 3 hearts, decreased in 2 and did not change in the remaining trial. There was thus no clear overall trend in this response. These results are summarized in Figure 3.2B: mean HR before Blebbistatin application ("Pre") was 103±9 bpm and mean HR during Blebbistatin exposure was 111±6bpm. Results of a t-test indicated no significant change in HR ($p=0.4252$) due to Blebbistatin exposure. In order to ensure that the passage of time was not affecting HR, controls were run with saline instead of Blebbistatin. Results of a t-test indicated no significant change in HR due to passage of time. ($p=0.4226$) Lack of a significant response in HR as a result of Blebbistatin application is in agreement with a recent finding by Stoyek et al (2022).

The application of Blebbistatin, and the consequent cessation of mechanical events in the cardiac cycle, should have effectively offloaded these receptors and thus reduced mechanically-related input to the ICNS. However, given the results of the experiments reported here, input from these receptors may not be a major driving force of autonomic tone within the isolated zebrafish heart. Yet this question remains open. The overall contribution of mechanoreceptors to ICNS operation may be more subtle than is apparent by the approach taken here; in this context a larger number of trials may uncover more details of this effect. In mammals, pioneering work by Gagliardi et al (1988), and studies done since then (for instance, Huang et al, 1993; Armour 2004) have claimed that mechanoreceptor input is important in HR control, but since direct evidence for the role of these afferents in generating autonomic tone has not been established, the full story is not yet known.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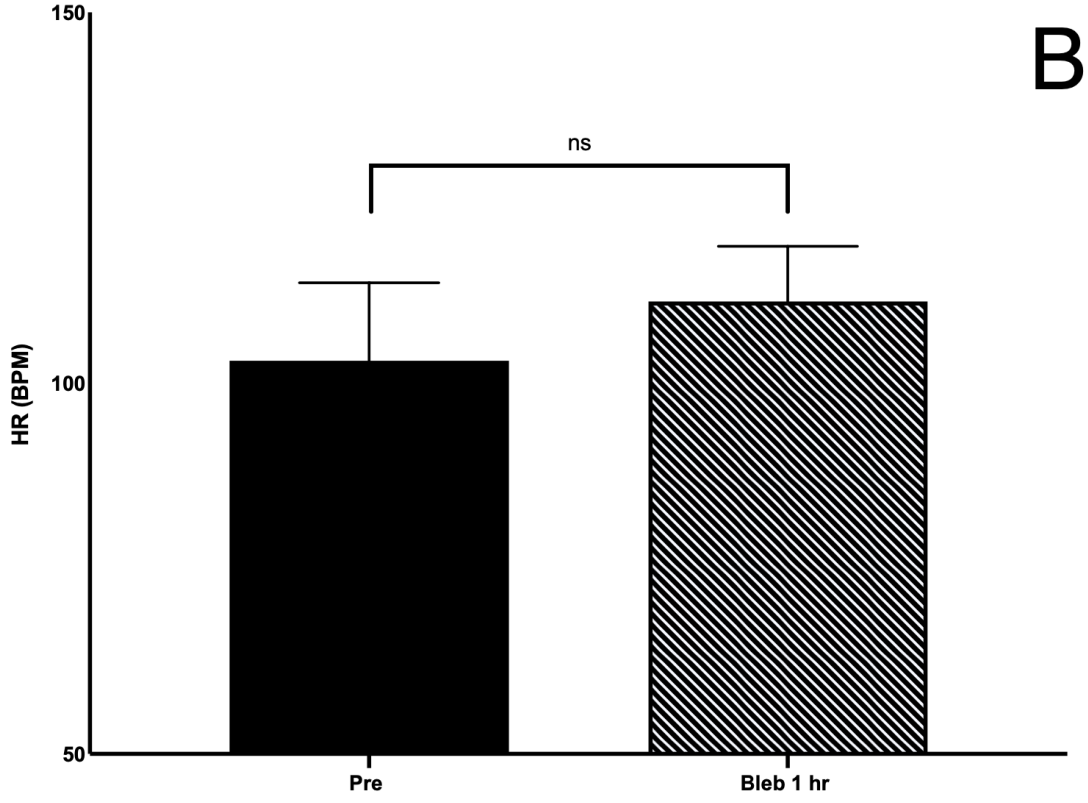
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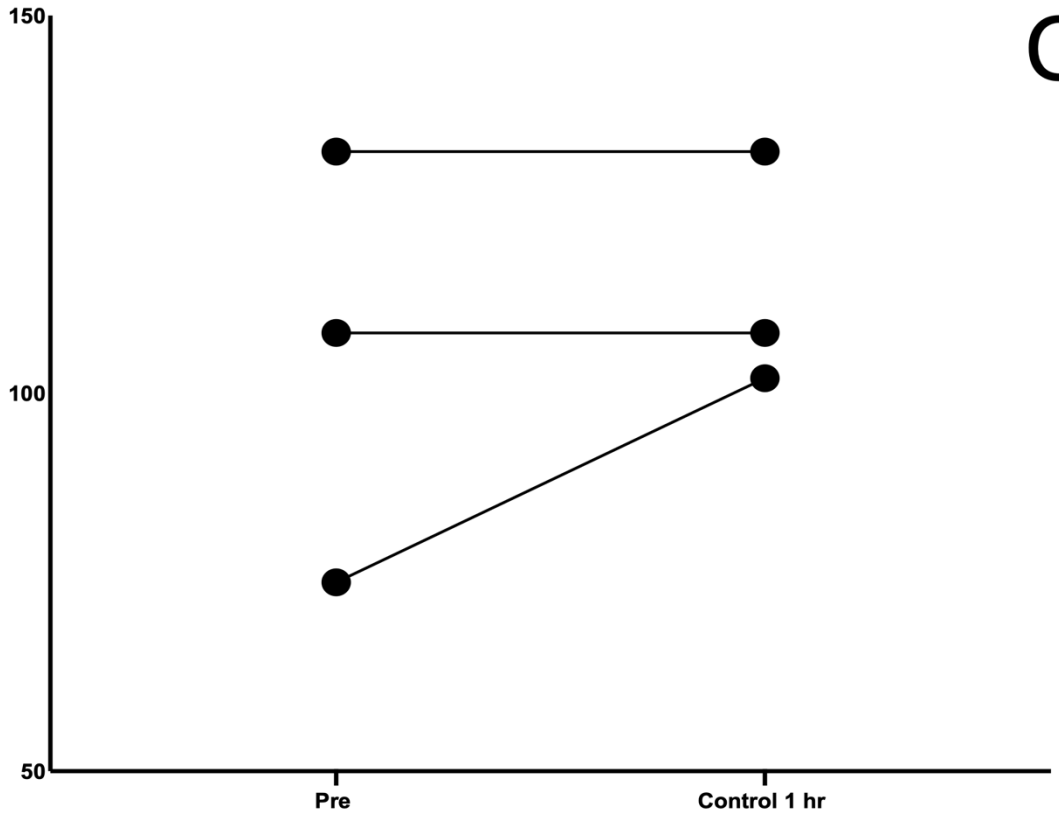
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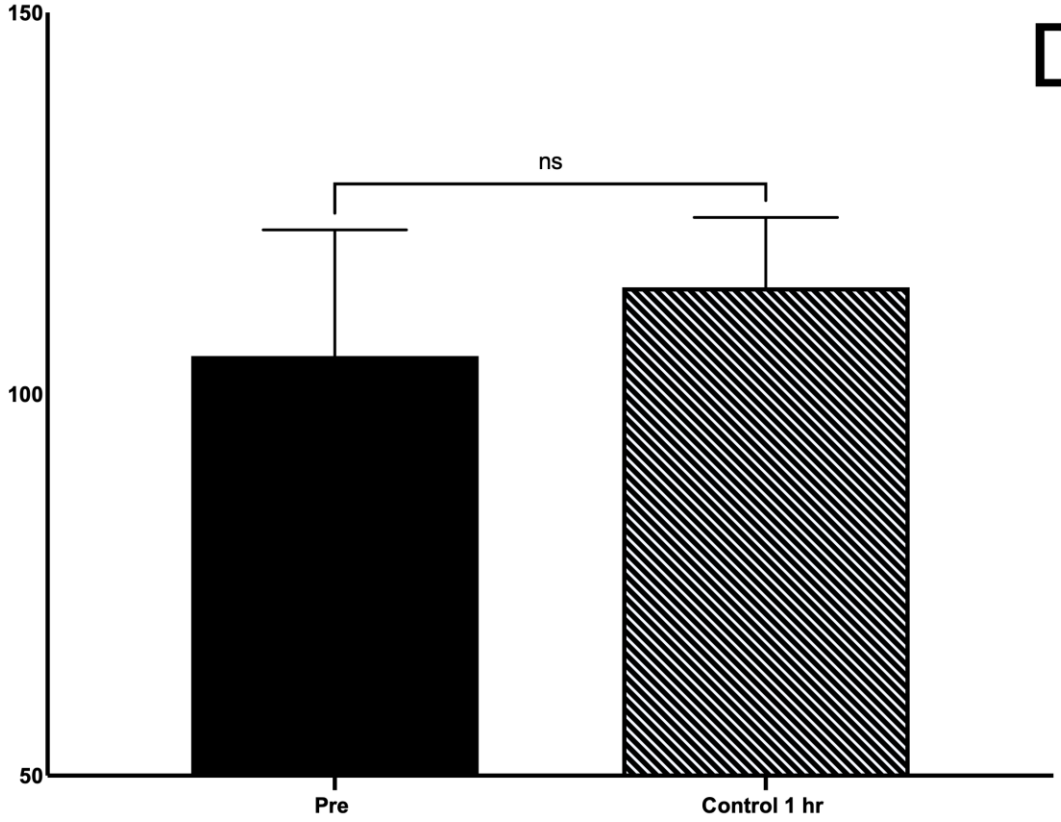
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Figure. 3.2. Effects of BLEB application A) Responses of 6 individual hearts to 1 hr application of BLEB at 30μM. Error bars represent mean ± 1 SEM. B) Bar graph summarizing data in panel A. C) Response of 3 individual hearts to 1hr of control saline application. D) Bar graph summarizing data in panel C. Application of BLEB resulted in no significant change in HR. (t-test, p=0.4252, n=6) Passage of time resulted in no significant change in HR. (t-test, p=0.4226, n=3)

