Winter 12-31-2015

Effects of Melatonin on Heartbeat and Possible Identification of a Melatonin Receptor in Drosophila Melanogaster

Tricia L. VanKirk
University of Maine, vankirkt@husson.edu

EFFECTS OF MELATONIN ON HEARTBEAT AND POSSIBLE IDENTIFICATION OF A MELATONIN RECEPTOR IN *DROSOPHILA MELANOGASTER*

By

Tricia L. VanKirk

B.A. University of Maine, 1994

A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Biological Sciences)

The Graduate School

The University of Maine

December, 2015

Advisory Committee:

Harold B. Dowse, Professor of Zoology and Cooperating Professor of Mathematics, Advisor

Mary S. Tyler, Professor of Zoology

Leonard J. Kass, Professor of Biological Sciences

Erik Johnson, Professor of Biological Sciences

Aaron Domina, Professor of Pharmacy

DISSERTATION ACCEPTANCE STATEMENT

On behalf of the Graduate Committee for Tricia L. VanKirk, I affirm that this manuscript is the final and accepted dissertation. Signatures of all committee members are on file with the Graduate School at the University of Maine, 42 Stodder Hall, Orono, Maine.

_____

Dr. Harold B. Dowse, Professor of Zoology Date

LIBRARY RIGHTS STATEMENT

In presenting this dissertation in partial fulfillment of the requirements for an advanced degree at The University of Maine, I agree that the Library shall make it freely available for inspection. I further agree that permission for "fair use" copying of this dissertation for scholarly purposes may be granted by the Librarian. It is understood that any copying or publication of this dissertation for financial gain shall not be allowed without my written permission.

Signature:

Date:

EFFECTS OF MELATONIN ON HEARTBEAT AND POSSIBLE IDENTIFICATION OF A MELATONIN RECEPTOR IN *Drosophila melanogaster*

By Tricia L. VanKirk

Dissertation Advisor: Dr. Harold B. Dowse

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
(in Biological Sciences)
December, 2015

Chapter 1 of this manuscript is a literature review that serves as an introduction to the entire dissertation. Chapter 2 examines the effects of the melatonin injection on heart rate and rhythmicity in *Drosophila melanogaster* Canton-S (wild-type) pupae and pupae bearing a variety of heart mutations. Chapter 3 investigates further the possible mechanisms of melatonin's ability to increase heart rhythmicity without significantly affecting heart rate. A melatonin antagonist, luzindole; a high-affinity melatonin agonist, 2-iodomelatonin and RNAi techniques are used to identify a possible melatonin receptor in *Drosophila melanogaster*.

An appendix contains a previously published manuscript detailing experiments performed at the beginning of my graduate studies before I began my work with melatonin. The paper is entitled *The Relationship of Heart Function to Temperature in Drosophila melanogaster and its Heritability* (Jennings et al., 2009).

ACKNOWLEDGMENTS

I would like to extend special thanks to all of my committee members, Dr. Harold "Dusty" Dowse, Dr. Mary Tyler, Dr. Len Kass, Dr. Erik Johnson and Dr. Aaron Domina. The cooperative dynamic of this group of people was instrumental in the completion of my degree. Abundant amounts of gratitude go out to my primary advisor, Dusty, who's patience knows no bounds and who stuck by me even when I thought the end was nowhere in sight. Somehow he always knew just the right amount to push.

I would also like to thank the University of Maine Graduate School for giving me the chance to discover my love for teaching. It was a path I had never considered until I had the opportunity to be a TA.

Immeasurable amounts of gratitude go out to my husband for listening to me ramble on about protocols and frustrations and for always offering alternative ways to approach my work. Having someone who shared in *and* understood my successes and failures throughout the process meant more to me than you could ever know. Thank you for always being my biggest fan.

Thank you to my children, Sarah, James and Ben for your support and understanding when I was not able to join you for weekend ski trips or wasn't there to help with homework or life's problems. I appreciate that you all realize how important it was for me to attain this goal.

Thank you to my parents for encouraging me and giving me the opportunity to go to college where I began this great adventure and for always believing in me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

MELATONIN AND ITS EFFECTS ON VERTEBRATE AND INVERTEBRATE PHYSIOLOGY

Cardiovascular disease is the leading cause of death (~598,000 per year) in the United States (Heron, 2013), thus elucidating causes is of principal importance for public health. Furthermore, there are several categories of this disease, including cardiomyopathy, conduction defects, hypertension, atherosclerosis, and venous malformations, many of which are caused by genes that are conserved and may act in common physiological processes or pathways (Bier & Bodmer, 2004). Acute coronary occlusion is the leading cause of morbidity and mortality in the Western world. According to the World Health Organization, it will be the major cause of death in the world by the year 2020 (Lopez & Murray, 1998). Cardiac events can result in damage to the intrinsic pacemaker cells of the heart, causing a variety of cardiac arrhythmias. Postoperative atrial fibrillation (AF) is a specific type of arrhythmia that is the most common complication after cardiac surgery (Zeriouh et al., 2014). Furthermore, it is well understood that cardiac arrhythmias are a common and often lethal manifestation of many acquired and inherited diseases affecting the cardiovascular system. Electrophysiologists have long sought to understand and treat the arrhythmogenic mechanisms of these diseases to sustain life and improve its quality (Delisle et al., 2004).

Drosophila **as a model organism for the study of cardiovascular disease**

The study of heart development and physiology in *Drosophila melanogaster* is intensifying rapidly as it continues to expand as a model system for studying cardiac problems generally and genetic influences in particular. Findings in the fly are directly applicable to the human heart as a growing number of genes have been identified with homologous function in both organisms (Bier & Bodmer, 2004). Although heart structure in *Drosophila* is very different from that of vertebrates, many of the basic elements for cardiac specification and differentiation are conserved. Of the 1682 human disease genes currently known, 74% have homologs in *Drosophila* and nearly a third of these genes (~500) are as highly conserved as genes known to be functionally equivalent between flies and humans (Bier & Bodmer, 2004). These *Drosophila* genes include homologs of genes causing a broad spectrum of human diseases ranging from neurological disorders and cancer to developmental defects, metabolic/storage disorders, cardiovascular disease, as well as genes required for function of the visual, auditory, and immune systems (Reiter et al., 2001). Because of the simplicity in its structure and availability of powerful genetic tools, the *Drosophila* heart has emerged as a model system for unraveling the basic genetic and molecular mechanisms of cardiac development, function, and aging (Qian et al., 2007).

Anatomy and physiology of the *Drosophila* heart

The *Drosophila* heart, or dorsal vessel, is located medially and dorsally in the hemocoel and transports hemolymph through the larval and adult body cavity (Miller, 1950; Rizki, 1978; Curtis et al., 1999). The anterior third of the dorsal vessel forms the aorta. The posterior segment, or heart proper, contains three pairs of openings called ostia to admit hemolymph (Rizki, 1978; Curtis et al., 1999; Wasserthal, 2007). Heart

pacemaking in *Drosophila* is myogenic (Dowse et al., 1995; Gu & Singh, 1995; Bodmer et al., 2005), and originates from a caudal pacemaker (Rizki, 1978), an assembly of ion channels, including those passing Ca^{2+} and K^{+}, interacting in an oscillatory manner (Dowse et al., 1995; Johnson et al., 1998; Bodmer et al., 2005). The possibility of a second, more anterior pacemaker in the region of the cardiovascular valve is suggested by reversals in contraction seen occasionally in larvae and consistently in early pupae (McCann, 1969; Rizki, 1978). Heartbeat is triphasic, consisting of contraction (systole), relaxation (diastole), and pause (diastasis) (Jones, 1977; Rizki, 1978; Curtis et al., 1999; Wasserthal, 2007). Heart rate is determined by the duration of diastasis (Markou & Theophilidis, 2000) and is modulated by temperature (Irisawa et al., 1993; Dowse et al., 1995), small peptide hormones (Johnson et al., 2000), and neurotransmitters (Miller, 1985; Dascal et al., 1986; Johnson et al., 1997; Hille, 2001). Cardioactive substances alter heart rate and rhythmicity by altering pacemaker ion channel kinetics either by direct binding or indirect modulation through signaling pathways (Johnson et al., 1997; Johnson et al., 2000; Johnson et al., 2002).

Four ion channel genes have been identified as necessary for normal heartbeat through analysis of mutant heartbeat and pharmacology (Johnson et al., 1998; Johnson et al., 2000). Three of the channels allow the passage of K^{+} and the other carries a Ca^{2+} current (Johnson et al., 2000). Johnson et al. (1997, 1998) proposed a model for the *Drosophila* cardiac pacemaker based on genetic and pharmacological data: A delayed-rectifier potassium channel current (I_{Kr}) containing an α subunit encoded by *ether à go-go* (*eag*) (Kaplan & Trout, 1969; Warmke et al., 1991a; Engel & Wu, 1992; Brüggemann et al., 1993) creates a hyperpolarizing leak. The decay of the

hyperpolarizing K^+ efflux has the effect of depolarizing sino-atrial cells so that a voltage-gated calcium channel, the α_1 subunit of which may be encoded by *cacophony* (*cac*) (Von Schilcher, 1976; Smith et al., 1996; Smith et al., 1998), opens to allow Ca^{2+} to enter the cell. This current is blocked by ω-conotoxin (ω-CgTx) MVIIC, an antagonist of N- (Ashcroft, 2000; Hille, 2001) and P/Q-type calcium channels, and importantly this agent is effective at disrupting the normal heartbeat of *Drosophila* (Olivera et al., 1994). Subsequently, this influx of calcium opens a calcium-activated potassium channel encoded by *slowpoke* (Elkins et al., 1986) and blocked by charybdotoxin (CTX) (Moczydlowski et al., 1988), and a fast voltage-gated potassium channel (K_V, A-type) encoded by *Shaker* (*Sh*) (Kaplan & Trout, 1969; Warmke & Ganetzky, 1994) and blocked by 4-aminopyridine (4-AP) (Thompson, 1977; Moczydlowski et al., 1988; Engel & Wu, 1992). The efflux of potassium repolarizes the membrane (Thompson, 1977; Elkins et al., 1986; Fozzard, 1986; Irisawa et al., 1993; Littleton & Ganetzky, 2000; Lipsius et al., 2001). The *eag* channel is also activated by the calcium depolarization, but in a more delayed fashion. The K^+ current carried by this channel type eventually triggers the pacemaker potential, thereby recommencing the oscillatory process.

Mutations affecting heart function in *Drosophila*

As noted above, there are several genes in *Drosophila* that encode ion channels and calcium signaling pathways involved in pacemaker function of the heart. Human homologues of these genes have also been identified. The gene *ether à-go-go* (*eag*) (Brüggemann et al., 1993) encodes a K^+ channel (Kaplan & Trout, 1969; Warmke et al., 1991; Bruggemann et al., 1993; Silverman et al., 2003). Flies bearing the *eag* mutant

exhibited heart beating irregularity at several test temperatures (Johnson et al., 1998). This gene has a homologue in humans known as HERG (Human *eag* Related Gene) (Warmke & Ganetzky, 1994), and mutations at this locus result in a cardiac arrhythmicity--Chromosome 7-linked long QT (LQT2) syndrome (Keating & Sanguinetti, 1996; Sanguinetti et al., 1996). Mutations in the *kumbakharna* gene in the fly, encoding the Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) produce severe cardiac defects (Sanyal et al., 2006) while several diseases that arise from defective SERCA function have been described in mammals; cardiac disorders are known to be associated with reduced SERCA2a function in cardiac muscles of higher vertebrates (MacLennan, 2000; Periasamy & Huke, 2001). It is well known that potassium channels are central to generation and possibly conduction of the heartbeat in *Drosophila*. Slowpoke (*slo*) is a mutation which encodes the pore-forming structural portion of a Ca^{2+} activated K^+ channel in *Drosophila melanogaster* (Atkinson et al., 1991). The role of the Ca^{2+}-dependent K^+ current (I_{CF}) is particularly critical in pacemaking of the heart (Johnson et al., 1998). The mutation *slo* virtually eliminates heartbeat by abolishing I_{CF} (Elkins et al., 1986; Johnson et al., 1998). Pallanck & Ganetzky (1994), report the cloning of mouse and human homologs of the *Drosophila melanogaster* calcium-activated potassium channel gene, *slowpoke* (*slo*). Both the human and mouse genes encode polypeptides that have more than 50% amino acid identities with their *Drosophila* counterpart. *no action potential* $^{temperature\ sensitive}$ (*nap*^{ts}) is a recessive mutation that affects the level of sodium channel activity and, at high temperature, causes paralysis associated with a loss of action potentials. *nap*^{ts} is a gain-of-function allele of *maleless* (*mle),* a gene required for X chromosome dosage compensation and male viability (Kernan et. al, 1991). *nap*^{ts} slows heartbeat and renders

it less rhythmic and prevents the heart from responding to temperature with an altered rate (Dowse et al., 1995). *mle* is highly homologous to human RNA helicase A and the bovine counterpart of RNA helicase A, nuclear helicase II, proteins implicated in the unwinding of RNA (Lee et al., 1997). The mutation *dunce* (*dnc*^{1}), encodes a cAMP-specific phosphodiesterase (PDE), which has adverse effects on learning and memory in *Drosophila.* cAMP-PDE also interacts with the calcium-calmodulin signaling pathway. This signaling pathway is also modulated by melatonin (Turjanski et al., 2004). Modulation of this signaling pathway changes the concentration of intracellular calcium. Calcium signaling is vital to heart function in both *Drosophila* and humans. Four human genes (DPDE1 through DPDE4) are closely related to the *dnc* locus of Drosophila (Bolger et al., 1993).

Homologies between vertebrate and *Drosophila* heart development

These parallels extend to heart development. The discovery of the homeobox transcription factor Tinman in *Drosophila* and its conservation in vertebrates provided the first evidence that heart development was controlled by homologous pathways in the animal kingdom (Bodmer, 1993; Azpiazu & Frasch, 1993). The NK-2 type homeobox-containing gene *tinman* (*tin*) (Kim & Nirenberg, 1989), is essential for early molecular events initiating cardiac development in *Drosophila* (Bodmer, 1993; Bier & Bodmer, 2004). A *tin* homolog, *NKX2-5*, has been strongly implicated as the cause of a developmental atrial septal defect and atrioventricular nodal dysfunction (Schott et al., 1998). The key determinants of cardiogenesis in the fly also play fundamental roles in early cardiac specification in the vertebrates, and they do so in similar ways (Bier & Bodmer, 2004). For example, in flies and vertebrates, Dpp/bone morphogenetic protein

(BMP)-TGF-h signals cross germ layers to maintain Tinman/NKX2-5 expression (Frasch, 1995; Schultheiss et al., 1997; Lockwood & Bodmer, 2002). A similar relationship exists between Tinman/NKX2-5 and Pannier/GATA-4 (Klinedinst & Bodmer, 2003; Garg et al., 2003). Moreover, mutations have been identified in humans in many of these conserved heart-specifying genes, such as NKX2-5, GATA-4, and TBX-5, which cause various forms of congenital heart disease (Basson et al., 1997; Schott et al., 1998; Garg et al., 2003). Because the control of early cardiac development and function is likely to be carried out by conserved networks of target genes, many congenital heart diseases, even those for which a major player has been identified, are likely to be multigenic (Ranade et al., 2003; Kraft & de Andrade, 2003).

Of particular interest to me are the effects of the indolamine melatonin (N-acetyl-5-methoxytryptamine) on the pacemaker of *Drosophila melanogaster*. Neurotransmitters and neurohormones are central to control of heart function in *Drosophila*. Prior research in our lab has shown that heart rate in the pupa of *D. melanogaster* is significantly increased by serotonin (5-hydroxytryptamine, 5-HT) (Johnson et al., 1998). Furthermore, four 5-HT receptors have been identified and sequenced in *Drosophila melanogaster*, strongly indicating a physiological purpose for this neurotransmitter (Saudou et al., 1992; Colas et al., 1995).

Because of the strong antagonistic relationship between serotonin and melatonin in regulating circadian rhythms (see below), and the fact that they are both synthesized from similar precursors, my interests focus on the effects that melatonin might have on the pacemaker activity and heart function of *D. melanogaster*. It was hypothesized that the acceleratory effect of serotonin would be paired with a deceleratory effect by melatonin. Surprisingly, unpublished studies of *D. melanogaster* and melatonin

performed by Evelyn Powers and me between 2009 and the present have shown that melatonin has no significant effect on heart rate, but significantly increases rhythmicity in hearts that were either beating normally or substantially arrhythmic before treatment with melatonin.

Melatonin

In 1958, Lerner et al. successfully isolated a substance from pineal extracts, which had been shown to lighten the skin color of tadpoles, frogs, toads, and fish (McCord & Allen, 1917; Kitay & Altschule, 1954). Melatonin, a ubiquitously present, low molecular weight molecule in living organisms, has a simple structure; however, it exhibits pleiotropic biological activities in species from bacteria to mammals (Hardeland et al., 2011). In vertebrates, the roles of melatonin are numerous and include: regulating circadian rhythm and acting as a neuromodulator, hormone, cytokine and biological response modifier (Man et al., 2011; Reiter, 1991). It also affects brain, immune, gastrointestinal, cardiovascular, renal, bone and endocrine functions, and acts as an oncostatic and anti-aging molecule (Yu et al., 1993; Pandi-Perumal et al., 2008; Slominski et al., 2008; Leja-Szpak et al., 2010; Celinski et al., 2011). Some of these activities are receptor-mediated, including via melatonin membrane receptors and nuclear receptors (Reppert, 1997; Dubocovich et al., 1999; Dubocovich & Markowska, 2005; Imbesi et al., 2009; Shiu et al., 2010). Other functions are melatonin receptor independent (Reiter, 1996; Tan et al., 2002) including the interactions of melatonin with reactive oxygen species (ROS) and those mediated by its bioactive metabolites (Silva et al., 2004, 2005; Mayo et al., 2005; Manda et al., 2007, 2008; Hardeland et al., 2009; Schaefer & Hardeland 2009). Melatonin is an indolamine which is biologically synthesized from the amino acid L-Tryptophan. Serotonin is a precursor to melatonin.

Melatonin release in mammals, birds and reptiles varies in a daily cycle, as does serotonin, and is well known for its key role in regulating circadian rhythms. In mammals, a circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus receives information from the retina about environmental light. The clock controls a rhythm of the enzyme hydroxyindole-O-methyltransferase (HIOMT), which O-methylates N-acetylserotonin to produce melatonin in the final step of melatonin synthesis. This information passes through the neural circuits of the superior cervical ganglia and controls rhythmic melatonin release by the pineal gland. Melatonin then travels through the blood stream and binds to melatonin receptors located throughout the body. Melatonin levels are highest at night, when serotonin levels are at their lowest. As we are exposed to daylight, serotonin levels increase and melatonin levels decline.

In addition to the pineal gland, some melatonin is synthesized in the Harderian gland of rats (Bubenik et al., 1976), retina of chickens (Iuvone et al., 2002), bone marrow of rats and humans (Conti et al., 2000), gastrointestinal tract of humans, birds and rodents (Lee & Pang, 1993) and lacrimal glands of Syrian hamsters (Mhatre et al., 1988). Rat and pig studies by Bubenik et al., 1996 confirmed early findings of Huether and coworkers (1992) that concentrations of melatonin are substantially higher in gastrointestinal tract tissues than in plasma. Interestingly, at any time of the day or night, the gut of humans contains at least 400 times more melatonin than the pineal gland (Chen et al., 2011). In humans endogenous melatonin is released from the pineal gland into the bloodstream and then into other bodily fluids, including cerebral spinal fluid, saliva, and bile (Head, 2005). Blood (endogenous) melatonin is mainly bound to albumin (70%) and to a lesser extent to orosomucoid (Morin et al., 1997).

Orosomucoid is a glycoprotein in the blood plasma that functions in carrying lipophilic compounds, such as melatonin. Circulating melatonin can reach all body tissues and is able to cross the blood–brain barrier to modulate brain activity (Hirata et al., 1974).

During the day human serum concentrations of the hormone are low (10-20 pg/ml), and significantly increase at night (80-120 pg/ml) with peak between 24:00 and 03:00 h. The onset of secretion is usually around 21:00-22:00 h and the offset at 07:00-09:00 h (Karasek et al., 1998).

Exogenous melatonin is rapidly absorbed in humans when taken orally and peak serum levels are observed at 60-150 minutes. Peak concentrations after oral dosing of 80mg of crystalline melatonin are significantly higher (350 to 10,000 times) than those seen with endogenous melatonin secretion (Waldhauser et al., 1984). Exogenous plasma melatonin displays a short half-life. Kinetic studies of circulating melatonin following intravenous or subcutaneous bolus administration demonstrated an elimination phase with a half –life ($t_{½}$) of 44 minutes in humans (Iguichi et al., 1982), 30 minutes in rhesus monkeys (Reppert et al., 1979), 20 to 24 minutes in rats (Ozaki et al., 1976; Gibbs & Vriend, 1981; Chan et al., 1984), and 13 to 14 minutes in Syrian hamsters (Vaughan et al., 1986). Endogenous melatonin is mainly metabolized in the liver to 6-OH-melatonin and excreted as conjugates of sulfuric and glucuronic acid (Kveder, 1961). About 90% of endogenous melatonin is excreted in urine as 6-sulfatoxymelatonin (aMT6s) with a plasma half-life of 10–45 minutes (Arendt, 1995). Exogenous and endogenous melatonin are both metabolized and excreted via the same pathways, but the bioavailability of exogenous melatonin from an oral dose ranges from 10-56 percent.

The low bioavailability of exogenous melatonin is thought to be a consequence of hepatic first-pass extraction, which converts melatonin to its metabolite before it enters the systemic circulation (Di et al., 1997).

Melatonin receptors

There have been two membrane bound melatonin receptors identified and characterized in humans, MT1 and MT2. MT1 was known as Mel1a, and MT2 as Mel1b (Dubocovich et al., 2010, 1998). Both of them belong to the family of G protein-coupled, seven transmembrane receptors (GPCRs) (Dubocovich et al., 2003; Witt-Enderby et al., 2003). However, melatonin is also lipophilic, which allows it to enter the nucleus directly and bind to nuclear receptors in the RORα/RZR family (Slominski et al., 2012).

In humans, MT1 receptors are expressed in the brain, cardiovascular system (including peripheral blood vessels, aorta and heart), immune system, testes, ovary, skin, liver, kidney, adrenal cortex, placenta, breast, retina, pancreas and spleen (Dubocovich & Markowska, 2005; Fischer et al., 2008; Pandi-Perumal et al., 2008; Slominski et al., 2005, 2008). In the brain, the receptor is predominantly found in the hypothalamus, cerebellum, hippocampus, substantia nigra and ventral tegmental area (Pandi-Perumal et al., 2008). MT2 receptors have been found in the immune system, brain (hypothalamus, suprachiasmatic nucleus (SCN)), retina, pituitary, blood vessels, testes, kidney, gastrointestinal tract, mammary glands, adipose tissue, and the skin (Dubocovich et al., 2005; Reppert et al., 1995; Roca et al., 1996; Slominski et al., 2005). It is highly likely that if the melatonin receptor is present in *D. melanogaster*, it will also be a GPCR.

At present, 35 GPCRs are functionally characterized in *Drosophila.* Another 14 receptors are predicted to be involved in neuropeptide signaling pathways, but their ligands are still unknown and therefore they are classified as "orphan" receptors (Caers et al., 2012).

Effects of melatonin

Melatonin's many effects in humans include its hypnotic/sedative effects, its ability to shift circadian rhythms when administered at appropriate times of the day, its ability to modulate the immune system by increasing T lymphocyte, natural killer cell, eosinophil and cytokine production (Lissoni et al., 1993), and its ability to slow cancer growth by inhibiting mitosis and inducing apoptosis of some cancer cells (Blask et al., 2002). Melatonin is a powerful antioxidant that stimulates gene expression and activities of superoxide dimutase, catalase, and glutathione peroxidase; all of which play a role in the metabolism of H_2O_2 which ultimately results in reducing the formation of the devastatingly toxic OH radical (Reiter, 1996). Owing to its antioxidant properties, melatonin also shows beneficial anti-aging effects in rats (Poeggeler, 2005). Melatonin may also act at the level of the mitochondrial respiratory chain to reduce electron leakage and, therefore, free radical generation (Reiter et al., 2002). The cardio-protective effects of melatonin (Tan et al., 2002) are of particular interest and will be discussed in more detail shortly.

Early melatonin research focused on its synthesis and metabolic pathways, specifically in relation to its day/night cycle, its improvement of thyroid function in peri-menopausal and menopausal women, its ability to lighten skin pigmentation in amphibians (McCord & Allen, 1917; Kitay & Altschule, 1954), its beneficial effects on behavioral and sleep disorders in human subjects, and its key roles in the modulation of

the endocrine system including regulation of GnRH (gonadotrophin releasing hormone) release, stimulation of oxytocin secretion, promotion of progesterone synthesis release, regulation of cortisol production, and promotion of androgen production (Balík et al., 2004; Schaeffer & Sirotkin, 1997; Tamura et al., 2008, 2009). Models used in early research included predominately humans and avian species, but most research on melatonin and its cardiovascular effects in mammals has been carried out in humans and rats.

Since the identification of melatonin by Lerner et al. (1958), it has been shown that melatonin is involved in the regulation of many physiological systems, including the cardiovascular system (Vazan et al., 2003;2005). Minimal research has been done on the effects of exogenous melatonin in the human cardiovascular system. The results obtained thus far show that melatonin improves cardiovascular health, however, the question remains as to whether melatonin's effects on cardiovascular function are antioxidant in nature or receptor mediated. It is well known that melatonin is a potent antioxidant, even more powerful than vitamins C and E. In 2002, Reiter and Tan showed that melatonin exhibits cardio-protective effects in rat hearts exposed to ischemia and then re-perfused. Cagnaccie et al., (1998) and Arangino et al., (1999) found that the oral administration of melatonin in comparison to placebo was able to reduce blood pressure, vascular reactivity (vasorelaxant effect), and catecholamines in healthy young women and men. These data suggest that melatonin administration may induce beneficial effects on the circulation of humans and subjects may be protected against cardiovascular accidents with regular melatonin supplementation. In the study completed by Cagnacci et al., (1998), the cardiovascular effects induced by the daytime administration of melatonin (1 mg) were compared with those of placebo in 17 young,

healthy, early follicular-phase women. Compared with placebo, the administration of melatonin modified, within 90 min, the pulsatility index (PI) of the internal carotid artery, abdominal aorta, and axillary artery. The pulsatility index is a measure of the variability of blood velocity in a vessel evaluated by color Doppler ultrasound. In a subsequent, two day study (Arangino et al., 1999), 14 normal, healthy men were given either 1 mg of melatonin or placebo on Day 1 and then, on Day 2, received the opposite treatment (as compared to the treatment they received on Day 1). Ninety minutes after supplementation on both days the researchers measured blood flow in a blood vessel (PI) in the arm as well as blood pressure in both groups. The administration of melatonin significantly reduced blood pressure, the PI in the internal carotid artery, and catecholamine levels within 90 minutes. Melatonin reduces blood pressure in young men (Arangino et al. 1999), reduces myocardial contractility via a specific cardiac receptor in rat papillary muscle (Abete et al., 1997), and increases the antioxidant (glutathione peroxidase) activity in the plasma and erythrocytes of spontaneously hypertensive rats (Girouard et al., 2004). In addition, decreased melatonin levels were reported in various pathological conditions including hypertension with "nondipper pattern" (which means that blood pressure is independent of the sleep-wakefulness cycle), aggravation of heart failure, ischemic heart disease, and in patients after acute myocardial infarction (Paulis & Simko, 2007). Also, patients with coronary heart disease have lower than normal melatonin levels (Dominguez-Rodriguez & Abreu-Gonzalez, 2010). Interestingly, incidence of sudden cardiac death is high in the morning hours when melatonin levels are low (Sahna et al., 2006).

Melatonin mechanisms of action

The mechanisms by which melatonin exerts its protective effects are not clearly understood. It is evident that melatonin is a very powerful antioxidant, and therefore has the ability to scavenge free radicals that can damage tissues. In recent years, research has shifted its focus to melatonin's powerful antioxidant properties as they relate to cardiovascular health. Melatonin is a powerful scavenger of reactive oxygen species (ROS), including the hydroxyl (Reiter, Tan et. al., 1998) and peroxyl radicals (Pieri et al., 1994) as well as singlet oxygen (Sewerynek et al., 1996) and nitric oxide (Noda et al., 1999). ROS are known to play a role in the formation of neurodegenerative and cardiovascular disease and cancer. The possibility exists that melatonin may have the ability to prevent such disease processes. In addition to scavenging ROS, melatonin stimulates the antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase (Head, 2005). Melatonin was also able to reduce lipid peroxidation more effectively than vitamin C or E (Gitto et al., 2001). Lipid peroxidation is the process whereby lipids undergo oxidative degradation. In this process, free radicals steal electrons from the phospholipids in the cell membrane and cause cell damage. The antioxidant properties of melatonin *in vivo* are demonstrated by the ability of melatonin to reduce ischemia-reperfusion injury in various organs of rats, including the heart (Tan et al., 1998), kidney (Sahna et al., 2005), brain (Cho et al., 1997) and liver (Sewerynek et al., 1996). Using the rat as a model, various experiments have been carried out in which pharmacological or physiological levels of melatonin have been used to protect the heart against ischemia-reperfusion (I/R) injury. This refers to the tissue damage that is caused after the blood supply returns to a tissue that has experienced a period of oxygen deprivation. In these studies, either exogenous melatonin was administered before,

during and after I/R injury to the heart, or the rats were pinealectomized to reduce endogenous levels of melatonin. The results showed a significant reduction in damage due to oxidative stress clearly owing to the concentration of melatonin. It was shown that the presence of melatonin reduced premature ventricular contractions and ventricular fibrillation, reduced ventricular tachycardia, reduced lipid peroxidation and lowered OH radical generation, reduced reperfusion arrhythmias and infarct volume and lowered mortality (Paulis & Simko, 2007). In addition to melatonin's protective effects of cardiac tissue, its protective effects of other tissue types have also been researched. In the *in vivo* brain (including the spinal cord), liver, kidney, gastrointestinal tract, lung and placenta, the amount of tissue damage and the severity of compromised function after I/R were shown to be reduced when melatonin was employed as an ameliorative agent. Furthermore, the damage inflicted by other agents that induce high oxidative stress is reduced when melatonin is exogenously administered (Paulis & Simko, 2007).

There is also evidence that melatonin's cardio-protective effects may be receptor mediated. In 2006, Rezanni *et al*., looked at the beneficial effects of melatonin in protecting against cyclosporine-A (CsA)-induced cardio-toxicity. When melatonin and CsA were injected along with luzindole, a melatonin receptor antagonist, it showed that the protective effects of melatonin were lost when the animals received the melatonin receptor antagonist. Melatonin's cardio-protective effects against myocardial ischemia/reperfusion (MI/R) injury in rats was were blocked by luzindole in experiments performed by Yu et al., in 2014.

Luzindole also abolished the short and long term cardio-protective effects of melatonin administered both before, during and after induced myocardial ischemia in rats (Lochner, 2006). Luzindole was also shown to block melatonin's ability to reduce blood pressure by binding to ML1 receptors in the anterior hypothalamic area (AHA) of the rat brain (Ding et al., 2001).

There are several non-specific mechanisms of melatonin action as well, which are unrelated to its antioxidant properties. Melatonin is thought to modulate the calcium/calmodulin signaling pathway either by changing intracellular Ca^{2+} concentration via activation of its G-protein–coupled membrane receptors, or through a direct interaction with calmodulin (CaM) (Turjanski et al., 2004). CaM is known to interact with a large number of proteins important for Ca^{2+} -dependent intracellular signaling, thus enabling the cell to control biological processes as diverse as muscle contraction, fertilization, cell proliferation, vesicular fusion, and apoptosis (Berridge et al., 1998). Satake et al., (1991) found that the inhibition of Ca^{2+} channels by melatonin causes vasorelaxation in the isolated rat aorta. Chen et al., (1993) found that there are time-dependent changes in $[Ca^{2+} + Mg^{2+}]$-dependent ATPase activity that are absent in pinealectomized or light-exposed rats, and that in-vitro studies of cardiac tissue incubated in the presence of melatonin over a wide range of doses showed that melatonin stimulated the Ca^{2+} pump. These findings suggest that the daily change in $[Ca^{2+} + Mg^{2+}]$-dependent ATPase activity in the sarcolemma of heart tissue is a result of the circadian rhythm in pineal melatonin production and secretion. These findings may be applicable to understanding normal cardiac physiology.

Melatonin research with *Drosophila*

Some melatonin research has been done using *Drosophila melanogaster* as a model. Arylalkylamine N-acetyltransferases (AANATs) are enzymes which catalyze the rate limiting step in melatonin synthesis in the vertebrate pineal gland. More specifically, conversion of serotonin (5-hydroxytryptomine; 5-HT) to N-acetylserotonin, the precursor of the circadian neurohormone melatonin, is catalyzed by serotonin N-acetyltransferase (AANAT) in a reaction requiring acetyl coenzyme A (AcCoA) (Hickman et al., 1999). Two different AANATs (AANAT1 and AANAT2) have been identified and characterized in *Drosophila melanogaster* (Hintermann & Meyer, 1995; Brodbeck et al., 1998; Amherd et al., 2000). A second enzyme, HIOMT, involved in O-methylating N-acetylserotonin in the final step of the melatonin synthesis pathway, has not yet been discovered in *Drosophila.*

Four receptors for 5-HT have been described in *Drosophila*: one shares sequence homology to the vertebrate 5HT receptor type 2 ($5HT_2R$; CG1056), one is similar to the vertebrate serotonin receptor type 7 ($5HT_7R$; CG12073) and two show high homology to vertebrate $5HT_1$ type receptor ($5HT_{1A}R$ and $5HT_{1B}R$; CG16720 and CG15113, respectively) (Silva et al., 2014). Computed gene (CG) numbers were used for genes identified during the annotation of the whole *Drosophila* genome sequence (Vaudry & Seong, 2014). *Drosophila melanogaster* synthesizes its own serotonin (Livingstone, 1981) and also has several clusters of serotonergic neurons located in its central nervous system (Valles, 1988).

Bonilla et al., (2006) found that melatonin was more effective than serotonin, lipoic acid and ascorbic acid at counteracting paraquat-induced oxidative stress in *D.*

melanogaster. In another pesticide exposure study, flies exposed to rotenone exhibited the neurodegenerative and behavioral deficits classically seen in Parkinson's disease (PD). Melatonin introduced into the feeding medium alleviated both symptomatic impairment and neuronal loss, supporting the idea that this agent may be beneficial in the treatment of PD (Coulom & Birman, 2004). In further examination of melatonin's antioxidant properties, a wild-type strain of *D. melanogaster* was exposed to the catalase inhibitor 3-amino-1,2,4-triazole in an effort to enhance oxidative stress from endogenous sources. While most of the flies that were fed inhibitor alone died, flies fed a combination of inhibitor and melatonin had a much higher survival rate (Coto-Montes and Hardeland, 1999). In addition to its protective effects, several studies have shown that melatonin-fed flies have a significantly increased lifespan (Li & Xu, 1997; Izmaylov & Obukhova, 1999; Bonilla et al., 2002; Anisimov, 2003; Terán et al., 2012).

The mechanism by which melatonin exerts its effects in *D. melanogaster* is not known. Because there had been no melatonin receptors identified in the fruit fly when I began my research, it was unclear whether melatonin acted only as a powerful antioxidant and free radical scavenger or had other, receptor-mediated effects that may be directly or indirectly regulating pacemaker activity. I proposed to conduct a series of experiments that would allow me further to explore melatonin's effects on pacemaker activity, emphasizing both genetics and pharmacology, and was eager to determine whether melatonin receptors are present in *Drosophila melanogaster*. Based on preliminary research in our lab that showed melatonin's effects on rhythmicity in wild-type hearts (Powers, unpublished), prior research identifying melatonin receptors and the effects of melatonin in various organ systems of human and other mammalian models,

and known homologies between the *Drosophila* and human genomes, I hypothesized that *Drosophila melanogaster* does have at least one melatonin receptor and that pacemaker activity is modulated by melatonin at the receptor level. If pacemaker activity is in fact modulated by melatonin at the receptor level in *Drosophila*, this will give us more clues about the mechanisms of melatonin's action in improving and protecting the human cardiovascular system.

Overview of research objectives

There has been no reported research to date that examines the antioxidant or other effects of melatonin on heart function in *Drosophila melanogaster*. An Honor's student in our lab carried out a series of experiments where wild-type *Drosophila* pupae were injected with varying concentrations of melatonin. Heart rate and rhythmicity were recorded and compared before and after injection. Results of these experiments showed no change in heart rate, but a significant increase in rhythmicity in previously arrhythmic hearts (Powers, unpublished). Although these results were not reported, they sparked my interest as to the mechanisms behind the rhythmicity increase.

The goals of my dissertation research have been twofold: 1. to examine the effects of melatonin on heart rhythmicity and frequency in various strains of *Drosophila melanogaster*, both wild-type and those bearing mutations affecting cardiac function. 2. to identify a melatonin receptor in *Drosophila melanogaster* and, therefore, elucidate whether melatonin's action is antioxidant related or receptor mediated. I believe that by combining genetics and pharmacology, the mechanism of melatonin action can be better understood.

Effects of melatonin on heart rate and rhythmicity

In chapter 1, I examined the response of Canton S (CS) (*Drosophila melanogaster* wild- type flies) to injections of varying concentrations of melatonin (0, 50 µM, 100 µM, 500 µM, 1000 µM and 2500 µM). With these data, I constructed a dose-response curve that allowed me to determine the most effective concentration of melatonin to use in subsequent experiments (1000 µM). Once this was determined, I injected the optimal concentration of melatonin into various strains of *Drosophila* that have a variety of different heart mutations, looking for a possible "rhythmicity rescue" of the mutation by melatonin. Since these mutations cause genetically-based alterations of heart physiology (usually pacemaker ion channels), rescuing an effect of a mutation with melatonin strongly implicates receptor-mediated activity of melatonin.

The mutations I tested were *no action potential* $^{temperature\ sensitive}$ (*nap*^{ts}), *Ca-P60A* $^{Kumbhakarna\ 170}$ (*SERCA*^{170}), *slowpoke* (*slo*), *dunce* (*dnc*^{1}) and *ether-a-go-go* (*eag*). These particular heart mutations involve variations either in ion channel activity or in intracellular calcium concentrations of the *Drosophila* pacemaker.

After my initial testing of the *Drosophila* mutant *slo*, I compared a dose response in this animal to that of CS wild-type flies by injecting *slo* pupae with the same concentrations of melatonin as described above (0, 50 µM, 100 µM, 500 µM, 1000 µM and 2500 µM).

The next set of experiments involved crossing the *slo* mutant with the *df* [*slo*] (*slowpoke* deficiency) strain. Deficiency stocks comprise chromosomal deletions which provide definitive null alleles for the genes they uncover on the sister chromosome and

are used to gauge the severity of any particular allele; indeed, the classical test for defining an amorphic or null allele is to assess whether the homozygous phenotype is as severe as when the allele is hemizygous with a deletion (Roote and Russell 2012). First generation (F1) progeny of this stock were injected with the optimal concentration of melatonin and the resulting rhythmicity index and frequency were analyzed.

My final set of experiments compared the effects serotonin, melatonin's precursor, with the effects of melatonin on heart rate and rhythmicity. Previous experiments carried out by Johnson et al.,1997, showed that serotonin significantly increased heart rate without increasing heart rhythmicity. Serotonin exerts its positive chronotropic effects by binding to 5-HT2 receptors that have been identified in the larval heart and aorta of *Drosophila melanogaster* (Majeed et al., 2014).

Melatonin receptor identification

The experiments described in the chapter 2 consist of a combination of pharmacological and genetic techniques designed to identify a possible melatonin receptor in *Drosophila melanogaster* and, therefore, determine whether melatonin's effects on heart rhythmicity are antioxidant related or receptor mediated.

Pharmacological experiments

Receptor identification often involves administration of known antagonists or agonists to the ligand in question. If melatonin is increasing heart rhythmicity in *Drosophila* by binding to a receptor, an antagonist acting at the receptor level, such as luzindole, will block these effects. Similarly, injection of a melatonin receptor agonist, such as 2-iodomelatonin, may mimic melatonin's effects.

The first set of experiments involved double injections of melatonin and luzindole in Canton-S, *slowpoke* and *nap*^{ts} *Drosophila* pupae. Injection Protocol: first inject pupa with 2500 µM luzindole, allow to rest for 1.5 minutes, then inject pupa with 1000 µM melatonin and immediately begin heart recording lasting for 10 minutes.

The second set of experiments involved injection of 1000 µM 2-iodomelatonin only in Canton-S and *slowpoke* pupae. Injection Protocol: an initial 10 minute pre-injection heart recording is followed by injection, then a 10 minute post-injection heart recording.

My final set of pharmacological experiments involved the injection of another known powerful antioxidant, ascorbic acid (vitamin C) in order to investigate further the mechanism of melatonin's effects on heart rhythmicity. Melatonin is a very powerful antioxidant that was found to be more effective than vitamin C or vitamin E in combating paraquat induced oxidative stress in *Drosophila melanogaster* (Bonilla et al., 2006). Canton-S (wild-type) and *slowpoke* (mutant) flies were injected with the 1000 µM ascorbic acid which was also dissolved in calcium free ringers with 1% ETOH. Heart rhythmicity index and frequency results were then compared to those of Canton-S and *slo* flies that were injected with calcium-ringers with 1% ETOH (controls). 2-iodomelatonin and melatonin.

RNAi Experiments

Ribonucleic Acid Interference (RNAi) is a process that permits elimination of expression of targeted gene sequences in an attempt to understand their function (Bellés, 2010). RNAi strains of orphan G-protein coupled receptors were crossed with a GMH5 driver that effectively silenced the gene in question in cardiac tissue. I chose four *Drosophila* orphan GPCRs to test: **CG4313, CG3171, CG13579, CG13575.** These specific strains were chosen because they come from a structural class of GPCRs that are known to bind substances that are structurally similar to melatonin.

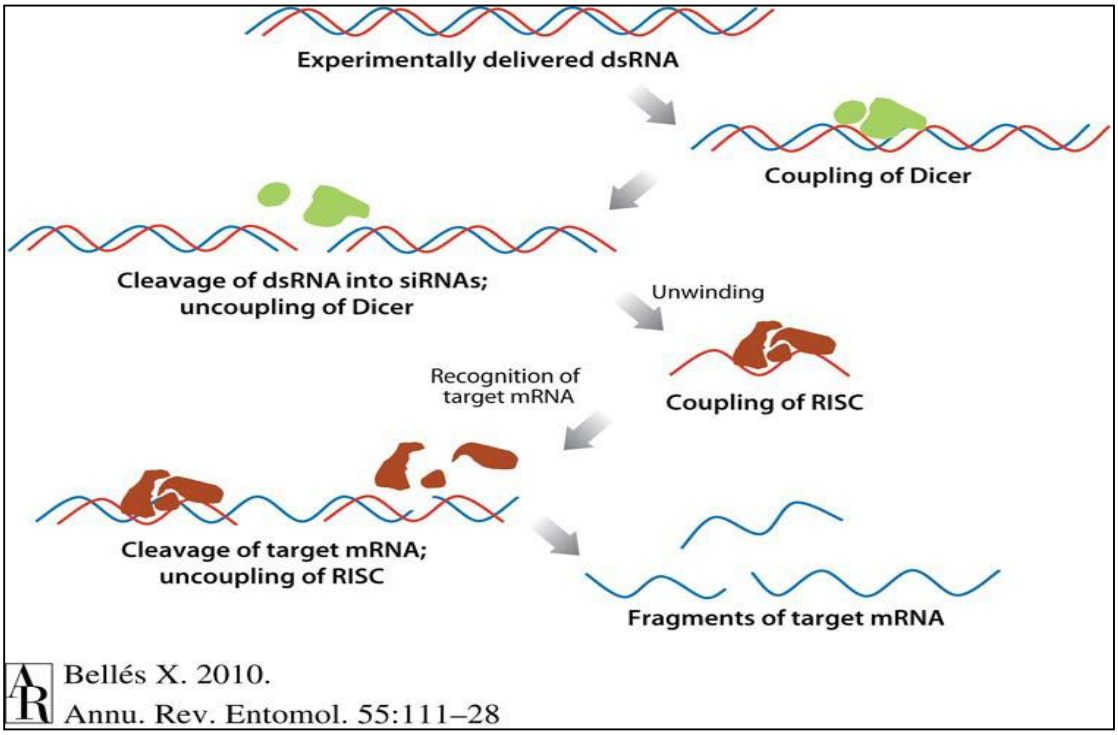


Figure I.1: The Generation of RNAi from dsRNA is completed as follows (a) the experimentally derived dsRNA binds to the enzyme dicer which cleaves the dsRNA at targeted sites (b). The coupling of a RISC to target mRNA creates cleavage (c) small subunits of mRNA (d) that are able to effectively silence targeted genes (Bellés, 2010).