CHAPTER 4 General Discussion

4.1 Summary of Key Findings

The major results of the research in this thesis demonstrate the presence of spontaneous activity within the ICNS in isolated hearts of zebrafish. Application of ATR resulted in tachycardia. In an isolated heart, efferent neurons with output to pacemaker cells exist, however isolation from the CNS means that they are receiving no signalling from outside of the heart. Blocking of M_2R should therefore have had no effect, however as we see a change in HR, this implies that there is parasympathetic tone within the heart that is acting through M_2R on the pacemaker cells to drive HR down. I have also demonstrated the presence of sympathetic tone. Application of TIM blocks β_2R which results in bradycardia, implying that sympathetic tone is present upstream to these receptors on the pacemaker cells causing an increase in HR. Increasing the temperature of the system not only increased HR but resulted in a trend toward modulation in the percentage change in HR by both ATR and TIM. Change in HR after ATR exposure went from an increase of 12% at 22°C to 9% at 27°C. Conversely change in TIM applicated tended to have a larger proportional HR effect at higher temperature (-19% at 27°C vs -7% at 22°C) This apparent modulation of sympathetic control of HR implies that the ANS itself may have a Q_{10}.

Zebrafish were used in this study because they are an excellent model for studying cardiac electrophysiology. The cardiac AP in a zebrafish is a closer representation of human cardiac APs than in some mammalian models (Vornanen and Hassinen 2016) and some of the dominant ion channels (I_{Na} and I_{Kr}) involved in myocyte function are conserved between humans and zebrafish (Echeazarra 2021). As well, unlike in mammalian models, the ICNS in zebrafish is visible and easily accessible (Stoyek et

al, 2016). My work represents an extension of the groundwork laid in Stoyek et al (2016) to further quantify the use of zebrafish as a model for studying human cardiac function.

The results of this thesis show that there is both sympathetic and parasympathetic tone present within the isolated heart. This tone is capable of modulation of spontaneous heart rate and exists independent of extracardiac neuronal input. The concept of autonomous function of the heart has been floated since the findings of Gagliardi et al (1988), who noted that local control of function by groups of neurons in cardiac ganglia seemed possible. The re-forming of myocyte and neural connections to create a functioning system in dissociated cardiac tissues (Horackova et al, 1993) further strengthened this concept. Here I have demonstrated further evidence in an isolated system that is capable of self-governance, validating the concept of the ICNS functioning as a stand-alone system.

Parasympathetic tone in the heart affects pacemaker cells via M_2R. Here I have shown that this influence can be blocked with the application of ATR. While the range of ATR dosage in the literature is broad, specific concentration related effects at the receptor level were unknown. I have shown the effects of direct ATR application at 3 defined concentrations on receptors in the heart. Concentration bracketing (3 μM, 10μM and 30 μM) of ATR was used to attempt to create a dose response relationship to determine if an optimal dose existed. I demonstrated that as little as 3 μM can cause significant cardioacceleration and that, while statistical determination of optimal dose was not possible at this time, visualization of these dose responses suggests that further experimentation will resolve this issue.

Sympathetic tone is transmitted to cardiac pacemaker cells via β-adrenergic receptors. While in mammals this mainly occurs via β_1 in teleosts β_2 may be the dominant subtype (Hugget et al, 2002). For this reason, TIM was chosen for my experiments as it is a non-specific β-blocker. While post-ganglionic parasympathetic efferent neurons are known to exist in the heart, in the classical model of the ANS, sympathetic postganglionic neurons are not. However, application of TIM in my experiments resulted in bradycardia, confirming the existence of these sympathetic postganglionic neurons. Concentration bracketing was also used in this study to attempt to determine an optimal dose of TIM. While this was not possible within the timeline of my study, I have found that as little as 3 μM of TIM is sufficient to cause cardioinhibition.

Increasing the temperature of the system during experimentation resulted in a significant overall increase in HR. The calculated Q_{10} of HR in my findings was 1.65, which falls within the typical range seen in a wide range of teleosts. As zebrafish are poikilotherms their internal temperature is determined by temperature of the system. This means that physiological processes that are affected by temperature are directly influenced by environmental temperature.

Increasing temperature also appeared to evoke a change in the influence of autonomic tone on HR. At elevated temperature there was a trend towards an increase in the amount of sympathetic tone seen, as well as an apparent decrease in the amount of parasympathetic tone. Sandblom and Axelsson (2011) found that at elevated temperatures, cholinergic (parasympathetic) tone tends to dominate, however this study was on whole body teleosts, so complicating affects from the CNS may be at play. My findings imply that the ANS itself (and therefore ICNS) may have a Q_{10}. The

mechanisms underlying this, however, were not clear. An increase in temperature will change ion channel gating which can result in an increase in conduction velocity through the axons and may increase neural discharge rate. As environmental temperature varies, the ANS may be more or less effective at regulation of cardiac output. During elevated temperatures the ICNS may play a larger role in cardiac control where the requirement for more precise autonomic control of HR may be necessary. This effect may also promote more efficient beat-by-beat control of cardiac function by the ICNS.

A further objective of this thesis was to determine if transmission of tone occurred via neurotransmission through nicotinic synapses. Nicotinic synapses were chosen as they are the most common synaptic mechanism driving neurons in the ICNS. Hexamethonium, a nicotinic antagonist was to be applied to the hearts to determine the role of nicotinic neurotransmission. Due to issues regarding unstable HR, the role of nicotinic synapses in transmission of tone was unable to be determined. Determination of their role should be given a high priority in future studies.

The results of the pharmacology of chapter 2 gave indirect evidence that there was neuronal activity within the ICNS projecting to pacemaker cells and I attempted to record neuronal APs with microelectrodes. However, APs could not reliably be found; attempting to produce evoked APs with vagal stimulation resulted in high electrical noise and no response to stimulation. Therefore, a different approach to find direct evidence of this tone is needed.

In mammalian models, study of the ICNS is difficult due to issues with visualization since the ICNS is embedded deep into the heart and is largely inaccessible for experimental manipulation (Stoyek et al, 2021). This issue is obviated in the zebrafish

heart where the entire ICNS is visible using a simple, basic preparation. Optical recording of spontaneous activity in the ICNS, via a voltage or calcium sensitive reporter and optogenetic activation of cardiac APs (Kopton et al, 2018) could allow for direct evidence of spontaneous neural activity.