CHAPTER 1

THE EFFECTS OF MELATONIN ON *Drosophila* HEART RATE AND RHYTHMICITY

Abstract

Melatonin (N-acetyl-5-methoxytryptamine), has been shown to have both cardio-protective and anti-arrhythmic properties in vertebrates largely owing to its powerful antioxidant properties. Very few studies have attributed improvements in cardiovascular health to the binding of melatonin to its receptors (MT1 and MT2). In the present study, micro molar concentrations of melatonin injected into the P1 pupae of *Drosophila melanogaster* wild type and the following heart mutants (*slo*, dnc^{1}, *eag*, $nap^{ts}$and $Ca\text{-}P60A^{Kum170}$) show acute and profoundly significant increases in heart rhythmicity across all subjects ranging from 54-104 % without significantly affecting heart rate of these animals. Effects of melatonin were also compared to injections of the melatonin precursor serotonin (5-hydroxytryptamine), where serotonin significantly increases heart rate in wild type and *slo* without significantly increasing heart rhythmicity. In conclusion, the acute antiarrhythmic effects of melatonin injection in *Drosophila melanogaster* are likely receptor-mediated based on the combination of collective data from the experiments performed in Chapters 1 & 2.

Introduction

There are a variety of causes of cardiovascular disease ranging from inherited conditions that alter heart structure or cause dysfunction in pacemaker ion channels, to ischemic cardiac events that result in damage to heart tissue from oxidative stress and

subsequent remodeling of heart structures. Furthermore, these diseases, whether inherited or acquired, are associated with different types of heart arrhythmias that alter overall function of the heart and often lead to sudden cardiac death. Clinical and pathologic findings suggest that patients with conditions such as coronary artery disease, cardiomyopathy, cardiac rhythm disturbances, or hypertensive heart disease are at high risk of SCD (Roberts, 1990). Abnormal heart rhythms, notably atrial and ventricular fibrillation, are leading causes of death and disability. Between 0.5 million and 1 million North Americans and Europeans die each year from sudden cardiac death (SCD), which causes 10–20% of all deaths among adults in the Western world (De Vreede-Swagemakers et al., 1997; Zheng et al., 2001). Prevention and treatment of cardiac arrhythmias include medical procedures such as cardioversion, implantable cardioverter defibrillators (ICDs) and catheter ablation; surgical procedures such as coronary artery bypass grafting; and the use of anti-arrhythmic medications. While some procedures like radio-frequency ablation can cure arrhythmias (Morady, 1999), available anti-arrhythmic drugs are incompletely effective in preventing arrhythmias, and such treatment carries risks, including actually promoting arrhythmia (Knollmann & Roden, 2008). An understanding of the underlying mechanisms of arrhythmias is essential to the future development of safe and effective preventative therapies.

The study of heart development and physiology in *Drosophila melanogaster* is intensifying rapidly as it continues to expand as a model system for studying cardiac problems generally and genetic influences in particular. Findings in the fly are directly applicable to the human heart as a growing number of genes have been identified with homologous function in both organisms (Bier & Bodmer, 2004). *Drosophila*

melanogaster is the only insect in which the molecular genetic basis of heart development process has been studied and reviewed in detail. *Drosophila* has an open circulatory system consisting of a simple, linear, tubular heart that circulates hemolymph through the larval and adult body cavity (Rizki, 1978). The transport of oxygen in the fruit fly is carried out by their highly branched tracheal system. In stark contrast, the vertebrate heart consists of an elaborate, closed system of chambers and vessels that transport blood through the body of the organism in order to provide oxygen to the tissues. Although the final heart structure in Drosophila is very different from that of vertebrates, many of the basic elements for cardiac specification and differentiation are conserved. The molecular components and basic control mechanisms of how the heart develops and how it functions are strikingly similar in vertebrates, other chordates, and insects (Bodmer, 1995; Bodmer & Venkatesh, 1998; Cripps & Olson, 2002; Harvey, 1996; Holland et al., 2003; Zaffran & Frasch, 2002). (See Section I Introduction for more detail about *Drosophila melanogaster* heart development.)

Melatonin (N-acetyl-5-methoxytryptamine), the main product of the pineal gland, is well known for its regulation of the biorhythms. Since the identification of melatonin by Lerner et al. (1958), it has been shown that melatonin is involved in the regulation of many physiological systems, including the cardiovascular system (Vazan et al., 2003;2005). Melatonin effects its response by either binding to one of its three receptors (MT1, MT2 or MT3), binding to nuclear receptors, or through its antioxidant activity. Very little research has been done attesting to melatonin's effects on heart arrhythmias. Only two studies have shown a relationship between melatonin and reduced heart arrhythmia in rats (Bertuglia & Reiter, 2007; Benova et al., 2015). The

former attributed melatonin's anti-arrhythmic effects to its acute effects on myocardial Cx43 channel's mediated electrical coupling via Cx43 phosphorylation; the latter attributed melatonin's anti-arrhythmic effects to its ability to prevent tissue damage after ischemia-reperfusion injury due to its powerful antioxidant activity. (See Section I Introduction for more detail on melatonin.)

In this study, I examined the effects of melatonin and serotonin on heart rate (frequency) and rhythmicity of *Drosophila melanogaster* wild-type and heart mutants when injected into early (P1) pupae. If melatonin is exerting its anti-arrhythmic effects via antioxidant mediated activity, it is expected that injection with serotonin will produce similar results, owing to its equally powerful antioxidant properties. The selected heart mutants (*slowpoke, ether-a-go-go, no action potential*$^{temperature\ \text{sensitive}}$, *dunce, sarco-endoplasmic reticulum* Ca^{2+}*-ATPase allele 170)* were chosen based on their arrhythmic phenotypes. (See Section I Introduction for a more detailed description of the aforementioned *Drosophila* mutants.)

Based on a smaller scale study previously performed in our lab, I predicted melatonin would increase heart rhythmicity without altering heart rate in *Drosophila* wild type flies and some *Drosophila* heart mutants. The following experiments were an attempt to elucidate the mechanism by which melatonin might be exerting its anti-arrhythmic effects by observing the responses of the different heart mutants. Previous experiments with *Drosophila melanogaster* have shown the acceleratory effects of injected neurotransmitters such as serotonin, octopamine, norepinephrine, and acetylcholine (Johnson et al., 1997), but there have been no published or reported experiments carried out with melatonin thus far.

Materials and Methods

Fly Cultures and Strains

All mutant and control strains were maintained in 250ml bottles in a 12:12 light: dark (L:D) cycle under uncrowded conditions in a humidified, temperature-controlled incubator at 25º on malt-molasses-cornmeal-agar medium. No population bottlenecks occurred. Propionic acid was added to the medium to control mold growth (Jennings et al. 2009).

Genetics

Homozygous individuals of the following *Drosophila melanogaster* strains were tested:

Canton-S (CS*)* wild-type

dunce (*dnc*^{1}) (Dudai et al., 1976)

ether-a-go-go (*eag*) (Wu et al., 1983; Drysdale et al., 1991; Brüggemann et al., 1993)

no action potential temperature sensitive (*nap*^{ts}) (Wu et al., 1978)

sarco-endoplasmic reticulum Ca^{2+}*-ATPase allele 170* (*Ca-P60A*^{Kum170}*)*

(Sanyal et al., 2005)

slowpoke (*slo*) (Elkins et al. 1986)

Reciprocal crosses were carried out with the mutant strain *slowpoke* and *df* [*slo*] (*slowpoke* deficiency). Crosses with deficiency stocks lead to deletions which provide definitive null alleles for the genes they uncover and are used to gauge the severity of any particular allele; indeed, the classical test for defining an amorphic or null allele is to assess whether the homozygous phenotype is as severe as when the allele is hemizygous with a deletion (Roote & Russell, 2012). One cross contained 10 *slowpoke* virgin females and 10 *df* [*slo*] males. The second cross contained 10 *df* [*slo*] virgin females and

10 *slowpoke* males. Reciprocal crosses were maintained in 250ml bottles under the same environmental conditions described above.

Ribonucleic Acid Interference (RNAi) is a process that permits elimination of expression of targeted gene sequences in an attempt to understand their function (Bellés 2010). Ten *slo* RNAi virgin females were crossed with 10 GMH5-Gal4 males in order to eliminate *slo* and mimic the *slo* mutation phenotype. This cross was maintained in a 250 ml bottle under the same environmental conditions described above. The heart-specific driver employed here is GMH5–Gal4, a 900-bp heart enhancer fragment 73 from the *tinman* gene (Bodmer, 1993; Venkatesh et al., 2000) that was cloned into the P{GaWB} vector upstream of the Gal4 sequences. This driver was enhanced with multiple copies of a UAS–Gal4 element allowing stronger myocardial expression when activated in late embryonic or during adult stages (Wessells & Bodmer, 2004).

Measurement of Heartbeat

All test subjects were collected at the third-instar larval stage just as they were entering the early pupal (P1) stage. At this point, they are translucent, making any heart movements transmitted to the surrounding organs easily visible. Each pupa was placed on a glass slide in a drop of distilled water and set in the light beam of an Olympus binocular microscope. The light source was powered by a DC supply to eliminate 60 Hz variation. The water served to stabilize and equilibrate temperature between the slide and the pupa, as well as to increase the brilliance of the illumination passing through the pupa. The pupa was adjusted so that one clear portion of the abdomen filled the field of view with just the surrounding fat bodies visible. The movement of these organs results in a variation in illumination that can be detected by a phototransistor. This signal is

amplified and recorded in a microcomputer through a DAS8 AD converter (Johnson et al., 1997). Temperature of the slide was maintained at 25˚C with a Sensortek TS-4 unit, however it was not possible to record temperatures within the animals. Recordings were done for 10 minutes pre-injection and again post-injection. Over the 10-minute time period for both pre-and post-injection recordings, heart frequency and heart rhythmicity data were recorded at 1-minute intervals, resulting in 10 separate readings. This allowed for analysis of the effects of injected substances over time. For pre-injection recordings each pupa was allowed 1.5 minutes to equilibrate to 25˚C after being placed under the microscope and before recording started. After injection of a substance, flies were immediately placed under the microscope and recording was started without the 1.5 minute equilibration time in order to observe the immediate effects of the injected substance.

Test of *Ca-P60A*^{Kum170} mutant strain specifics

Both pre and post injection heartbeat were recorded for 10 minutes at 25˚C for the *Ca-P60A*^{Kum170} mutant strains. In addition to the protocol for the other subjects, *Ca-P60A*^{Kum170} mutants were also exposed to a 2.5 minute heat shock at 41˚C either before or after melatonin injection; in between pre- and post-injection heartbeat recordings.

For pre-injection heat shock, each pupa was placed in a glass vial and then submerged in a 41˚C water bath for 2.5 minutes. The pupa was then immediately transferred to a glass slide, injected with melatonin and placed under the microscope for post-injection recording. For post-injection heat shock, each pupa was first injected, then immediately placed in a glass vial and subjected to the 41˚C water bath for 2.5 minutes. The pupa was subsequently transferred to a glass slide and immediately placed

under the microscope for post injection recording. Post-injection pupae were not allowed any time to acclimate before recording to allow observation of any immediate effects that injected substances might have.

Pharmacology of melatonin and ascorbic acid

Melatonin is insoluble in water and must initially be dissolved in ethyl alcohol. For consistency, both melatonin and ascorbic acid were first dissolved in 1 % ethyl alcohol and then added to Calcium-free *Drosophila* Ringer's solution (Ashburner, 1989). Concentrations of 2500 µM, 1000 µM, 500 µM, 100 µM, and 50 µM melatonin were prepared to assay dose response. A single concentration of 1000 µM ascorbic acid was prepared. Based on these results, the standard concentration used for ensuing experiments of both melatonin and ascorbic acid injections was 1000 µM.

Solutions were perfused directly over the pacemaker and caudal region of the dorsal vessel by injection into the caudal end of the animal (Johnson et al., 1997). Glass electrodes were used for injection and were pulled on a World Precision Instrument PUL-100 micropipette puller. Tips were gently broken to assure sharpness.

Following a 10 minute pre injection recording, the pupa was placed under an Olympus dissecting scope for injection. A World Precision Instrument Nanoliter Injector (model # A203XVY) was calibrated to administer 46 nl volume of liquid. A micromanipulator was used to position the glass electrode in the dorsal posterior region near the caudal region of the heart. Heartbeat was recorded for 10 minutes before injection and for 10 minutes immediately (within 1 minute) after injection of a substance as delineated above.

Analysis of heartbeat data

Data from individual flies taken at 25˚C were analyzed by autocorrelation (Chatfield, 1980) and Maximum Entropy Spectral Analysis (MESA) (Ulrych & Bishop, 1975; Dowse, 1989; Levine et al., 2002; Dowse, 2009). Estimations of heart rate frequency were based both on the spectral analysis and on inspection of the raw data plots and autocorrelation for verification.

Regularity of the heartbeat was quantified from the autocorrelation analyses. Rhythmicity in the signal results in recurring peaks of positive and negative correlation and the decay envelope reflects regularity in the signal (Chatfield, 1980). The height of the second peak was expressed as a fraction of the height of the zeroth lag, (i.e., a coefficient of correlation; see Dowse et al., 1995; Levine et al., 2002; Dowse, 2009 for details). We refer to this coefficient as "rhythmicity (RI)." We commonly observe that with more skipped beats, irregular intervals between beats, periods of heartbeat cessation, and rate variations tracks the level of irregularity in RI (Johnson, Ringo, & Dowse, 2000; Levine et al., 2002; Dowse, 2009).

The program was set up to measure heart rate frequency and heart rhythmicity over a 10 minute period. At each minute over the 10 minute period, a value for frequency and rhythmicity was calculated. This program allows us to examine the effects of injected substances over a longer period of time allowing estimates of when the effects began and how long they last (see figure 1.1).

In order to further explore the way in which the heartbeat was altered by the injection of melatonin, we wrote software to sort through the recorded data to pinpoint and record the duration of the interval between each peak in the signal and the next.

Intervals in which the signal equaled or exceeded the mean of the absolute value of the time series were identified sequentially and a simple bubble sort was applied to identify the highest value in this time frame. Its time was recorded. Thus we are able to visualize the "Inter-Beat Interval" or IBI as a function of time. Fig 1.1g-j shows an example of this analysis, the IBI for a Canton-S pupa recorded for 10 minutes and plotted as a function of time.

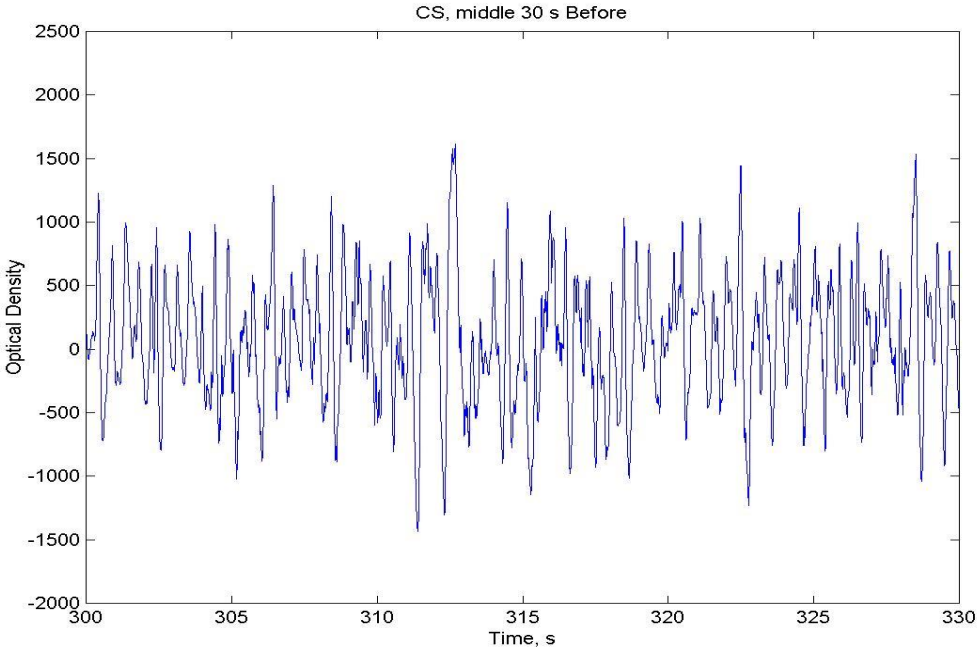
Because each fly was used as its own control and more than two measurements were carried out on the same subject Repeated Measures Analysis of Variance (ANOVA) tests were used to compare data before injection and after injection for each *Drosophila* strain tested. Kolmogorov-Smirnov goodness of fit tests were used to show that all data were normally distributed. Both tests were carried out within the statistical software R.

Results

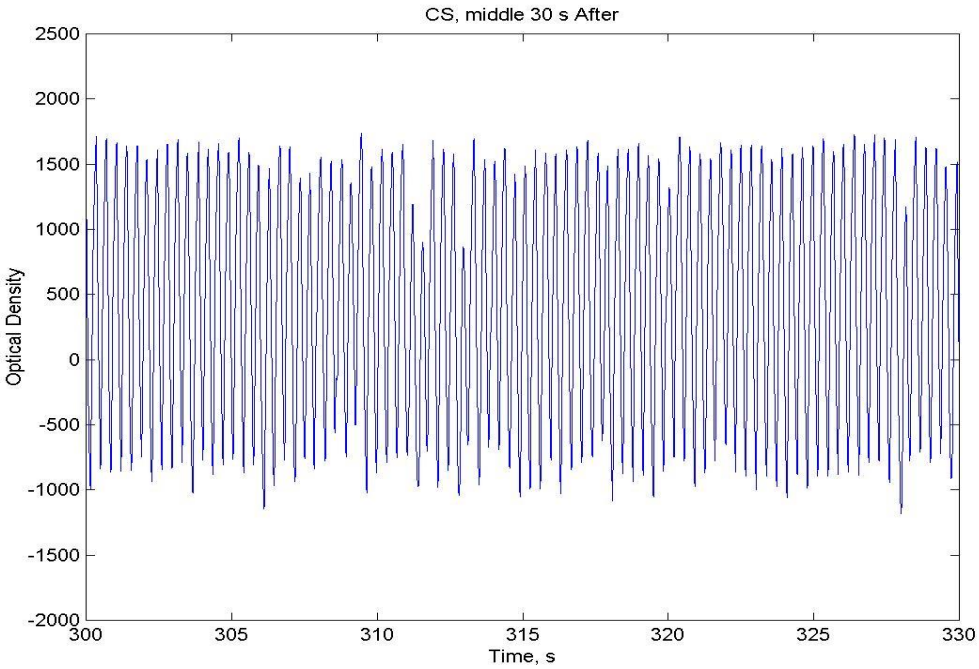
Data Analysis Figures

Figure 1.1 Full analysis of the heartbeat of a CS pupa before and after injection with 1000μM melatonin. **a, b** Raw data optical recording: a 30-s segment starting at the midpoint of the raw data before **(a)** and after **(b)** melatonin injection shows this difference in more detail. This 30-s segment is representative of the 4 minute segments indicated in **(k)**. **c, d** Autocorrelation for these segments used to assess regularity of heart rhythm before **(c)** and after **(d)** melatonin injection. **e, f** Spectral analysis (MESA) for heart rate (frequency) of these segments starting at the midpoint before **(e)** and after **(f)** melatonin injection. **g, h** Interbeat Interval (IBI) analysis shows this change in a more objective fashion. **(g)** (before) and **(h)** (after) show the IBI analyses for both records in their entirety. The change in regularity is easily seen after a period of "wash in". Note the development of the more regular beat after the wash-in period. **i, j** 30-s segments starting at the midpoint of the data show the IBI analysis at higher magnification before **(i)** and after **(j)** injection. The difference in the variability of IBI is striking. Autocorrelation and MESA for these segments **(c)** and **(d)** and **(e)** and **(f)** are remarkable and clearly track the change in IBI. Together, these analyses point towards the increase in RI stemming from the change in variance in IBI. **(k)** Shows the average (CS pupae, N=30) rhythmicity index (RI) for each minute; 10 minutes before injection and 10 minutes after injection. The horizontal bar over minutes 4-7 and 15-18 indicate time segments used for comparison in all experiments.

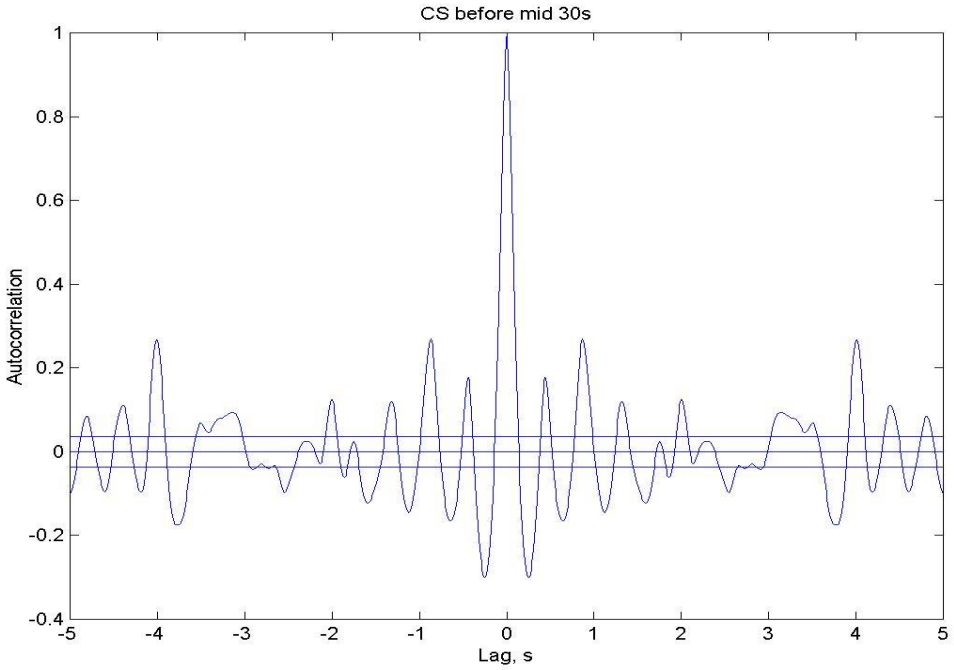
a. Raw data optical recording: a 30-s segment starting at the midpoint of the raw data before melatonin injection



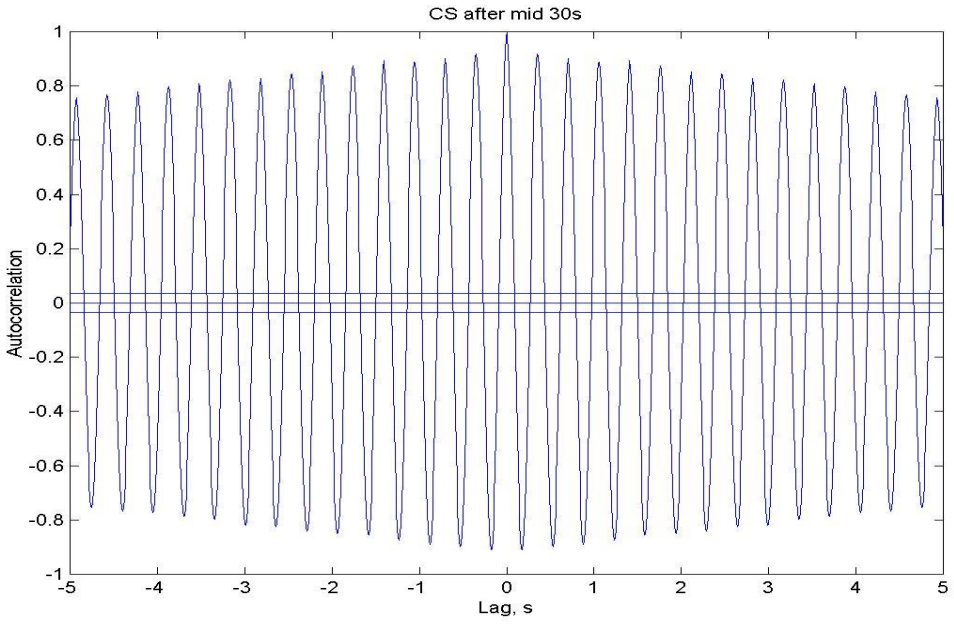
b. Raw data optical recording: a 30-s segment starting at the midpoint of the raw data after melatonin injection



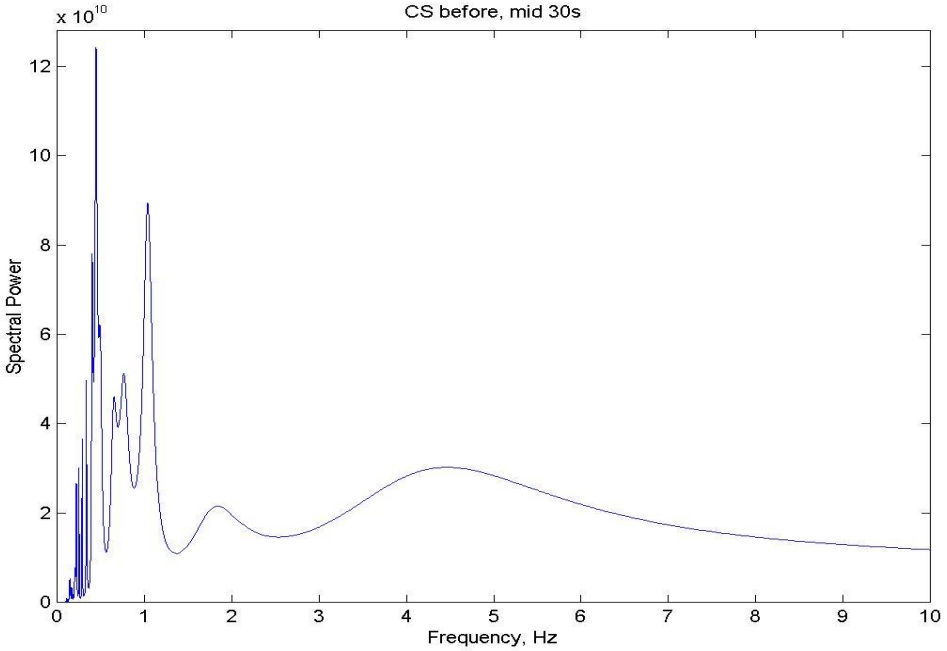
c. Autocorrelation used to assess regularity of heart rhythmn before melatonin injection



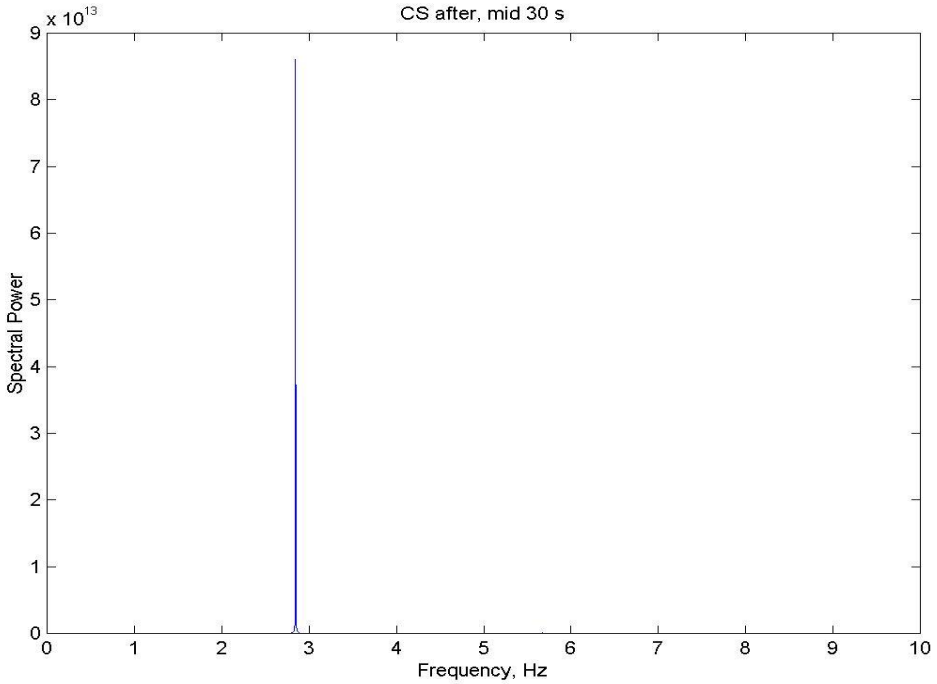
d. Autocorrelation used to assess regularity of heart rhythmn after melatonin injection



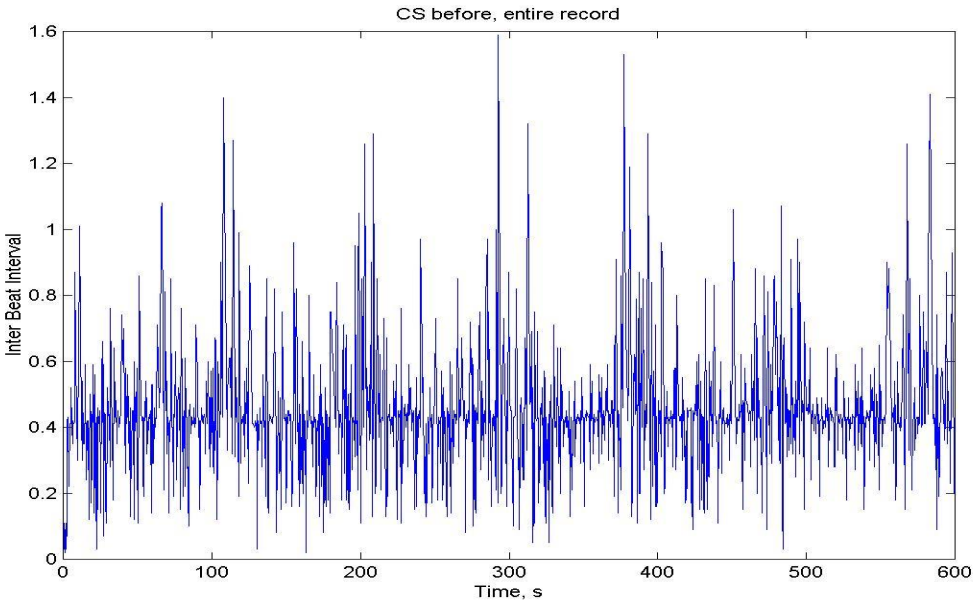
e. Spectral analysis (MESA) for heart rate (frequency) of these segments starting at the midpoint before melatonin injection



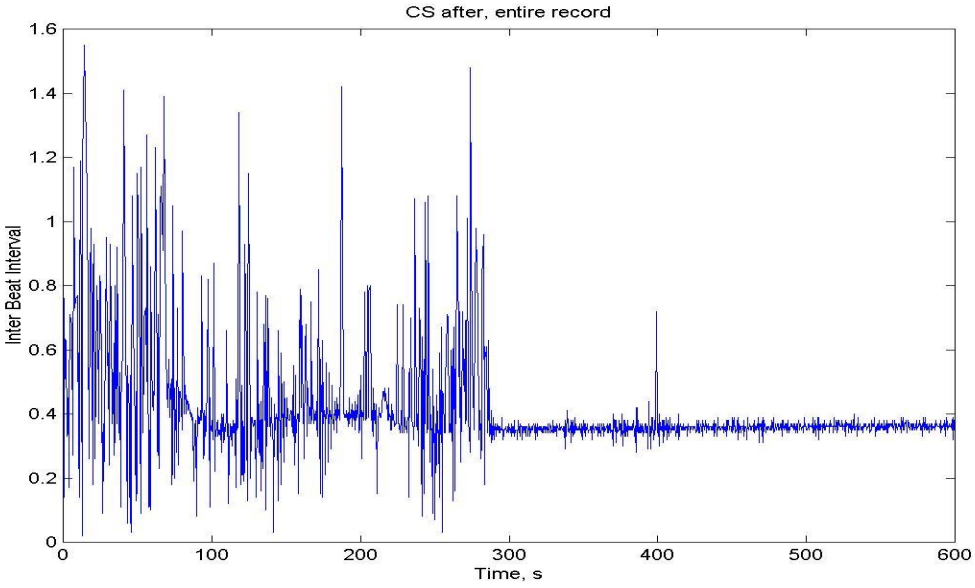
f. Spectral analysis (MESA) for heart rate (frequency) of these segments starting at the midpoint after melatonin injection



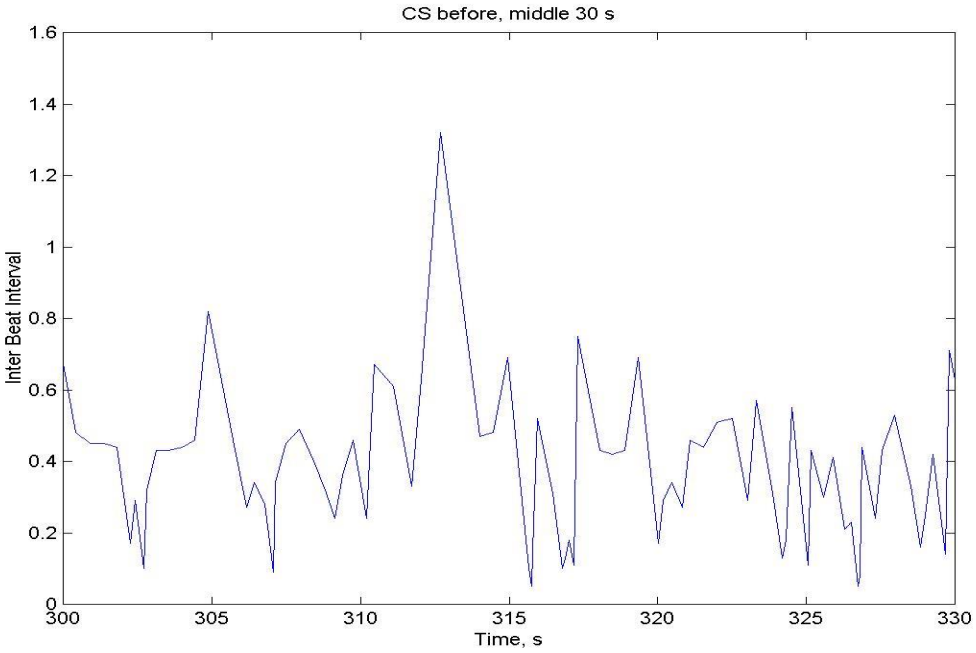
g. Interbeat Interval (IBI) analysis of entire 10 minute recording before melatonin injection



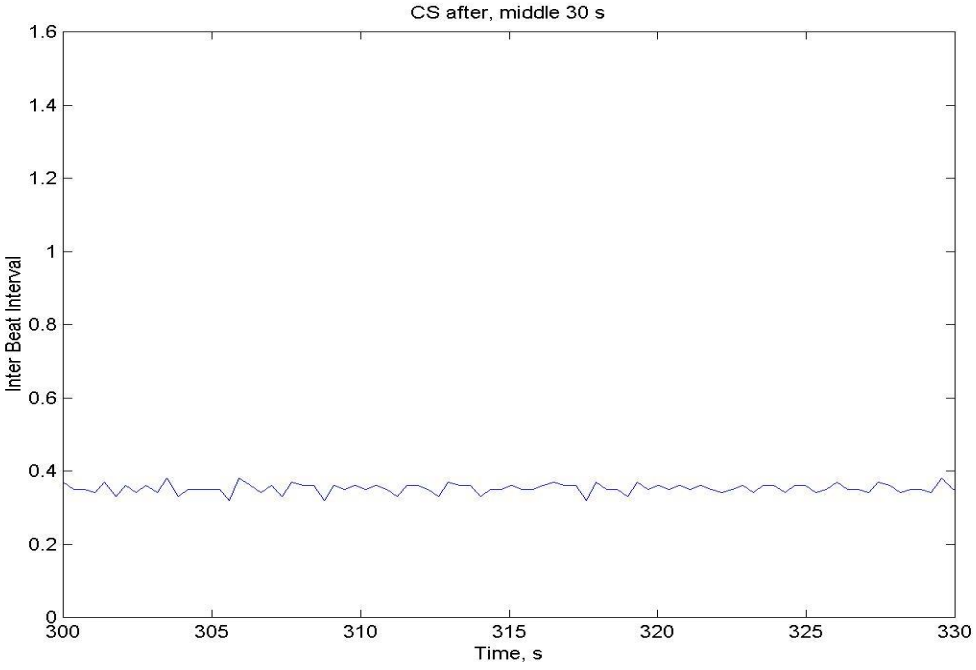
h. Interbeat Interval (IBI) analysis of entire 10 minute recording after melatonin injection



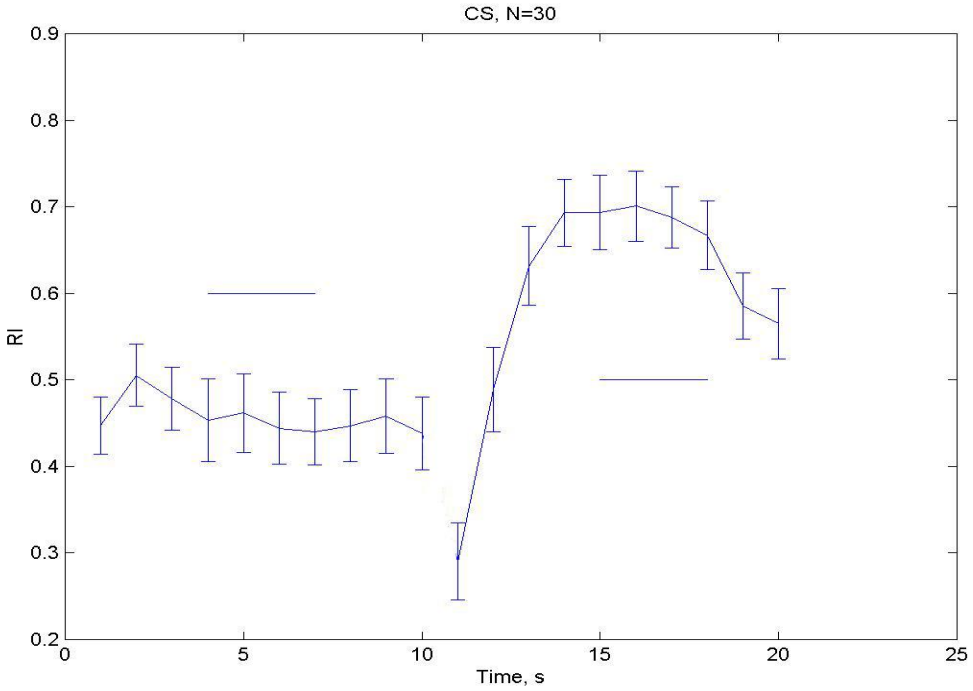
i. IBI analysis at higher magnification; 30-s segment starting at the midpoint before melatonin injection



j. IBI analysis at higher magnification; 30-s segment starting at the midpoint before melatonin injection



k. Shows the average (CS pupae, N=30) rhythmicity index (RI) for each minute; 10 minutes before injection and 10 minutes after injection



Dose response of injected melatonin in wild type and *slowpoke* pupae

Both wild type and *slowpoke* pupae were injected with 50, 100, 500, 1000 and 2500 µM concentrations of melatonin. Wild-type (Canton-S) pupae showed significant increase in heart rhythmicity with injections of 500, 1000 and 2500 µM melatonin with % increase in heart rhythmicity of 32 %, 47 % and 39% respectively ($p < 0.05$) as compared to rhythmicity before melatonin injection. Melatonin injections of 0 µM (control injections of Ca^{+2} free ringers and Ca^{+2} free ringers with 1% ethanol), 50 µM and 100 µM in wild type pupae showed no significant change in heart rhythmicity ($p > 0.05$) (Figure 1.2). *Slowpoke* (*slo*) pupae showed significant increase in heart rhythmicity with injections of 100, 500, 1000 and 2500 µM melatonin with % increase in heart rhythmicity of 19 %, 77%, 98% and 40% respectively ($p < 0.05$). Melatonin injections of 0 µM (control injections of Ca^{+2} free ringers and Ca^{+2} free ringers with 1% ethanol), and 50 µM *slo* pupae showed no significant change in heart rhythmicity ($p > 0.05$) (Figure 1.3). There was no significant change in heart rate in either wild type or *slo* pupae across all injected concentrations of melatonin ($p > 0.05$). The peak response for both wild type and *slo* pupae was with injection of 1000 µM melatonin. This was chosen as the standard dose used in all experiments.

Figure 1.2 Canton-S melatonin dose response curve

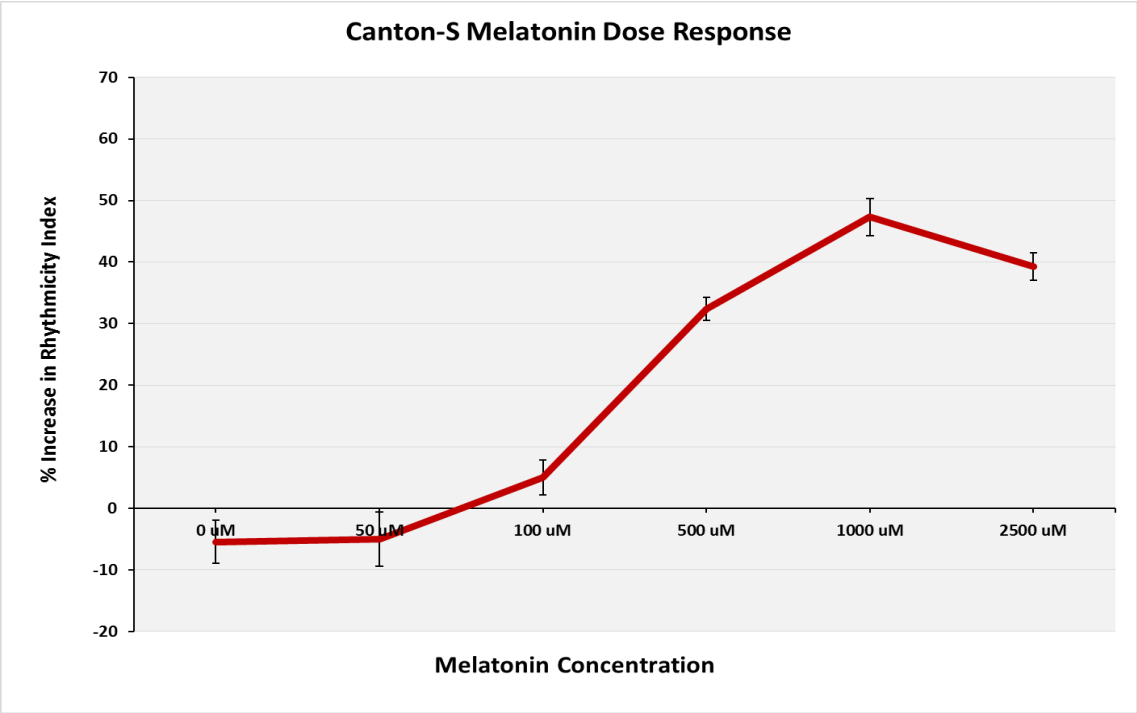
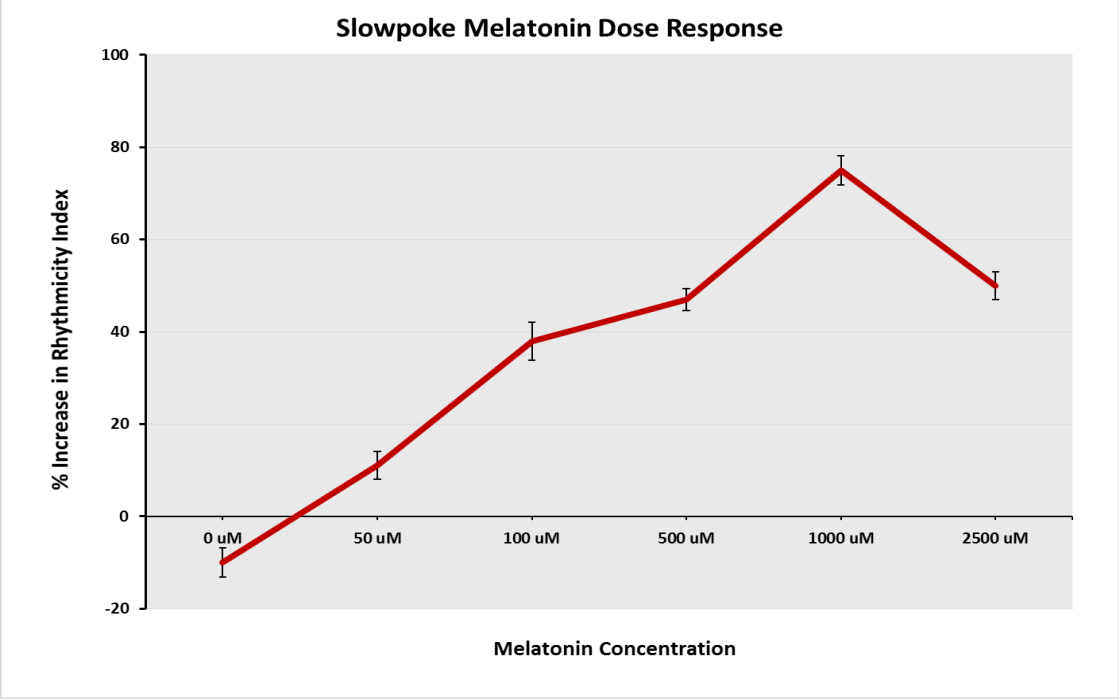


Figure 1.3 Slowpoke melatonin dose response curve



Effects of melatonin on heart rate and rhythmicity of *Drosophila melanogaster* wild- type and heart mutant pupae

Both wild type and heart mutant pupae injected with 1000 µM melatonin showed significant increase in heart rhythmicity ranging from 54 to 104% (Table 1.1 and Fig 1.4). Canton S, *eag*, *slo*, *nap*^{ts}, and *slo x df* [*slo*]^{d} pupae showed a significant *decrease* in heart rate ranging from 2 to 12 % ($p < .05$), but all other strains showed no significant change in heart rate (Table 1.2 and Fig. 1.5).