An additional objective of this thesis was to determine the source of autonomic tone within these isolated hearts. It was proposed by Gagliardi et al (1988) that mechanosensory afferents exist in the heart and have the capacity modulate rate. I attempted to address this issue by eliminating such afferent input by stopping contraction with blebbistatin. However, HR responses to blebbistatin were not significant. Therefore, the role of these mechanosensory afferents remains a mystery. Further work needs to be done to uncover the source of this tone, with additional studies of the role of mechanosensory afferents.

Teleosts are ancient, having evolved during the late Cambrian to early Ordovician period (Shu et al, 1999). However, these species have a complete, functioning autonomic nervous system with sympathetic and parasympathetic control of all organs including the heart. This system has been conserved through vertebrate evolution, therefore conclusions from my work are generalizable to neurocardiology in all vertebrates.

4.2 Limitations of Thesis Research

Limitations on this thesis work fell into two categories: “non-experimental limitations” due to extenuating circumstances of the health of adult zebrafish from the Faculty of Medicine CORES Zebrafish Facility (“CORES”) and fish from alternate sources (Section 4.2.1); and limitations due to issues associated with the pharmacological agents in the experimental design. These are discussed in the sections below.

4.2.1 "Non-Experimental" Limitations

Work on this thesis began in the Fall of 2019. Shortly into my second semester the university was shut down and I was unable to perform experiments due to safety precautions during the COVID-19 pandemic. This resulted in a gap of 5 months with no laboratory work (for further detail of this see Appendix B). When I returned to the laboratory to resume experiments there was a marked decrease in success rate of my experiments. Hearts would frequently be arrhythmic, with dropped beats being common, and rate would quickly deteriorate until hearts were non-viable. Not only were the hearts not responding to drugs which previously worked but obtaining rhythmic rate at all became difficult.

These problems were also occurring in other research groups in the Faculty of Medicine. A number of possibilities were considered in trying to solve this issue. The most obvious course of action was to assume that problems arose within the basic experimental protocols used by individual laboratories. Included among these possibilities were: issues with the purified water or the chemicals used to make up standard saline and perfusate solutions; fish handling and anesthetic techniques; and unstable, aged, or contaminated pharmacological agents. To address these issues, I obtained water from different sources (various water purification systems in Medical Neuroscience department, Physiology and Biophysics department and Pharmacology department). 3 different saline recipes were tried (HEPES buffered, Bicarbonate buffered and ZIRC ZFIN Ringers), made up with fresh compounds purchased for making up the saline solutions. New MS222 anesthetic was obtained, and different anesthetic techniques were attempted (variations on the standard MS222 procedure, and the use of ice to cool

the animals for euthanasia). Fresh pharmacological agents were bought from the distributor and were stored in dark humidity-controlled containers to reduce any chance of degradation or contamination. None of these attempts improved the viability of the isolated hearts. Upon consultation with colleagues in other laboratories using zebrafish and with personnel managing the CORES Facility it was agreed that the issue was likely due to a decrease in the health of the fish for unknown reasons. Health of the hearts of fish from the Facility continued to trend downwards, and as a result so did success and quality of experiments for all users.

In the spring and summer of 2021 adult and larval zebrafish samples were sent to the ZFIN facility in Portland, Oregon, USA for testing. During this period of time, I began the process of sourcing fish from outside of the CORES facility. Sources of fish included those from the Aquatron Facility at Dalhousie University (25), the NRC zebrafish research facility in Halifax (45), and from the laboratory of Tamara Franz-Odendaal at Mount Saint Vincent University (20). During this period new batches of fertilized zebrafish eggs were imported from ZIRC and were raised in the CORES facility. However, while I was able to obtain fish from these sources, I was not able to obtain as many fish as needed to complete in full the studies outlined in Chapter 3. In addition, not all of the fish from these alternate sources turned out to be viable in my experiments, further hindering progress.