Control pupae were injected with *Drosophila* calcium free ringers solution or calcium free ringers solution with 1% ethanol. Controls showed no significant change in heart rhythmicity or in heart rate ($p > 0.05$).

Due to the initial period of time that it took for melatonin to exert its effects, minutes 4 through 7 were chosen as significant values for calculation of the means taken pre and post injection for both heart rate and rhythmicity. This protocol was used across all measurements except those involving a heat shock protocol (see fig. 1.1k).

Table 1.1 Effects of melatonin injection on heart rhythmicity in wild-type and heart mutants

Genotype	Meltonin Injected µmol-1[a]	Mean RI Pre[b] 25´C	Mean RI Post[c] 25´C	% Change	N	+/- SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	0.449	0.694	+54%	30	0.02/0.02	(1,29) 158.7	<2e-16 ***
dnc[1]	1000	0.404	0.702	+74%	10	0.04/0.04	(1,9) 86.4	2.00e-13 ***
eag	1000	0.287	0.584	+104%	5	0.04/0.04	(1,4) 120.7	1.16e-11 ***
nap[ts]	1000	0.406	0.746	+84%	30	0.02/0.01	(1,29) 338.6	< 2e-16 ***
slo	1000	0.357	0.681	+91%	42	0.02/0.01	(1,41) 61.4	9.29e-14 ***
slo x df [*slo*] [d]	1000	0.418	0.733	+76%	20	0.02/0.02	(1,19) 341.6	< 2e-16 ***
df [*slo*] *x slo*[e]	1000	0.379	0.711	+87%	11	0.02/0.02	(1,10) 292.1	<2e-16 ***
sloRNAi x GMH5[f]	1000	0.305	0.570	+87%	5	0.03/0.02	(1,4) 86.1	4.89e-10 ***

[a]Concentration of melatonin injected

[b]Mean Rhythmicity Index pre-injection minutes 4-7

[c]Mean Rhythmicity Index post-injection minutes 4-7

[d]*slo* virgin females crossed with *df* [*slo*] males

[e]*df* [*slo*] virgin females crossed with *slo* males

[f]*slo*RNAi virgin females crossed with *GMH5*-Gal4 males

*** $p < 0.001$ comparing before injection to after melatonin injection within each strain

Figure 1.4 Effects of 1000 µM melatonin injection on wild-type and heart mutants

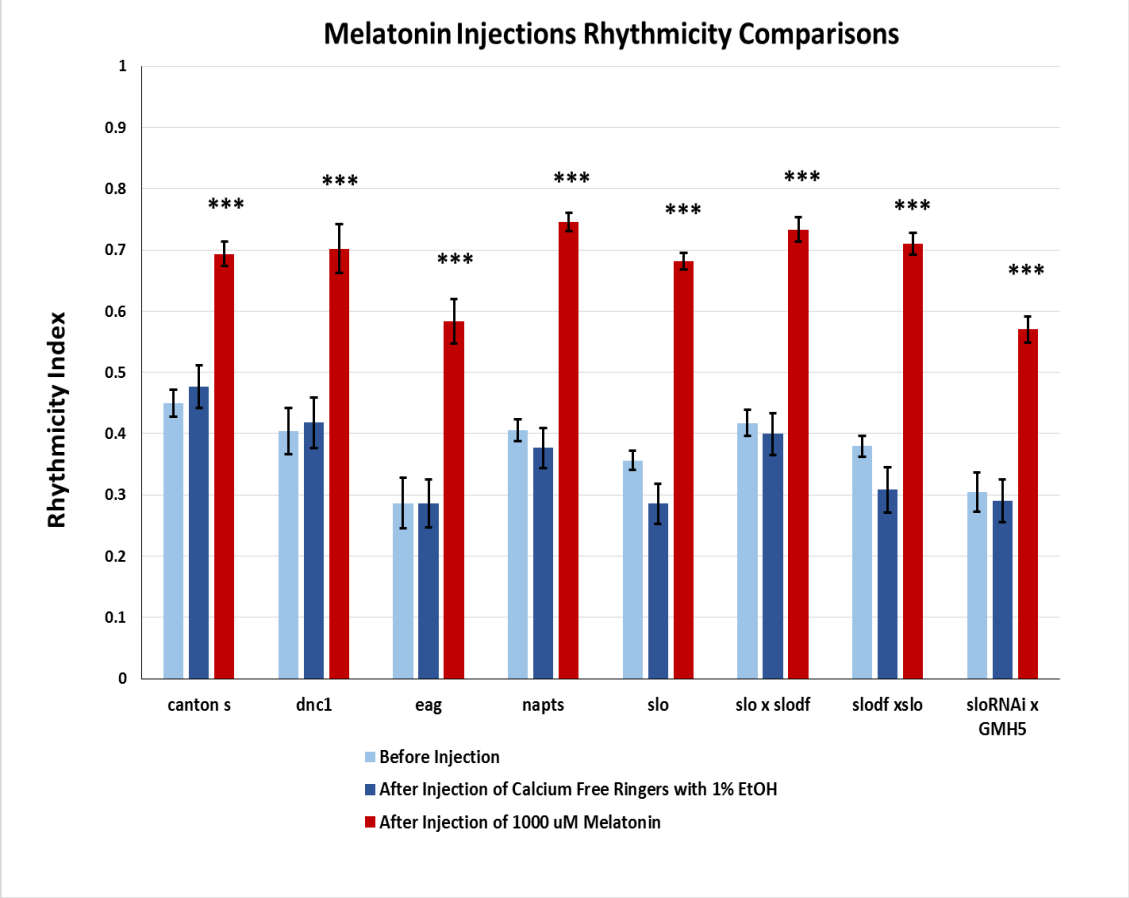


Table 1.2 Effects of melatonin injection on heart rate in wild-type and heart mutants

Genotype	Meltonin Injected µmol-1[a]	Mean Heart Rate Pre[b] 25´C	Mean Heart Rate Post[c] 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	2.28	2.21	- 3 %	30	0.02/0.03	(1,29) 6.1	0.0144 *
dnc^{1}	1000	2.04	1.98	- 3%	10	0.03/0.04	(1,9) 1.3	0.251
eag	1000	1.89	1.80	- 5%	5	0.03/0.04	(1,4) 5.4	0.027 *
nap^{ts}	1000	2.29	2.01	- 12%	30	0.05/0.05	(1,29) 19.1	4.69e-05 ***
slo	1000	2.37	2.28	- 4%	42	0.02/0.02	(1,41) 14.7	0.00015 ***
slo x df [*slo*][d]	1000	2.46	2.41	- 2%	20	0.04/0.04	(1,19) 5.7	0.0199 *
df [*slo*] *x slo*[e]	1000	2.16	2.12	- 2%	11	0.05/0.04	(1,10) 0.4	0.514
sloRNAi x GMH5[f]	1000	1.81	1.72	- 5%	5	0.05/0.04	(1,4) 2.3	0.140

[a]Concentration of melatonin injected

[b]Mean heart rate pre-injection minutes 4-7

[c]Mean heart rate post-injection minutes 4-7

[d]*slo* virgin females crossed with *df* [*slo*] males

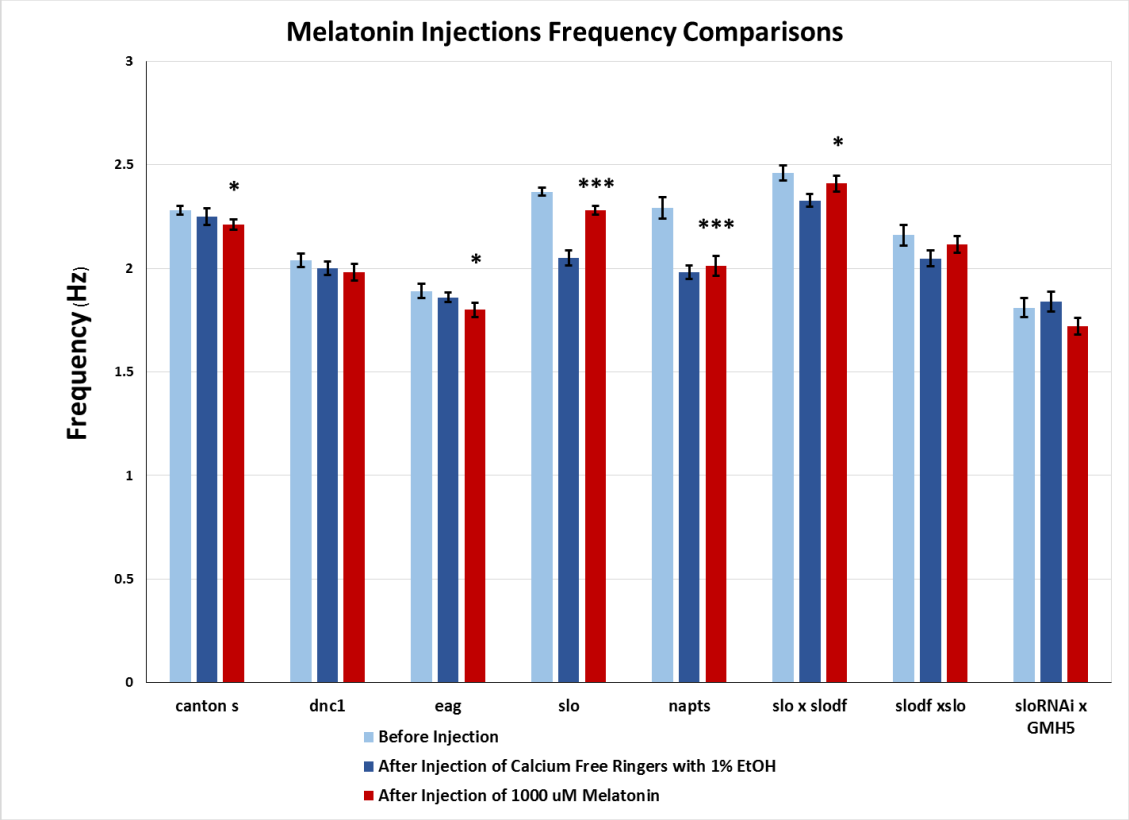
[e] *df* [*slo*] virgin females crossed with *slo* males

[f]*slo*RNAi virgin females crossed with *GMH5*-Gal4 males

* $p < 0.05$ comparing before injection to after melatonin injection within each strain

*** $p < 0.001$ comparing before injection to after melatonin injection within each strain

Figure 1.5 Effects of melatonin injection on heart rate in wild-type and heart mutants



Effects of melatonin injection on heart rhythmicity of *Ca-P60A^{Kum170}* (SERCA ^{170}) mutants before and after 2.5 minute heat shock at 41˚C

Both wild-type and SERCA^{170} pupae showed a significant decrease in heart rhythmicity with 2.5 minute 41˚C heat shock alone ($F(1,4) = 153.6$; $p < 0.001$ and $F(1,4) = 32.6$; $p < 0.001$). Injections of 1000 µM melatonin before and after heat shock showed significant increase in heart rhythmicity in both wild-type and SERCA^{170} strains (Melatonin injection before heat shock: $F(1,4) = 9.4$; $p < 0.05$ and $F(1,9) = 51.1$; $p < 0.001$; Melatonin injection after heat shock: $F(1,4) = 30.1$; $p < 0.001$ and $F(1,9) = 28.0$; $p < 0.001$). (Fig. 1.6 and 1.7).

For recording where melatonin was injection before the 2.5 minute heat shock, minutes 1-10 of pre and post injection were compared and statistically analyzed. For recordings where melatonin was injected after the 2.5 minute heat shock, minutes 7-10 of pre and post injection were compared and statistically analyzed.

Comparison of wild-type to SERCA ^{170} mutants showed a similar trend where significant increase in heart rhythmicity occurred across the entire 10 minute recording with melatonin injection before heat shock, while significant increase in heart rhythmicity didn't begin until minute 7 with melatonin injections after heat shock, and continued to increase from that point (Fig. 1.8 and 1.9).

Control pupae were injected with *Drosophila* calcium free ringers solution with 1% ethanol. Controls showed no significant change in heart rhythmicity with injection before or after 2.5 minute heat shock at 41˚C ($p > 0.05$).

Figure 1.6 Effects of 1000 µM melatonin injection on SERCA ^{170} pupae before and after 2.5 minute heat shock

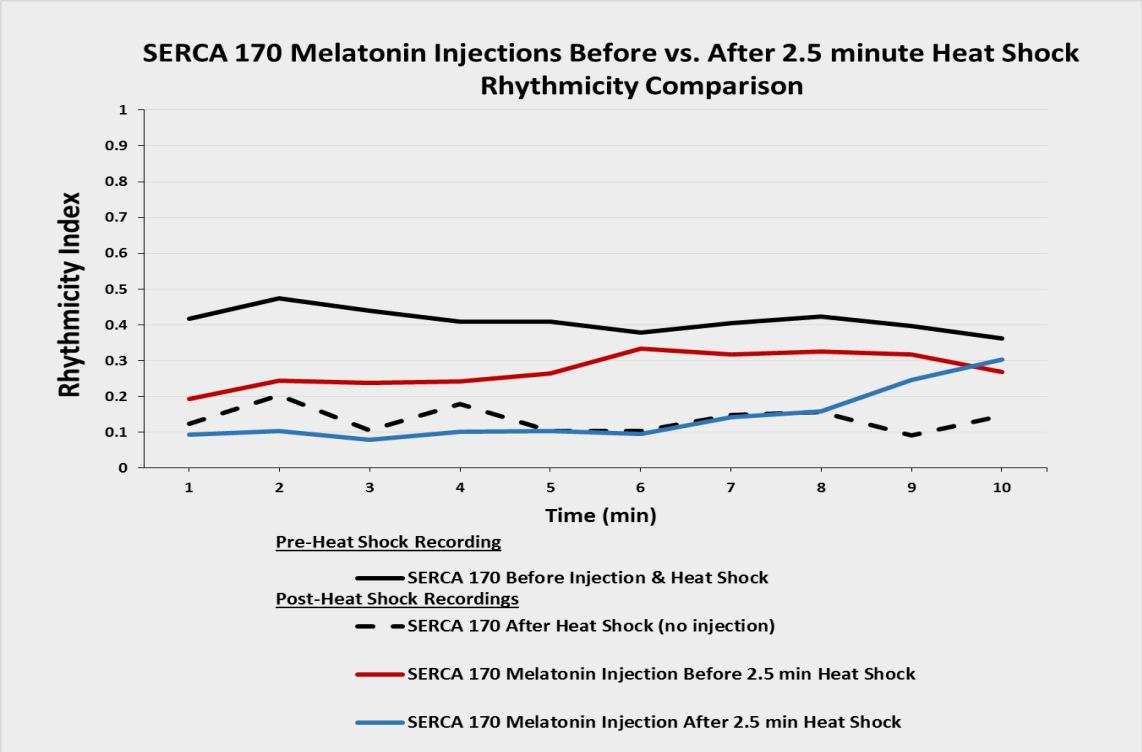


Figure 1.7 Effects of 1000 µM melatonin injection on Canton-S pupae before and after 2.5 minute heat shock

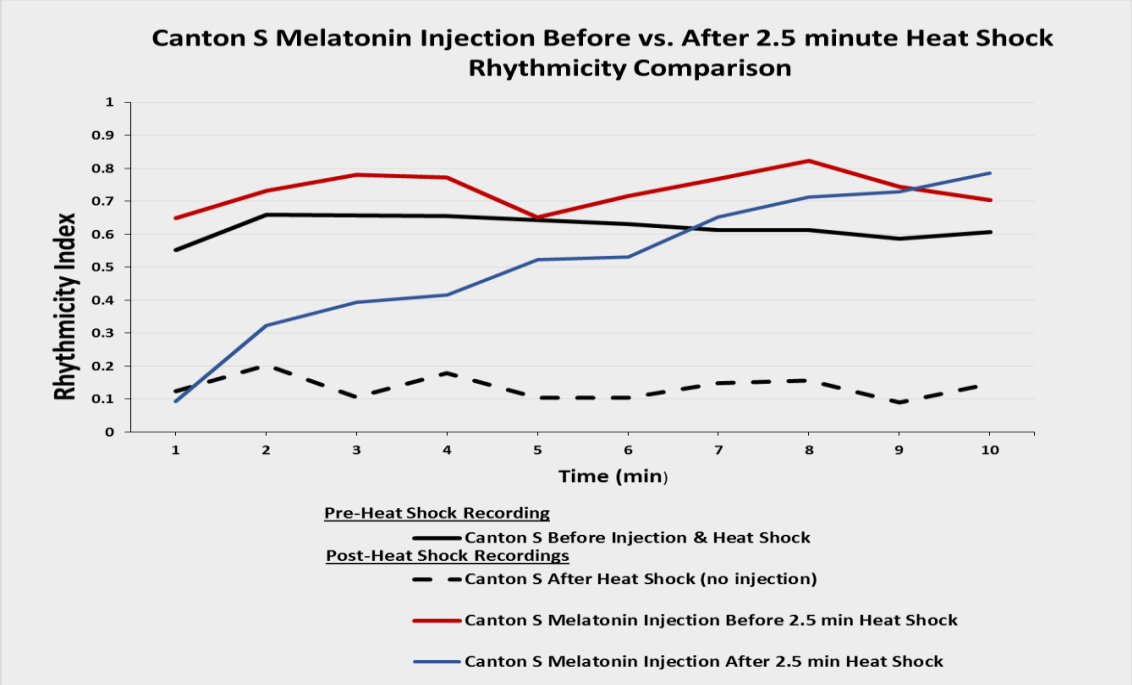


Figure 1.8 Canton-S vs. SERCA ^{170} 1000 µM melatonin injection after 2.5 minute heat shock

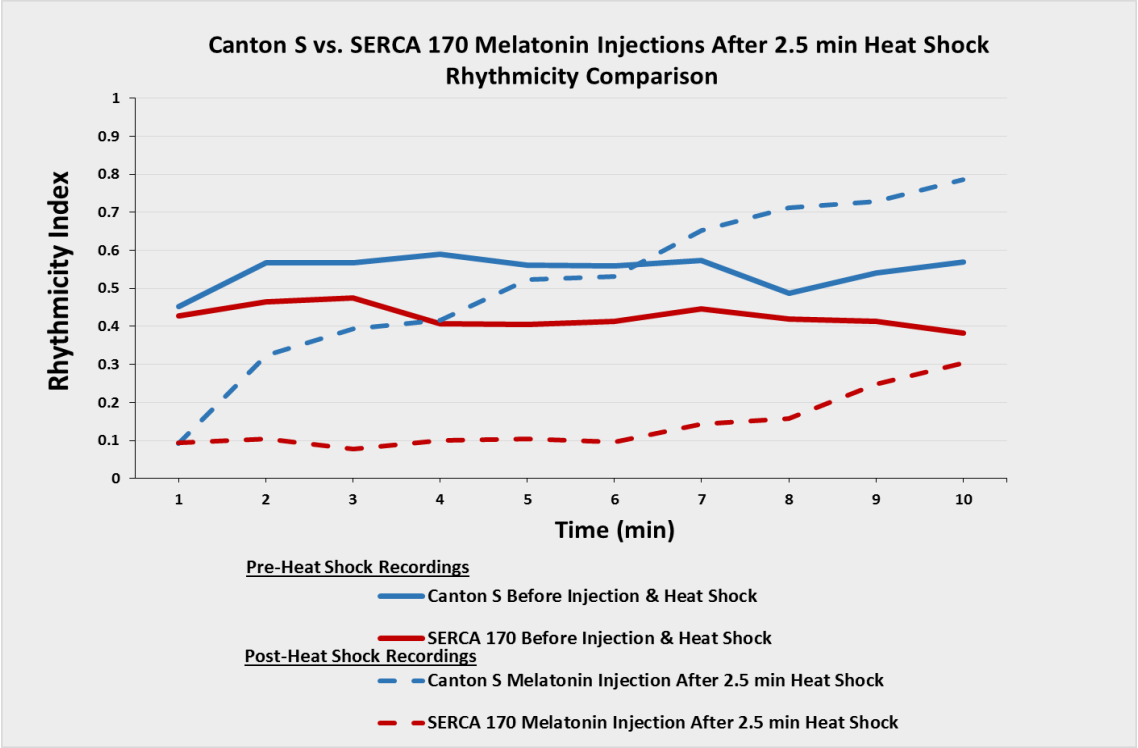
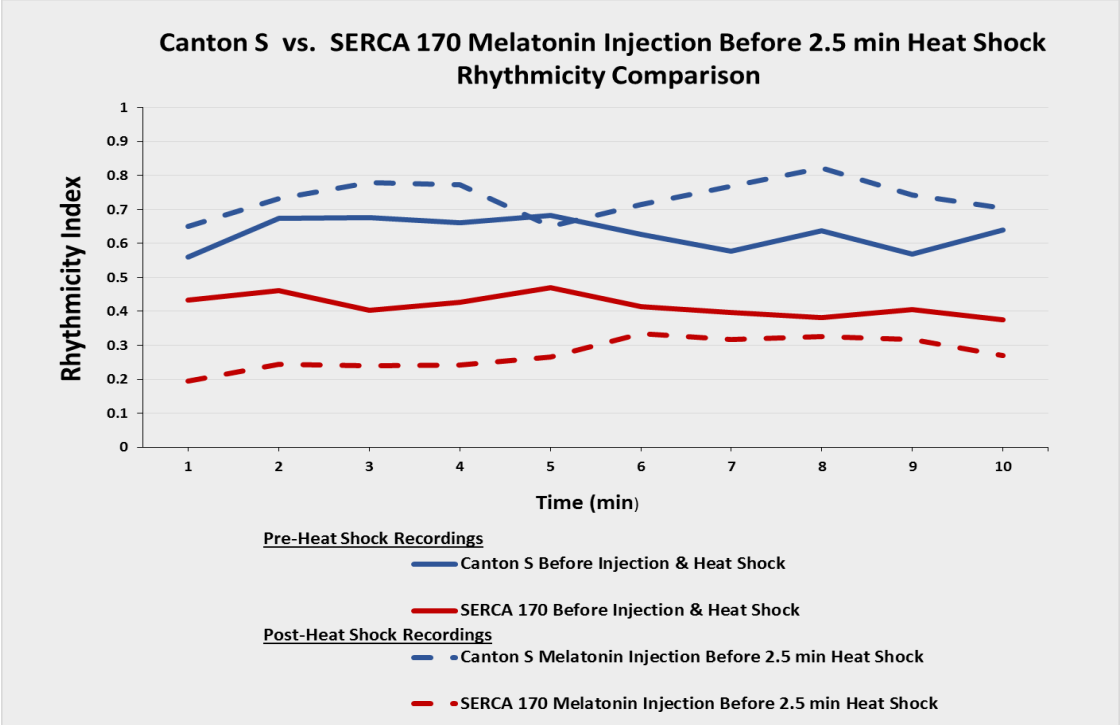


Figure 1.9 Canton-S vs. SERCA ^{170} 1000 µM melatonin injection before 2.5 minute heat shock



Effects of Serotonin on heart rate and rhythmicity of wild type and *slowpoke* mutants

Injection of 1000 µM serotonin in both wild-type and *slowpoke* pupae had no significant effect on heart rhythmicity ($p > 0.05$) (Table 1.9 and Fig 1.10).

Injections of 1000 µM serotonin did significantly increase heart rate in both wild-type and *slowpoke* pupae ranging from 28 to 31 % ($p < 0.001$) (Table 1.10 and Fig 1.11).

Results of serotonin injection are opposite of results of melatonin injection whereby melatonin increases heart rhythmicity without increasing heart rate, serotonin increases heart rate without increasing heart rhythmicity.

Table 1.3 Effects of 1000 µM serotonin injection on heart rhythmicity in wild-type and *slowpoke* pupae

Genotype	Serotonin Injected µmol-1[a]	Mean RI Pre 25´C	Mean RI Post 25´C	% Change	N	+/- SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	0.543	0.585	+7%	5	0.04/0.03	(1,4) 2.2	0.150
slowpoke	1000	0.190	0.211	+11%	5	0.03/0.03	(1,4) 0.2	0.671

[a]Concentration of serotonin injected

[b]Mean Rhythmicity Index pre-injection minutes 4-7

[c]Mean Rhythmicity Index post-injection minutes 4-7

Figure 1.10 Effects of 1000 µM serotonin injection on heart rhythmicity in wild-type and *slowpoke* pupae

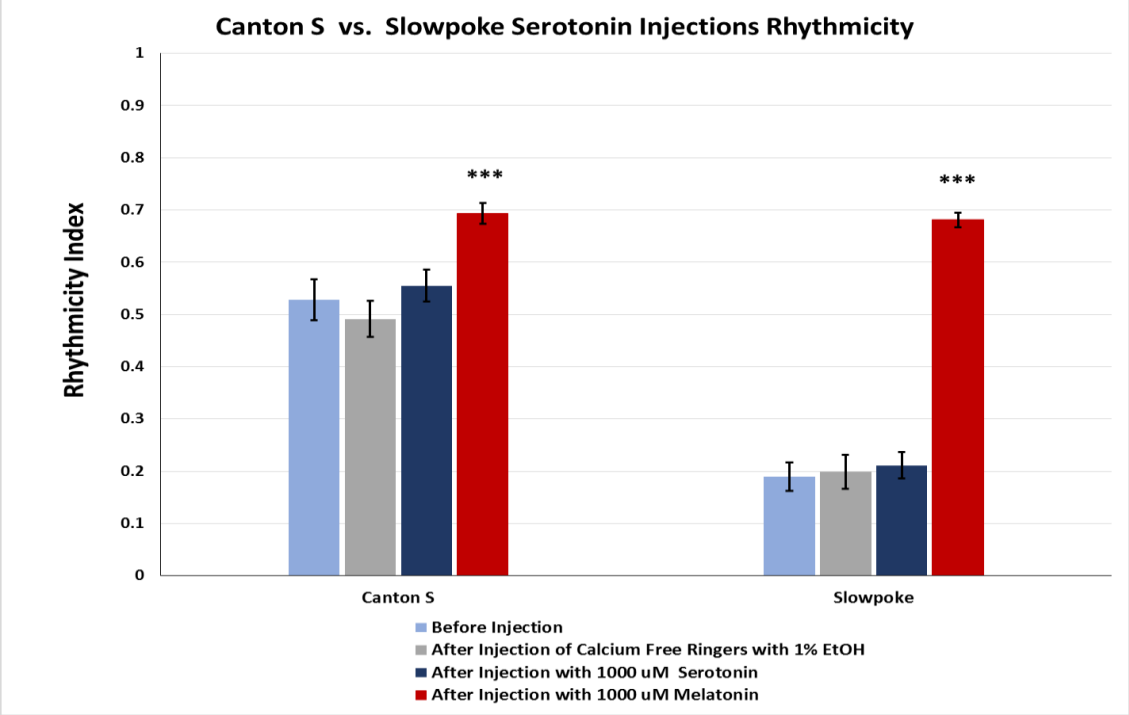


Table 1.4 Effects of 1000 µM serotonin injection on heart rate in wild-type and *slowpoke* pupae

Genotype	Serotonin Injected µmol-1[a]	Mean Heart Rate Pre 25´C	Mean Heart Rate Post 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	2.37	3.11	+ 31%	5	0.06/0.05	(1,4) 35.7	1.98e-06 ***
slowpoke	1000	2.27	2.91	+ 28%	5	0.04/0.04	(1,4) 473.1	< 2.2e-16 ***

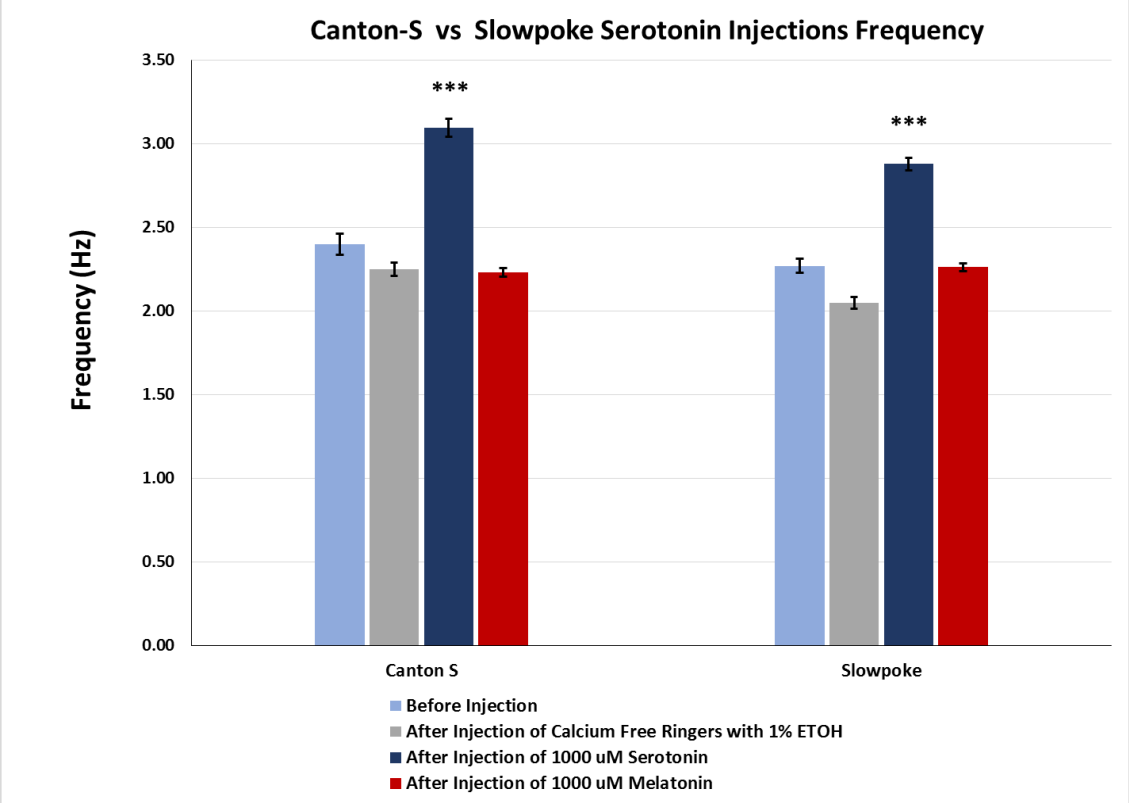
[a]Concentration of serotonin injected

[b]Mean heart rate pre-injection minutes 4-7

[c]Mean heart rate minutes 4-7

*** $p < 0.001$ comparing before injection to after serotonin injection within each strain

Figure 1.11 Effects of 1000 µM serotonin injection on heart rate in wild-type and *slowpoke* pupae



Discussion

Heart rhythmicity in *Drosophila melanogaster* is remarkably and consistently increased by injection of melatonin in wild type pupae (P1) and pupae exhibiting a variety of heart mutations. Percentage increase in heart rhythmicity ranged from 54% in wild-type to 104% in *eag* progeny. Curiously, melatonin significantly increased heart rate by 7% in *eag*, but did result in a significant decrease in heart rate in Canton S, *slo*, *eag*, nap^{ts}, and *slo x df* $[slo]^{d}$ pupae. Data from injections with serotonin, melatonin's precursor, also supported the previous findings of Johnson et al., 1997, that serotonin significantly increases heart rate in wild-type and *slo* pupae by 31% and 28% respectively without significantly affecting heart rhythmicity.

Both melatonin and serotonin exhibit powerful antioxidant properties owing to their ability to scavenge damaging free radicals produced by reactive oxygen species (ROSs) (Bonilla et al., 2006; Gülçin, 2008; Oxenkrug, 2005; Gitto et al., 2001; Head, 2005; Pieri et al., 1994). Serotonin was found to have higher DMPD (N, N–dimethyl-p-phenylendiamine) radical scavenging and cupric ions (Cu^{2+}) reducing activity than melatonin because of its phenolic group (Gülçin, 2008). While several studies in vertebrates have attributed melatonin's cardio protective properties to its antioxidant activity (Reviews: Reiter & Tan, 2003; Tengattini et al., 2007), serotonin has not been utilized in such experiments because, unlike melatonin, it also increases heart rate. It is also important to note that studies showing anti-arrhythmic effects of melatonin in

hamsters and rats were in conjunction with oxidative stress caused by ischemia-reperfusion damage. Melatonin was able to reduce oxidative stress caused by ischemia-reperfusion injury, and thereby reduce the incidence of post-ischemic-reperfusion induced ventricular tachycardia (VT) and ventricular fibrillation (VF) (Bertuglia & Reiter, 2007).

The experiments carried out in this study report on the immediate anti-arrhythmic effects of melatonin when injected directly over the dorsal vessel and pacemaker of *Drosophila* pupae with no oxidative stress incurred by the animal. Also, if melatonin's anti-arrhythmic effects are owing to its antioxidant properties, one would expect serotonin to have similar anti-arrhythmic effects, which is not the case.

In support of melatonin's anti-arrhythmic effects being receptor mediated and not antioxidant mediated, I designed experiments to inject concentrations of ascorbic acid (vitamin C) into *Drosophila* pupae. Results of ascorbic acid injections showed a 5% and 24% decrease in heart rhythmicity in wild type and *slo* pupae, respectively (See Chapter 2 Results).

Based on the above results and discussion and results of ascorbic acid experiments in chapter 2 of this manuscript, I believe that it is highly unlikely that melatonin's anti-arrhythmic effects in *Drosophila melanogaster* are antioxidant mediated. Instead, I hypothesize that melatonin produces these effects by binding to a melatonin receptor and initiating a signaling pathway of events that ultimately lead to increased heart rhythmicity.

While this hypothesis is well supported by the experiments performed in this study and experiments carried out in Chapter 2 of this manuscript, outside speculation regarding melatonin's ability to modulate heart rhythmicity through receptor binding is limited and somewhat ambiguous. Since heart rate and rhythmicity in vertebrates and *Drosophila* are both primarily regulated by pacemaker activity, it makes sense to turn our attention to possible direct effects of melatonin on pacemaking. The pacemaker in both organisms comprises an ensemble of ion channels. The underlying biophysics of the oscillatory behavior may vary in detail, but the overall similarities are striking and insect research has already carried over into understanding vertebrate mechanisms with implications for human health (Bodmer et al., 2005).

Pacemaking in these organisms is dependent on Ca^{+2} and K^{+} ion currents but not dependent on Na^{+} (Irisawa et al., 1993; McCann & Sanger, 1969; McCann, 1963, 1969; Miller, 1985). Many of the heart mutants that I chose to test in these experiments have altered heart rate and/or heart rhythms due to mutations in genes that encode both Ca^{+2} and K^{+} ion channels and calcium signaling pathways involved in pacemaker function of the heart. Human homologues of these genes have also been identified (see Introduction). As mentioned above, heart rhythmicity is increased with injection of melatonin in wild type and all heart mutants tested. There does appear to be a positive correlation between percentage increase in heart rhythmicity and the potassium channel mutations *ether-a-go-go* and *slowpoke* with heart rhythmicity increase ranging from 91-104%, which would indicate that melatonin may be directly affecting K^{+} ion channels in a way that mitigates the effect of the mutated channel.

I also tested a mutation that affects the level of sodium channel activity and, at high temperature, causes paralysis associated with a loss of action potentials. nap^{ts} is a gain-of-function allele of *maleless* (*mle)*, a gene required for X chromosome dosage compensation and male viability (Kernan et. al, 1991). nap^{ts} slows heartbeat and renders it less rhythmic and prevents the heart from responding to temperature with an altered rate (Dowse et al., 1995). It has also been shown that even at permissive temperature, nap^{ts} suppresses the effects of a variety of mutations (*Shaker, ether-a-go-go, Hyperkinetic*) that affect potassium channels and cause increased membrane excitability (Ganetzky & Wu, 1982). Melatonin did increase heart rhythmicity in nap^{ts} mutants by 84%. Even though pacemaking in both vertebrates and invertebrates has been shown to be independent of Na^+ ion currents, it is possible that this gain-of-function allele may be exerting its effects on the heart by interacting with potassium and/or calcium ion channels in the *Drosophila* pacemaker.

The other two mutations that I tested were not specific mutations of calcium channels, but rather were ones that affect calcium transport into the sarcoplasmic reticulum ($Ca\text{-}P60A^{Kum170}$) and the calcium-calmodulin signaling pathway via interactions with a cAMP-specific phosophodiesterase encoded by dnc^1.

$Ca\text{-}P60A^{Kum170}$ is an EMS-induced mutant that affects sarco-endoplasmic reticulum calcium ATPase (SERCA), a membrane protein that pumps free cytosolic calcium into intracellular stores. Abnormalities in the amount or structure of the SERCA protein have been linked to cardiac malfunction in mammals. One of the most consistently observed abnormalities in patients with ventricular arrhythmias is the

impaired ability to handle intracellular calcium due to changes in ryanodine receptor (RyR) and sarco-endoplasmic reticulum Ca^{2+} - ATPase (SERCA) (Wolk, 2000).

This *Drosophila* SERCA mutant shows a severe decrease in heart rhythmicity after being subjected to a 2.5 minute heat shock at 41˚C (Sanyal et al., 2006). When these pupae were injected with melatonin before heat shock, they showed an increase in heart rhythmicity of 137%. When these pupae were injected with melatonin after heat shock, they showed an increase in heart rhythmicity of 123%. I attribute the difference in the results between the two protocols to the fact that melatonin had a longer time to exert its effects when injected before heat shocking as wild type flies subjected to the same protocols showed a similar trend (170% increase before and 158% after). Based on these results and previous experiments indicating the importance of SERCA and RyR in regulating intracellular calcium levels of myocardial cells and maintenance of heart rhythmicity, melatonin may have an effect on intracellular calcium levels through another pathway or may be affecting ryanodine receptors indirectly. Experiments with *Drosophila* ryanodine receptor mutants were not performed in this study, but would be of interest in the investigation of melatonin's effects in future experiments.

Results in this study showed only a 74% increase in heart rhythmicity in dnc^1 mutants. The mutation *dunce* (dnc^1), encodes a cAMP-specific phosphodiesterase (PDE), which interacts with the calcium-calmodulin signaling pathway (Walter & Kiger, 1984). Turjanski et al., 2004, found that melatonin can modulate this signaling pathway by changing the concentration of intracellular calcium via activation of its G protein-coupled receptors. It is possible that melatonin is exerting its anti-arrhythmic effects through this signaling pathway, but no conclusion can be drawn from this result.

Recent research has reported that long term administration of melatonin to ventricular fibrillation (VF)-prone spontaneously hypertensive (SHR) and normotensive Wistar rats confers protection from lethal arrhythmia because of the increase in the VF threshold. The antiarrhythmic effect of melatonin was associated with the enhancement of myocardial Connexin 43 (Cx43) gene expression as well as the total levels of Cx43 protein and its functional phosphorylated forms in both SHR and Wistar rat hearts (Benova et al., 2013, 2015). In addition to these findings, Ai et al. 2000, found that Wnt-1 is a specific and potent inducer of Cx43 expression in cardiomyocytes and that this effect results in enhanced accumulation of Cx43 protein and formation of functional gap junction channels. Interestingly, the gene product of *wingless* (*wg*), the *Drosophila* Wnt homologue, has been shown to be crucial for normal heart development, where it appears to be absolutely required for specifying the heart progenitors (Park et al., 1996, 1998). It is also known that mononucleate cardiomyocytes that comprise the heart tube in invertebrates such as *Drosophila* are electrically connected by gap junctions formed by innexin (Connexin is the vertebrae homologue). Perhaps melatonin is having a similar effect on the innexins in the *Drosophila* heart.

Although it is highly likely that melatonin is producing its anti-arrhythmic effects by binding to a melatonin receptor, the possible mechanisms that affect rhythmicity are diverse and complex. Future research should first focus on positive identification and characterization of a melatonin receptor in *Drosophila* (See Chapter 2). Melatonin's anti-arrhythmic pathway would be open to further study were such a receptor to be identified and characterized.

CHAPTER 2

ANALYSIS OF MELATONIN ACTION AND POSSIBLE IDENTIFICATION OF A MELATONIN RECEPTOR IN *Drosophila melanogaster*

Abstract

Melatonin injection into P1 pupae of *Drosophila melanogaster* results in an acute and profoundly significant increase in heart rhythmicity ranging from 47-151% without altering heart rate of the organism. In the present study luzindole, a non-competitive melatonin antagonist; 2-iodomelatonin, a high affinity melatonin agonist; and ascorbic acid, a powerful antioxidant are injected into *Drosophila* wild-type and heart mutant pupae in order to investigate melatonin's anti-arrhythmic mechanisms. Animals injected with luzindole and melatonin showed no significant increase in heart rhythmicity in wild type or heart mutant pupae showing that luzindole was able to block melatonin's anti-arrhythmic effects. Animals injected with 2-iodomelatonin showed an 11 % and 15 % greater increase in heart rhythmicity than melatonin in wild type and *slowpoke (slo).* Wild type and *slo* pupae injected with ascorbic acid showed a significant *decrease* in heart rhythmicity. Results from all three injected substances provide strong support for the hypothesis that melatonin's anti-arrhythmic effects are receptor mediated as opposed to antioxidant mediated. On the basis of these findings, an attempt to identify a possible melatonin receptor in *Drosophila melanogaster* was made by using RNA interference (RNAi) and loss of function tests with four different *Drosophila* orphan G protein-coupled receptors (*CG4313, CG3171, CG13579, CG13575*). While *CG3171, CG13579,* and *CG13575* showed no loss of function (i.e. heart rhythmicity still increased with melatonin injection by 27 %, 26 % and 33 %

respectively), *CG4313* exhibited loss of function (no significant increase in heart rhythmicity with melatonin injection). This finding indicates that the *CG4313* orphan receptor may be a melatonin receptor in *Drosophila melanogaster.*

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), an indolamine which is biologically synthesized from the amino acid L-Tryptophan, is the methoxy derivative of serotonin. The discovered molecule was named melatonin because of its effects on melanin ('mela-') aggregation in the melanocytes of amphibians (Lerner et al., 1958) and its derivation from serotonin ('-tonin'). Melatonin is present in the earliest life forms and is found in all organisms including bacteria, algae, fungi, plants, insects, and vertebrates including humans (Tan et al., 2003). Although melatonin is typically referred to as a hormone, the diversity of actions that it effects do not always comply with the conventional definition of a hormone. A hormone is defined as a molecule that is synthesized in an organ, released into a bodily fluid (usually the bloodstream) from where it travels to another cell or group of cells were it acts via specific receptors to mediate its effects. Many of melatonin's affects are not receptor mediated. It is well known that melatonin is a potent antioxidant, even more powerful than vitamin E (Pieri et al., 1994). The ability of melatonin and its metabolites to expunge free radicals and related reactants possibly involves all of the following actions: a) direct detoxification of radicals and radical products, b) stimulation of the activities of several antioxidative enzymes, c) inhibition of the activities of pro-oxidative enzymes, d) promotion of the synthesis of glutathione, another essential antioxidant, e) synergistic actions with other antioxidants, and f) mitochondrial actions of melatonin that reduce free radical

generation (Reiter et al., 2007). Melatonin also acts as an autocoid when it is released by a cell and acts on another cell in the immediate vicinity (Tan et al., 2003). In mammals, melatonin can also signal intracellular processes via activation of two high-affinity G-protein-coupled receptors designated MT1 and MT2 (Dubocovich et al., 1983; Dubocovich & Markowska, 2005; Reppert et al., 1996). See Introduction for more detail on melatonin receptors.

Often in preliminary experiments with melatonin, it is unknown whether its observed effects are antioxidant or receptor mediated. This is especially true if melatonin receptors have not yet been identified and characterized in the organism that is being tested, as is the case with *Drosophila melanogaster*. Serotonin (5-hydroxytryptamine; 5-HT) is a precursor to melatonin. Four receptors for 5-HT have been described in *Drosophila*: one shares sequence homology to the vertebrate 5HT receptor type 2 ($5HT_2R$; CG1056), one is similar to the vertebrate serotonin receptor type 7 ($5HT_7R$; CG12073) and two show high homology to vertebrate $5HT_1$ type receptor ($5HT_{1A}R$ and $5HT_{1B}R$; CG16720 and CG15113, respectively) (Silva et al., 2014). *Drosophila melanogaster* synthesizes its own serotonin (Livingstone, 1981) and also has several clusters of serotonergic neurons located in its central nervous system (Valles, 1988).