4.2.2 Experimental Issues

As I discussed in Chapter 2 Sections 2.1.1, 2.1.2, in zebrafish, little is known regarding the use and outcomes of the antagonists I choose for this study. Use of ATR has been explored in teleosts but actual effect at the receptors is unknown. In addition,

reports of usage of TIM in teleosts are sparse. TIM was chosen for my experiments because it is a broad spectrum β-adrenergic blocker, meaning it blocks both β_1 and β_2 receptors. While β_2 is the dominant receptor in the teleost heart, it is possible that there are some β_1 receptors present which could be contributing to the effects seen. Furthermore, regarding ATR, while the likelihood of other muscarinic receptors being blocked by ATR and contributing to HR change is less than for TIM it is still possible. I attempted to mitigate the possibility of other muscarinic receptors affecting HR by applying specific muscarinic subtype antagonists (Pirenzepine, Gallamine, 4-DAMP) however, this was during the stage where HR was unreliable, so results of these tests were inconclusive.

Another experimental limitation was that in my hands both ATR and TIM had either long wash out times, or after their initial application, HR did not return to baseline. Consequently, there were inconsistent results when repeated doses were applied to the same heart. For this reason, only one run of any agent was used per heart, and only one agent was used with any given heart (with the exception of the agonist-antagonist experiments described in Chapter 2). This issue prevented the recording of matched data on multiple drug concentrations in the same hearts, or at different temperatures, so preventing the use of more powerful statistical analysis methods (ANOVA, multiple-means comparisons etc.).

4.3 Future Directions

4.3.1 Receptor Subtype Availability and Sensitivity

An obvious first choice for future directions of work arising from my studies is confirming the range of sympathetic and parasympathetic receptor subtypes present and

the effects of subtype-specific activation in the zebrafish heart. This could be readily achieved through the use of subtype-selective antagonists. Should additional sub-types than those I targeted be present in the zebrafish heart, investigating their sensitivity and contribution to autonomic tone would be a logical further follow up to my work.

4.3.2 Direct Evidence of Autonomic Tone

The results of the pharmacology experiments in my study have shown indirect evidence of autonomic tone. Therefore, attempting to observe and record direct evidence of this tone would be a logical next step, which I was unable to complete.

Calcium and voltage sensitive proteins allow for real time imaging of neuronal-specific activity (Demaurex, 2005, Akemann et al, 2012) so their use with the ICNS would allow for monitoring spontaneously occurring neural activity across the whole population of neurons in the heart. Monitoring calcium dynamics within the ICNS and then measuring changes in both dynamics and HR following application of autonomic antagonists would provide direct evidence of autonomic tone.

4.4 Conclusions

The use of zebrafish as a model for human cardiac function presents an opportunity for greater understanding of cardiac control. The ICNS in zebrafish is visible and accessible for manipulation; furthermore, many aspects of cardiac electrophysiology in zebrafish closely resemble humans (Stoyek et al, 2022, Vornanen and Hassinen, 2016). Despite this, our understanding of neurocardiology, not only in zebrafish, but in any teleost, species is lacking. Here I have demonstrated the existence of spontaneous activity within the isolated zebrafish heart. This tone is both sympathetic and parasympathetic in nature and exists independent of extracardiac input.

I have also demonstrated the relative influence of autonomic tone on HR through the use of pharmacological antagonism. Scant literature exists on the effects of TIM, or most pharmacological agents in zebrafish. Furthermore, reports of the effects of ATR in this species mainly consist of whole-body experiments, meaning cardiac responses to specific drug concentrations at this organ remain unknown. As stated by Barros et al. (2008), our lack of understanding of pharmacological agents in zebrafish is a severe limitation in this field. Therefore, my body of work has provided new insight to help illuminate cardiac control by the nervous system.

Overall, my findings represent a step forward in the concept of the ICNS functioning as a stand-alone system. The presence of autonomic tone in the heart and its ability to modulate rate independently of external input, as well as the potential for alterations in ANS modulation in response to temperature change is evidence of this. Further understanding of the stand-alone function of the ICNS will eventually allow for more precise heart therapeutics in humans.

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APPENDIX A Building the Tools of Electrophysiology

Carbon Fibre Electrodes

Carbon fibre electrodes were built using a modified version of the technique described by Millar (1992).