Conversion of serotonin to N-acetylserotonin, the direct precursor of the melatonin, is catalyzed by serotonin N-acetyltransferase (AANAT) in a reaction requiring acetyl coenzyme A (AcCoA) (Hickman et al., 1999). Two different AANATs (AANAT1 and AANAT2) have been identified and characterized in *Drosophila melanogaster* (Hintermann & Meyer, 1995; Brodbeck et al., 1998; Amherd et al., 2000),

but it is unknown whether *Drosophila* makes endogenous melatonin. Because *Drosophila* and vertebrate serotonin receptors and vertebrate melatonin receptors all belong to the G protein-coupled 7-transmembrane family, it is likely that if they exist, melatonin receptors in *Drosophila* would also be of this type. Analysis of the completed Drosophila genomic sequences (Brody & Cravchik, 2000; Adams et al., 2000) identified >100 genes encoding G protein-coupled receptors (GPCRs). Further analysis indicated that 44 such receptors are likely to have peptide ligands and that the majority of these are derived from ancestors for mammalian peptide GPCR genes (Hewes & Taghert, 2001). At present, 35 peptide amine GPCRs are functionally characterized in *Drosophila.* Another 14 GPCRs are predicted to be involved in neuropeptide signaling pathways, but their ligands are still unknown and therefore they are classified as "orphan" amine receptors (Caers et al., 2012).

The use of melatonin agonists and antagonists is a good first approach to elucidating melatonin's effects. Luzindole is a melatonin receptor antagonist for the human MT_1 and MT_2 receptors (pK_i 6.2-6.8 and pK_i 7.6-8.1 respectively) (Dubocovich, 1988). Luzindole has been used in a number of experiments and acts by blocking melatonin's effects, which supports melatonin's receptor mediated actions (Blask et al., 2002; Dubocovich et al., 1998; Leja-Szpak et al., 2010; Rezzani et al., 2006; Roth et al., 1999; Yu et al., 2014). 2-[^{125}I]iodomelatonin is a selective, high-affinity ligand for the identification and characterization of melatonin receptor sites. The binding affinity of 2-[^{125}I]iodomelatonin to human MT_1 and MT_2 receptors displays values of pK_i 10.2-11.0 and pK_i 9.7-10.3 respectively (Audinot et al., 2003). The specific binding of 2-[^{125}1]iodomelatonin fulfills all the criteria for binding to a receptor site, being stable,

reversible, saturable, and of high affinity (Dubocovich & Takahashi, 1987). 2-[^{125}I]iodomelatonin binding acts by mimicking the effects of melatonin and has been used to identify melatonin receptors in a variety of peripheral tissues in vertebrates (Pang et al., 1993).

Another deorphanization tool is a loss-of-function approach called RNA interference (RNAi). This allows us to eliminate targeted gene sequences in an attempt to understand their function (Bellés, 2010). (See Section I Introduction for more details on RNA interference.) *Drosophila* RNAi is cell-autonomous, and because of this, targeted expression of RNAi constructs using the Gal4/UAS system (Brand & Perrimon, 1993) can be used for cell- or tissue-specific interrogation of gene function. Indeed, this approach has been used extensively.

Typically, different Gal4-driver lines are crossed with UAS-target gene lines, and the effects of tissue-specific gene expression are examined in the progeny. The Gal4-UAS system provides a number of distinct advantages including promoter expression patterns when using UAS-β-galactosidase (UAS-lacZ) or UAS-green fluorescent protein (GFP) as a tissue marker; ectopic expression in a variety of tissues, and the tissue-specific effects of specific gene knockdown using UAS-RNAi lines (Dietzl et al., 2007; Haley et al., 2009). For example, tinC-GAL4 or GMH5-Gal4 can be used to drive cardiac-specific transgenes to express recombinant cDNA to examine ectopic protein expression or RNAi to examine specific gene knockdown in the heart (Neely et al., 2010; Taghli-Lamallem et al., 2008). Although these drivers do capture the heart it is important to note that they may also include other sites of expression in the animal.

The goals of the experiments carried out in this study are twofold: 1. To clarify whether melatonin's anti-arrhythmic effect (shown in Chapter 1) are antioxidant or receptor mediated 2. To identify a specific melatonin receptor in *Drosophila melanogaster.*

The first group of experiments examining melatonin's antioxidant vs. receptor mediated effects involved a series of co-injections of luzindole antagonist and melatonin into *Drosophila* early pupae (P1), and a second series of injections of high affinity agonist 2-[^{125}I]iodomelatonin or ascorbic acid (vitamin C). The second group of experiments attempted to implicate a *Drosophila* orphan receptor using paired specific receptor RNAi strains crossed with the GMH5-Gal4 heart specific driver and then injecting melatonin. A knock-down of the orphan receptor combined with loss-of-function with melatonin injection (no increased heart rhythmicity) would implicate a melatonin receptor

Materials and Methods

Fly Cultures and Strains

All mutant and control strains were maintained in 250ml bottles in a 12:12 light: dark (L:D) cycle under uncrowded conditions in a humidified, temperature-controlled incubator at 25º Con malt-molasses-cornmeal-agar medium. No population bottlenecks occurred. Propionic acid was added to the medium to control mold growth (Jennings et al., 2009).

Genetics

Homozygous individuals of the following *Drosophila melanogaster* strains were tested:

Canton-S (CS) wild-type

no action potential $^{temperature\ sensitive}$ (*nap*^{ts}) (Wu et al., 1978)

slowpoke (*slo*) (Elkins et al., 1986)

RNAi strains and crosses

The heart-specific driver employed here is GMH5–Gal4, a 900-bp heart enhancer fragment 73 from the *tinman* gene (Bodmer 1993; Venkatesh et al., 2000) that was cloned into the P{GaWB} vector upstream of the Gal4 sequences. This driver was enhanced with multiple copies of a UAS–Gal4 element allowing stronger myocardial expression when activated in late embryonic or during adult stages (Wessells & Bodmer, 2004).

RNAi strains of *Drosophila melanogaster* of G-protein coupled orphan receptors tested:

CG 4313 RNAi (FlyBase ID: FBgn0025632)

CG 3171 RNAi (FlyBase ID: FBgn0046687)

CG 13579 RNAi (FlyBase ID: FBgn0014445)

CG 13575 RNAi (FlyBase ID: FBgn0034996)

Ten virgin females of each RNAi strain were collected and crossed with ten GMH5 males. In addition, a reciprocal cross of ten GMH5 virgin females and ten *CG4313* RNAi males was carried out based on the results of possible melatonin receptor identification from the original *CG4313* RNAi cross. Crosses were maintained in 250ml bottles under the same environmental conditions described above.

First generation (F1) pupae from these crosses were injected with 1000 µM melatonin and heart rhythmicity and frequency were recorded. If melatonin injection still caused an increase in rhythmicity, the silenced orphan receptor was eliminated. If melatonin injection did not cause an increase in rhythmicity, the orphan receptor was considered a candidate melatonin receptor.

Measurement of Heartbeat

See Materials & Methods, Chapter 1

Pharmacology of melatonin, luzindole and 2-[^{125}I]iodomelatonin and L-ascorbic acid

Melatonin is insoluble in water and must initially be dissolved in ethyl alcohol. For consistency, melatonin, luzindole, 2-[^{125}I]iodomelatonin and L-ascorbic acid were first dissolved in 1 % ethyl alcohol and then added to Calcium-free *Drosophila* Ringer's solution (Ashburner, 1989). Concentrations of 2500 µM, 1000 µM, 500 µM, 100 µM, and 50 µM melatonin were prepared to assay dose response.

Luzindole is a melatonin receptor antagonist for the human MT_1 and MT_2 receptors (pK_i 6.2-6.8 and pK_i 7.6-8.1 respectively) (Dubocovich, 1988).

2-[^{125}I]iodomelatonin is a selective, high-affinity ligand for the identification and characterization of melatonin receptor sites. The specific binding of 2-[^{125}I]iodomelatonin fulfills all the criteria for binding to a receptor site, being stable, reversible, saturable, and of high affinity (Dubocovich & Takahashi, 1987). The binding affinity of 2-[^{125}I]iodomelatonin to human MT_1 and MT_2 receptors displays values of pK_i 10.2-11.0 and pK_i 9.7-10.3 respectively (Audinot et al., 2003). Single

concentrations of 2500 µM luzindole, and 1000 µM 2-[^{125}I]iodomelatonin were prepared. Based on the melatonin dose assay results, the standard concentration used for ensuing experiments of melatonin and 2-iodomelatonin injections was 1000 µM. A higher dose of (2500 µM) was used for luzindole to ensure that any melatonin receptors present would be completely blocked by the antagonist.

Melatonin exhibits both receptor mediated and antioxidant mediated effects. As a powerful antioxidant, melatonin has been shown to be more effective than vitamin C (ascorbic acid) and vitamin E in combating paraquat induced oxidative stress in *Drosophila melanogaster* (Bonilla et al., 2006). Injections with ascorbic acid in *Drosophila* wild-type and *slowpoke* pupae will help to ascertain whether melatonin's effects on heart rhythmicity are receptor or antioxidant mediated.

Solutions were perfused directly over the pacemaker and caudal region of the dorsal vessel by injection into the caudal end of the animal (Johnson et al., 1997). Glass electrodes were used for injection and were pulled on a World Precision Instrument PUL-100 micropipette puller. Tips were gently broken to assure sharpness.

Following a 10 minute pre-injection recording, the pupa was placed under an Olympus dissecting scope for injection. A World Precision Instrument Nanoliter Injector (model # A203XVY) was calibrated to administer 46 nl volume of liquid. A micromanipulator was used to position the glass electrode in the dorsal posterior region near the caudal region of the heart. Heartbeat was recorded for 10 minutes before injection and for 10 minutes immediately (within 1 minute) after injection of a substance as delineated above.

Specifics of tests with luzindole

In addition to the protocol for melatonin and 2-[^{125}I]iodomelatonin, CS, *slo* and *nap^{ts}* were subjected to the following protocol. After pre injection recording of heartbeat, P1 pupae were injected with 46 nL of 2500 µM luzindole, then allowed 1.5 minutes of rest, and then injected with 46 nL of 1000 µM melatonin before post injection heartbeat recording.

Owing to its clear, well-known and immediate effects of increased heart rate, I used epinephrine and a beta-blocker (metoprolol) to establish the double injection protocol. An initial 10 minute pre-injection recording of heart rate and rhythmicity was performed.

Analysis of heartbeat data

See Materials & Methods, Chapter 1

Results

Effects of injections of melatonin alone and double injections with luzindole and melatonin in wild type, *slowpoke* and *nap^{ts}* pupae

Both wild-type and pupae bearing heart mutations injected with 1000 µM melatonin showed significant increase in heart rhythmicity ranging from 54 to 91% (Table 2.1 and Fig. 2.1)

When the same strains were injected first with 2500 µM luzindole, allowed to rest for 1.5 minutes, and then injected with 1000 µM melatonin, a significant *decrease* in heart rhythmicity was observed in wild-type pupae ($F(1,14) = 18.6$; $p < 0.001$), but no significant increase in heart rhythmicity (Table 2.2 and Fig. 2.1).

Table 2.1 Effects of melatonin injection on heart rhythmicity in wild-type and heart mutants

Genotype	Meltonin Injected µmol-1[a]	Mean RI Pre 25´C	Mean RI Post 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	0.449	0.694	+ 54%	30	0.02/0.02	(1,29) 158.7	<2.2e-16 ***
nap^{ts}	1000	0.406	0.746	+ 84%	30	0.02/0.01	(1,29) 338.6	< 2.2e-16 ***
slo	1000	0.357	0.681	+ 91%	42	0.02/0.01	(1,41) 61.4	9.29e-14 ***

[a]Concentration of melatonin injected

[b]Mean Rhythmicity Index pre-injection minutes 4-7

[c]Mean Rhythmicity Index post-injection minutes 4-7

*** $p < 0.001$ comparing before injection to after melatonin injection within each strain

Table 2.2 Effects of double injection of luzindole and melatonin in heart rhythmicity in wild-type and heart mutants

Genotype	Luzindole/ Meltonin Injected µmol-1	Mean RI Pre 25´C	Mean RI Post 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	2500/1000	0.583	0.480	- 18 %	15	0.03/0.03	(1,14) 18.6	3.78e-05 ***
nap^{ts}	2500/1000	0.338	0.288	- 8 %	5	0.03/0.05	(1,40 .001	0.970
slo	2500/1000	0.378	0.346	-15%	10	0.03/0.03	(1,9) 3.7	0.0583

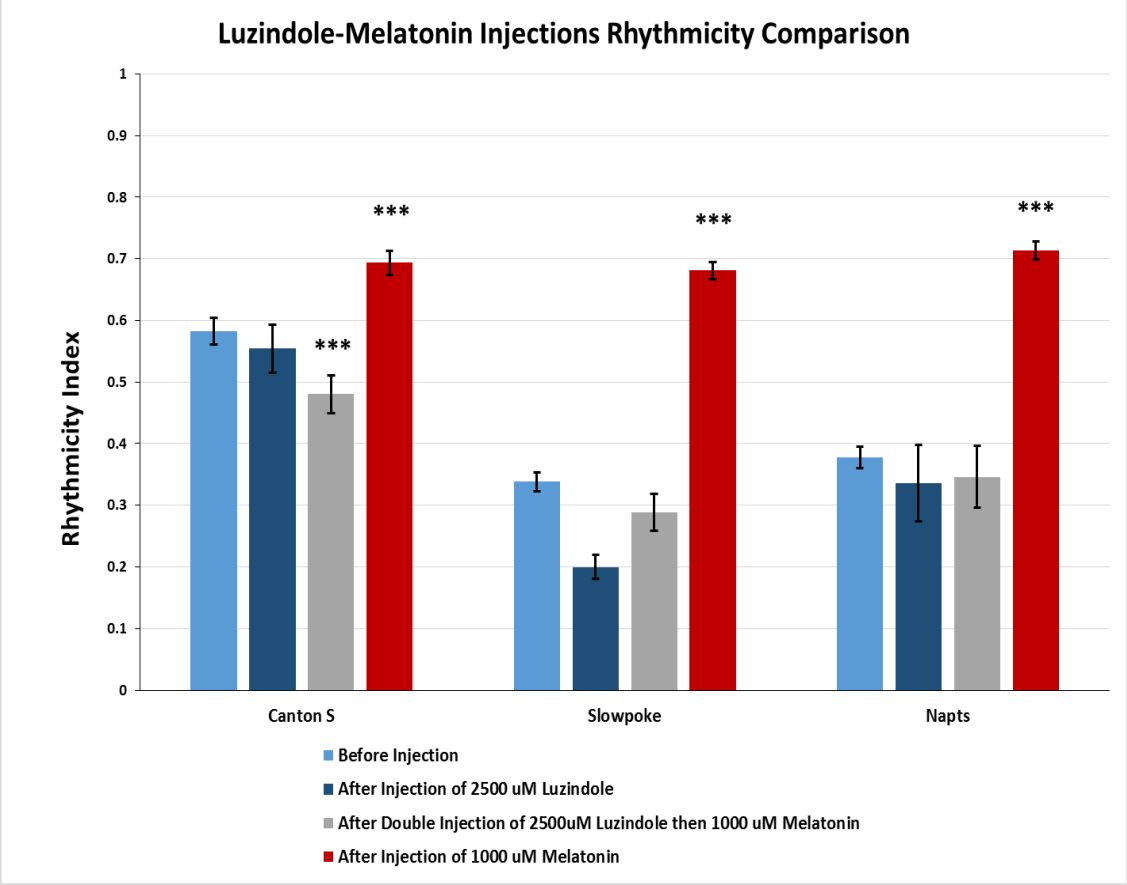
[a]Concentrations of luzindole/melatonin injected

[b]Mean Rhythmicity Index pre-injection minutes 4-7

[c]Mean Rhythmicity Index post-injection minutes 4-7

*** $p < 0.001$ comparing before injection to after luzindole/melatonin injection within each strain

Figure 2.1 Effects of melatonin alone and luzindole/melatonin double injections on heart rhythmicity in wild-type and heart mutants



Effects of ascorbic acid, 2-iodmelatonin and melatonin on heart rate and rhythmicity in wild type and *slowpoke* pupae

Injection with 1000 µM 2-iodomelatonin, a high affinity melatonin agonist, showed significant increase in heart rhythmicity between 53 % and 141 % in wild type and *slowpoke* pupae respectively ($p < 0.001$) (Table 2.3 and Fig. 2.2, 2.3).

Ascorbic acid (vitamin C) is a powerful antioxidant. When 1000 µM concentrations were injected into wild type and *slowpoke* pupae, no significant change in heart rhythmicity was observed (Table 2.4, Fig 2.2, 2.3).

Injections of 2-iodomelatonin had no significant effect on heart rate in either wild type or *slowpoke* pupae (Table 2.5, Figs. 2.4, 2.5).

Injection of ascorbic acid showed significant *decrease* in heart rate in both wild type and *slowpoke* pupae ($p < 0.001$) (Table 2.6, Figs. 2.4, 2.5).

Table 2.3 Effects of 2-Iodomelatonin injection on heart rhythmicity in wild-type and *slowpoke* pupae

Genotype	2-Iodomelatonin Injected µmol-1[a]	Mean RI Pre 25´C	Mean RI Post 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	0.519	0.796	53%	5	0.03/0.01	(1,4) 30.3	6.92e-06 ***
slowpoke	1000	0.256	0.618	141%	5	0.03/0.01	(1,4) 139.9	2.08e-12 ***

[a]Concentration of 2-Iodomelatonin injected

[b]Mean Rhythmicity Index pre-injection minutes 4-7

[c]Mean Rhythmicity Index post-injection minutes 4-7

*** $p < 0.001$ comparing before injection to after 2-iodomelatonin injection within each strain

Table 2.4 Effects of ascorbic acid injection on heart rhythmicity in wild-type and *slowpoke* pupae

Genotype	Ascorbic Acid Injected µmol-1[a]	Mean RI Pre 25´C	Mean RI Post 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	0.551	0.513	-7 %	10	0.03/0.03	(1,9) 0.13	0.718
slowpoke	1000	0.244	0.198	-19 %	10	0.03/0.02	(1,9) 1.6	0.217

[a]Concentration of ascorbic acid injected

[b]Mean Rhythmicity Index pre-injection minutes 4-7

[c]Mean Rhythmicity Index post-injection minutes 4-7

Figure 2.2 Effects of ascorbic acid, 2-iodomelatonin and melatonin on heart rhythmicity in wild-type pupae

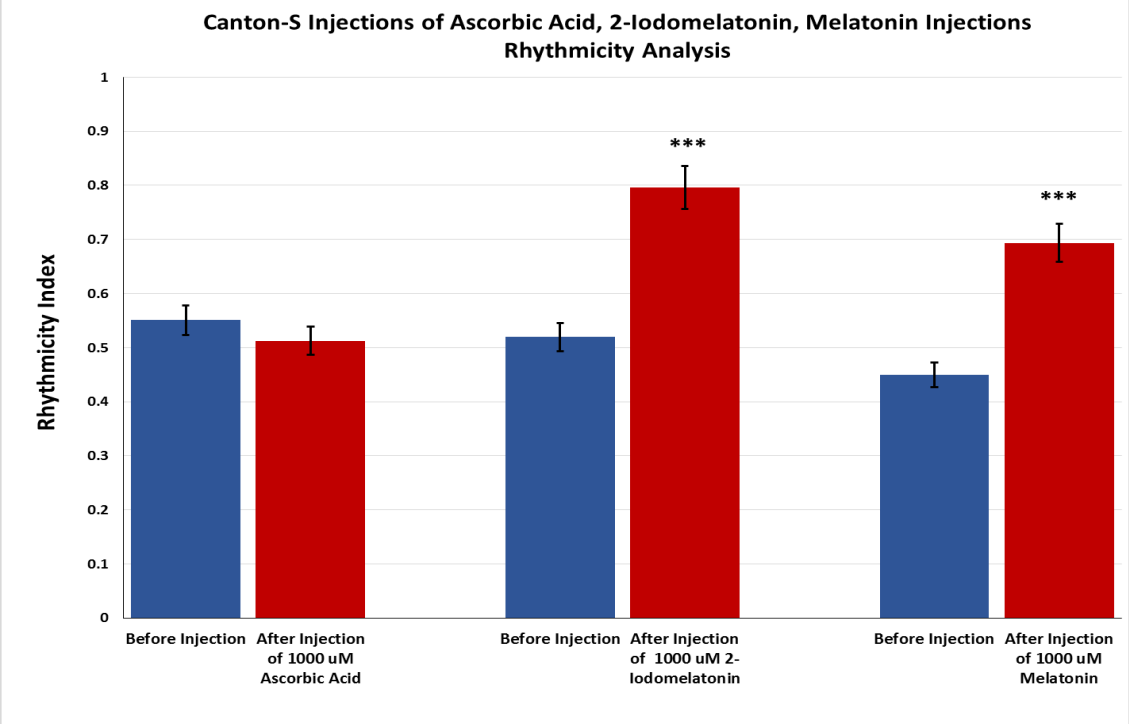


Figure 2.3 Effects of ascorbic acid, 2-iodomelatonin and melatonin on heart rhythmicity in *slowpoke* pupae

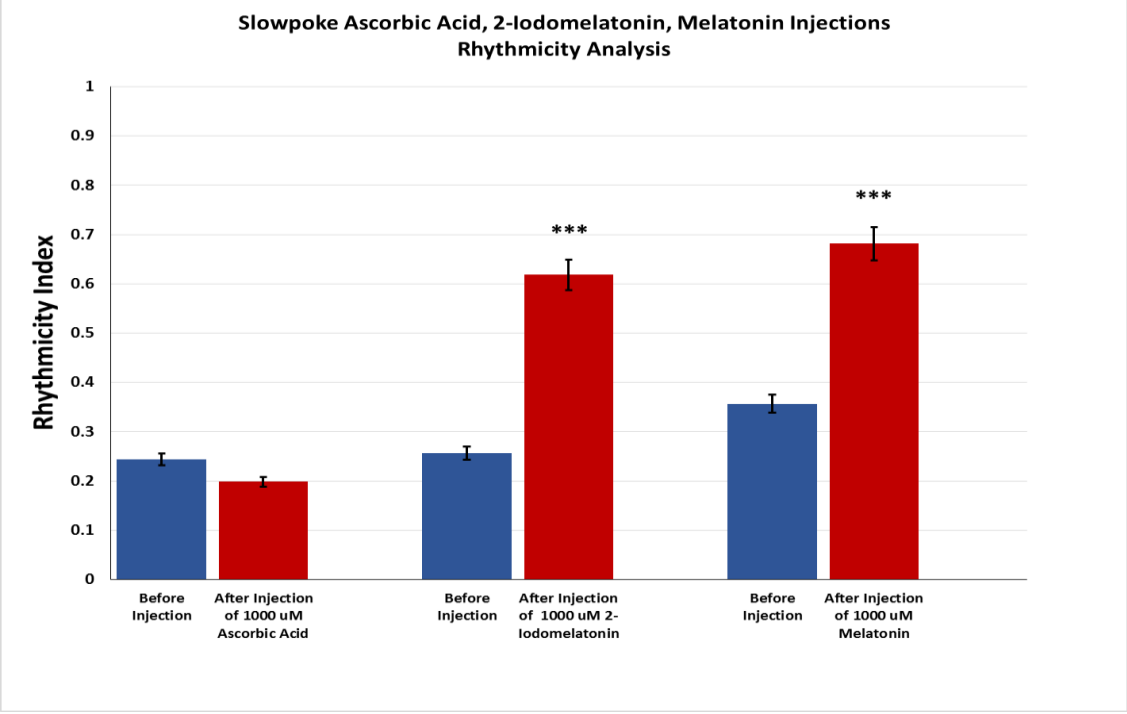


Table 2.5 Effects of 2-iodomelatonin injections on heart rate in wild-type and *slowpoke* pupae