Fire polished micropipettes (1.0mm outer diameter, 0.5mm inner diameter, 10 cm length; Sutter Instruments, Novato, CA, USA) were filled with acetone (Fisher Scientific) via capillary action. A 10 μ diameter carbon fibre (donation from Quinn lab) was inserted into the pipette using Teflon tipped forceps. Acetone allowed for the fibre to slide easily into the pipette as it reduced the resistance of friction between the fibre and the pipette. The pipette was then set aside to allow for the acetone to evaporate out.

Pipettes were then inserted off-set into a micropipette puller (Model P-87, Sutter Instruments) and pulled to coat the fibre with glass. (Off-set insertion allowed for control of size of resulting 2 pipettes). As the pulling resulted in the fibre remaining in only 1 of the 2 pipettes, the fibre was trimmed depending on the excess sticking out of the tip and the other pipette was discarded.

Insulation was stripped from the end of fine copper wire and then dipped in nickel print (MG Chemicals, Burlington, ON, Canada) and inserted into the back end of the pipette, making contact with the fibre. The particles of the nickel in the compound provided electrical conduction between the copper wire and the carbon fibre. The wire was then sealed to the pipette using cyanoacrylate. The opposite end of the wire was then stripped and attached to a micro board pin with solder for connection the recording head-stage.

During subsequent recording attempts it was noted that longer pipettes resulted in higher noise level as the part of the electrode not submerged in saline acted as an antenna so smaller versions of these electrodes were made in an attempt to create a clearer signal.

Artefact Suppressing Suction Electrodes

Due to the high degree of noise created by artifacts from stimulating pulses during vagal stimulation, artefact suppressing suction electrodes were created. The idea for a separate “artefact suppressor” lead was reported by Stys (1992).

Micropipettes were pulled and the tips were broken off and abraded so the resulting opening at the tip of the pipette was just wide enough to reliably attach to tissue by applying suction (this was found through trial and error). The pipette was held by a microelectrode holder with a port (Model ESW-F10P; Warner Instruments, Holliston, MA, USA).

A silver chloride wire was then wrapped around the pipette and dipped in the perfusate to act at the secondary electrode. The recording end of this secondary wire was positioned near the tip of the suction electrode. Its position allowed for recording of any noise that was present near the suction electrode which could then be compared to the recording electrode and filtered out. This acts as an additional bath ground as the noise at the recording electrode may be significantly different than at the remote bath ground depending on distance.

Two lengths of insulated signal were stripped and soldered onto the plug end of the microelectrode holder, and onto the silver wire. These were then attached to the into head stage.

Appendix B Timeline

The following is provided to indicate timing of experiments in relation to fish health and fish sources.

September 2019- March 2020

During this period, I performed my initial experiments with ATR and TIM, confirming their effects, as well as applying Musc and ISO. My pilot HEX trials took place during this period as well. Fish were being used from the CORES facility and hearts were viable.

March 23, 2020 - July 5th, 2020

During this period of time, I was not present in the lab due to the COVID-19 pandemic restricting access to the facilities.

July 2020-October 2020

Returned to the lab July 5th, I began with agonist-antagonist trials. In September I broke my collar bone and was unable to run fish due to the lifting involved and I was incapacitated for 6 weeks, however, I was able to work with my hands, and I took this time to build the carbon fibre electrodes.

November 2020- January 2021

I continued to do ATR and TIM trials. I initially attempted doing a reduced-prep set up, removing the ventricle and reducing tissue in the atria to attempt to cut any non-necessary components out of the system. I also attempted atrium to ventricle depolarization tests, seeing if there was any degree of electrical blockage in the heart. Fish still from CORES facility.

February 2021

Fish from the Aquatron arrived and I went back to agonist-antagonist trials.

March 2021- May 2021

Began experimentation using different concentrations of antagonists as well as effects of heat. Fish used were from CORES.

June 2021- July 2021

Experiments here were a continuation of the concentration and temperature experiments. Fish during this time were from ZIRC and NRC

August 2021

Continued with concentration and temperature. Using 3mpf fish from CORES.

September 2021-October 2021

Experiments using vagal stimulation and recording of APs were attempted during this time. Fish were from CORES

November 2021-February 2022

Experiments here were finishing temperature and concentration effects, and testing of blebbistatin. Fish during this time were from MSVU and CORES.

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