Genotype	2-Iodomelatonin Injected µmol-1	Mean Heart Rate Pre 25´C	Mean Heart Rate Post 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	2.26	2.27	0%	5	0.06/0.04	(1,4) 0.01	0.906
slowpoke	1000	2.11	2.13	+1%	5	0.04/0.06	(1,4) 1.2	0.322

[a]Concentration of 2-Iodomelatonin injected

[b]Mean heart rate pre-injection minutes 4-7

[c]Mean heart rate minutes 4-7

Table 2.6 Effects of ascorbic acid injections on heart rate in wild-type and *slowpoke* pupae

Genotype	Ascorbic Acid Injected µmol-1	Mean Heart Rate Pre 25´C	Mean Heart Rate Post 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
Canton-S *(+)*	1000	2.39	2.25	-6 %	10	0.04/0.04	(1,9) 22.2	1.38e-05 ***
slowpoke	1000	2.18	1.86	-15 %	10	0.04/0.05	(1,9) 17.3	9.71e-05 ***

[a]Concentration of ascorbic acid injected

[b]Mean heart rate pre-injection minutes 4-7

[c]Mean heart rate minutes 4-7

*** $p < 0.001$ comparing before injection to after ascorbic acid injection within each strain

Figure 2.4 Effects of 2-iodomelatonin, ascorbic acid and melatonin injections on heart rate in wild-type pupae

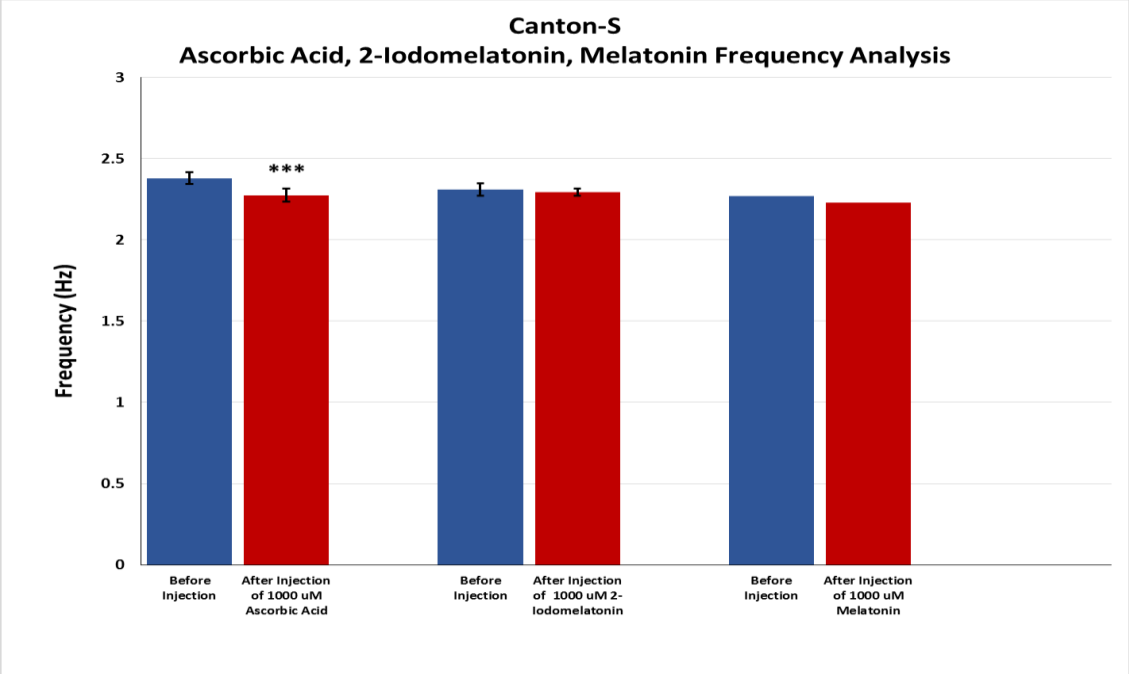
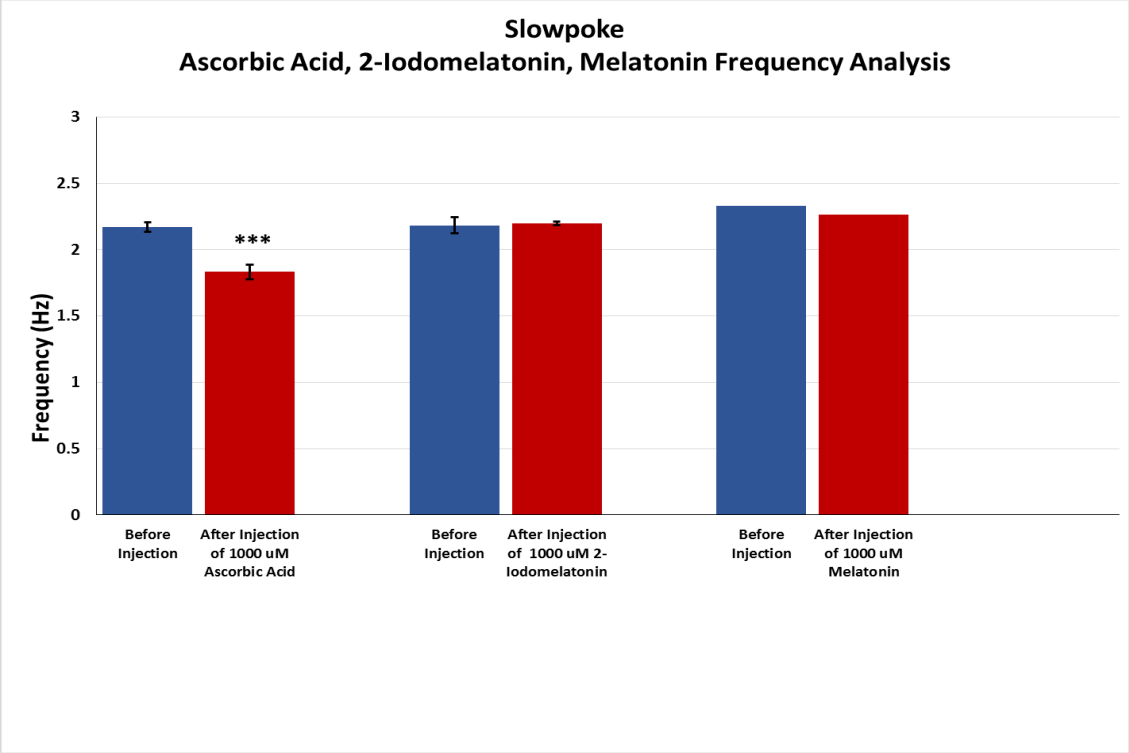


Figure 2.5 Effects of 2-iodomelatonin, ascorbic acid, and melatonin injections on heart rate in *slowpoke* pupae



Effects of melatonin injection on four different orphan receptor RNAi strains crossed with GMH5-Gal4 heart driver

Repeated measures ANOVAs showed significant effects of melatonin injection in *CG3171, CG13579* and *CG13575* orphan receptor strains ($p < 0.05$). This correlates strongly with the results of absolute % increase in rhythmicity ranging from 20 to 38 % in these three strains. These results indicate that no loss of function with melatonin injection (Table 2.7, Fig. 2.6).

Upon initial testing, *CG4313* showed promising results based on the strong but not significant trend and 20 animals were tested (10 each of reciprocal crosses). Results of melatonin injection with this orphan receptor RNAi continued to show no increase in heart rhythmicity ($p > 0.05$) (Table 2.7, Fig. 2.6). This loss of function result implicates that his *CG4313* is a highly likely candidate for a melatonin receptor in *Drosophila melanogaster.*

Table 2.7 Effects of melatonin injection on heart rhythmicity in orphan receptor RNAi crossed with GMH5-Gal4 driver

Orphan Receptor RNAi	Meltonin Injected µmol-1[a]	Mean RI Pre[c] 25´C	Mean RI Post[d] 25´C	% Change	N	+/-SEM Pre/Post	F Value	P Value
CG4313[a]	1000	0.580	0.588	+ 2%	10	0.04/0.03	(1,9) 0.12	0.730
CG4313[b]	1000	0.507	0.494	- 2%	10	0.04/0.04	(1,9) 0.13	0.720
CG3171	1000	0.680	0.817	+20 %	5	0.03/0.04	(1,4) 12.2	0.00161 **
CG13579	1000	0.649	0.817	+26 %	5	0.05/0.03	(1,4) 33.7	3.08e-06 ***
CG13575	1000	0.491	0.679	+38%	5	0.07/0.04	(1,4) 14.2	0.000791 ***

[a] orphan receptor RNAi virgin females crossed with GMH5 Gal 4 males

[b] GMH5 Gal4 virgin females crossed with orphan receptor RNAi males

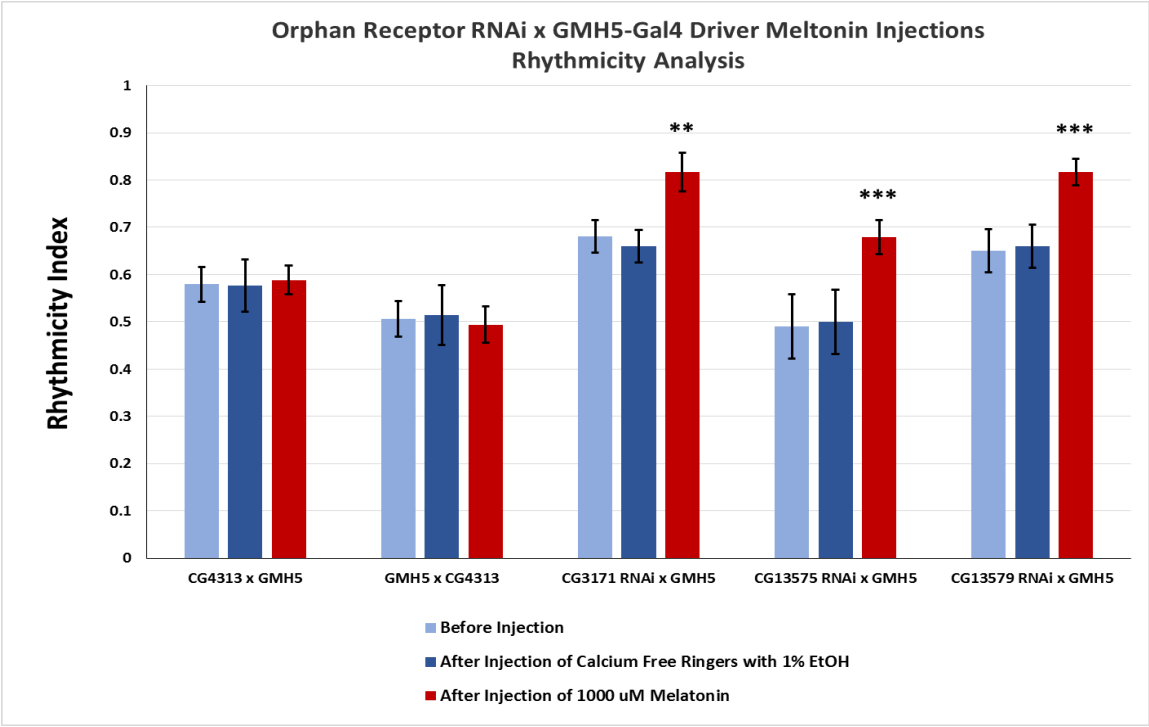
[c] Mean Rhythmicity Index pre-injection minutes 4-7

[d] Mean Rhythmicity Index post-injection minutes 4-7

** $p < 0.01$ comparing before injection to after melatonin injection within each strain

*** $p < 0.001$ comparing before injection to after melatonin injection within each strain

Figure 2.6 Effects of melatonin injection on heart rhythmicity in orphan receptor RNAis crossed with GMHG-Gal 4 driver



Discussion

In this set of experiments, I showed that melatonin's ability to increase heart rhythmicity in *Drosophila melanogaster* is a result of melatonin receptor binding. In the process of elucidating melatonin's effects, I also discovered an orphan G protein-coupled receptor that is highly likely to be a melatonin receptor. Evidence for these findings stem from the results of three different experimental approaches. I showed:

1. A highly specific response to melatonin, 2. Effective blocking of melatonin's effects with its antagonist luzindole, 3. Mimicking of melatonin's effects with its high-affinity agonist 2-iodomelatonin, and 4. Loss of function using RNAi techniques to silence a specific orphan GPCR.

The effects of the hormone melatonin on heart rhythmicity in *Drosophila melanogaster* are profound. Though it is well known that *Drosophila* has receptors for and produces its own serotonin, which is melatonin's precursor, evidence for the presence of melatonin receptors or endogenous production of melatonin in the fruit fly has not been presented to date. Elucidation of whether melatonin's effects on *Drosophila's* cardiovascular system are antioxidant mediated or receptor mediated is crucial to unraveling the mystery of the mechanisms of this hormone.

In a previous set of experiments (Chapter 1) I demonstrated that melatonin was able to increase heart rhythmicity by 54-104% without affecting heart rate in both wild type and heart mutant strains of *Drosophila melanogaster*. This was an interesting result, especially when compared to serotonin's opposing effects of increasing heart rate without significantly affecting heart rhythmicity (Johnson et al., 1997; VanKirk,

unpublished manuscript). Since a melatonin receptor had not yet been identified in *Drosophila,* it was not clear how melatonin was producing these effects. A handful of studies have been carried out in vertebrates reporting the anti-arrhythmic properties of melatonin (Benova et al., 2013, 2015; Bertuglia & Reiter, 2007; Diez et al., 2009; Vazan et al., 2005), but most researchers attributed this effect to melatonin's ability to scavenge reactive oxygen species (ROSs) produced during myocardial injury.

It seemed a logical next step to introduce a melatonin antagonist in my experimental design in order to gain a better understanding of melatonin's activity. In this set of experiments I performed double injections of the non-competitive melatonin antagonist, luzindole and melatonin. Not only did luzindole completely abolish melatonin's anti-arrhythmic effects in wild type, *slo* and nap^{ts} pupae, it also showed a slight , but non-significant decrease in heart rhythmicity when injected alone in wild-type and nap^{ts} pupae and a significant decrease in heart rhythmicity in *slo* pupae. This would make sense if *Drosophila* makes melatonin endogenously as luzindole would be blocking its effects as well. This antagonist effect of luzindole can only be exerted through receptor-mediated activity, which strongly implicates the presence of melatonin receptors in *Drosophila melanogaster.*

In a similar group of experiments designed to support the earlier findings, I chose to inject wild type and *slo* pupae with the high-affinity melatonin agonist, 2-Iodomelatonin. Results of these experiments showed that 2-Iodomelatonin increased heart rhythmicity in *slo* pupae by 50% more than melatonin, respectively. These results are consistent with the fact that 2-Iodomelatonin shows higher binding affinity to melatonin receptors (MT1 and MT2) than melatonin itself (Audinot et al., 2003). As

was the case with luzindole, 2-Iodomelatonin is only able to exert its effects by binding to a melatonin receptor, which these results strongly support. This is another clear indication that a melatonin receptor exists in *Drosophila melanogaster.*

Melatonin's cardio-protective effects are known to be antioxidant-related (Reviews: Reiter & Tan, 2003; Tengattini et al., 2007). At present there are several lines of evidence which suggest that an increased intake of antioxidants, vitamins, ascorbic acid (C), tocopherols (E), and beta-carotene (A), have some protective role in the coronary heart disease and other cardiovascular problems (Zhang et al., 2014). In support of melatonin's anti-arrhythmic effects being receptor-mediated and not antioxidant- mediated, I designed experiments to inject concentrations of ascorbic acid (vitamin C) into *Drosophila* pupae. Results of ascorbic acid injections showed a 5% and 24% decrease in heart rhythmicity in wild type and *slo* pupae, respectively. Even though Eslami et al., 2007, report that ascorbic acid, when used as an adjunct to ß-blockers, is highly effective at reducing post-operative atrial fibrillation, its antioxidant properties do not seem to have the same effect on the *Drosophila* heart. Instead, these results disprove the hypothesis that melatonin's anti-arrhythmic effects are antioxidant related. Furthermore, studies in vertebrates implicating that melatonin's anti-arrhythmic effects are antioxidant-mediated did not include the use of luzindole in their experimental design (Benova et al., 2013, 2015; Bertuglia & Reiter, 2007; Diez et al., 2009; Vazan et al., 2005).

Mammalian melatonin receptors are members of the G protein-coupled receptor family. GPCRs represent the largest superfamily and most diverse group of mammalian transmembrane proteins. The main characteristic feature of these proteins is that they

share a common seven-transmembrane (7TM) configuration (Tang et al., 2012). A broad range of ligands can bind to GPCRs, which result in a variety of signal transduction pathways that influence numerous physiological pathways in the body. GPCRs are the most prominent family of pharmacological targets in biomedicine (Lappano & Maggiolini, 2011).

The rhodopsin-like (Class A) receptors are the largest and most extensively studied subfamily of GPCRs. Both serotonin (5-HT) receptors and melatonin receptors in vertebrates and serotonin receptors in *Drosophila* fall into this category, so it is reasonable to assume that possible melatonin receptors in the fruit fly would also be of this type. The *Drosophila melanogaster* genes *CG4313, CG3171, CG13575* and *CG13579* code for putative neuropeptide receptors belonging to class A are still orphans (Hauser et al., 2006). Bloomington stock center has RNAi strains for the aforementioned genes that can knock down the gene of interest in specific tissues and be used for loss of function tests.

In my final set of experiments I crossed the RNAi strains of four different *Drosophila* orphan GPCRs (*CG4313, CG3171, CG13575, CG13579*) with the heart specific Gal 4 driver, GMH5 in order to silence the orphan receptor in question and carry out loss of function tests. Injection of melatonin into *CG3171, C13575* and *CG13579* crosses all showed significant increase in rhythmicity. Because RNA interference knocks down the gene coding the orphan receptor, an increase in rhythmicity with melatonin injection indicates that the melatonin receptor is still present and is not coded for by the targeted gene. However, injections of melatonin into the *CG4313* silenced pupae showed no significant increase in heart rhythmicity, which

indicates that the *CG4313* orphan receptor could be a melatonin receptor. Interestingly, of the four orphan receptors tested, the *CG4313* orphan receptor also shows the highest homology (~ 30%) to the human melatonin receptor.

In summary, the combination of pharmacology and genetics described above provide data that support the receptor mediated anti-arrhythmic effects of melatonin and the presence of a melatonin receptor in *Drosophila melanogaster*. Coupling a ligand such as melatonin to an orphan receptor will provide invaluable information as to the signaling pathway that underlies melatonin's anti-arrhythmic effects.

LITERATURE CITED

Abete P, Bianco S, Calabrese C, Napoli C, Cacciatore F, Ferrara N, Rengo F. 1997. Effects of melatonin in isolated rat papillary muscle. FEBS Lett. 412:79–85.

Adams et. al. 2000. The genome sequence of Drosophila melanogaster. Science. 287:2185–2195.

Ai Z, Fischer A, Spray DC, Brown AMC, Fishman GI. 2000. Wnt-1 regulation of connexin43 in cardiac myocytes. J. Clin. Invest. 105:161–171.

Amherd R, Hintermann E, Walz D, Affolter M, Meyer U a. 2000. Purification, cloning, and characterization of a second arylalkylamine N-acetyltransferase from Drosophila melanogaster. DNA Cell Biol. 19:697–705.

Anisimov VN. 2003. Effects of Exogenous Melatonin--A Review. Toxicol. Pathol. 31:589–603.

Arangino S, Cagnacci a, Angiolucci M, Vacca a M, Longu G, Volpe a, Melis GB. 1999. Effects of melatonin on vascular reactivity, catecholamine levels, and blood pressure in healthy men. Am. J. Cardiol. 83:1417–9.

Arendt J. 1995. Melatonin and the mammalian pineal gland. Springer.

Ashburner M. 1989. Drosophila: A Laboratory Manual: 002.

Ashcroft FM. 2000. Ion Channels and Disease. Academic Press.

Ashton K, Wagoner AP, Carrillo R, Gibson G. 2001. Quantitative trait loci for the monoamine-related traits heart rate and headless behavior in Drosophila melanogaster. Genetics 157:283–94.

Atkinson NS, Robertson G a, Ganetzky B. 1991. A component of calcium-activated potassium channels encoded by the Drosophila slo locus. Science 253:551–555.

Audinot V, Mailliet F, Lahaye-Brasseur C, Bonnaud A, Le Gall A, Amossé C, Dromaint S, Rodriguez M, Nagel N, Galizzi J-P, et al. 2003. New selective ligands of human cloned melatonin MT1 and MT2 receptors. Naunyn. Schmiedebergs. Arch. Pharmacol. 367:553–561.

Azpiazu N, Frasch M. 1993. Tinman and bagpipe: Two homeo box genes that determine cell fates in the dorsal mesoderm of Drosophila. Genes Dev. 7:1325–1340.

Balík a., Kretschmannová K, Mazna P, Svobodová I, Zemková H. 2004. Melatonin Action in Neonatal Gonadotrophs. Physiol. Res. 53.

Basson CT, Bachinsky DR, Lin RC, Levi T, Elkins JA, Soults J, Grayzel D, Kroumpouzou E, Traill TA, Leblanc-Straceski J, et al. 1997. Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. Nat. Genet. 15:30–5.

De Belle JS, Hilliker AJ, Sokolowski MB. 1989. Genetic localization of foraging (for): a major gene for larval behavior in Drosophila melanogaster. Genetics 123:157–63.

Bellés X. 2010. Beyond Drosophila: RNAi in vivo and functional genomics in insects. Annu. Rev. Entomol. 55:111–28.

Benova T, Knezl V, Viczenczova C, Bacova S, Radosinska J, Tribulova N. 2015. Acute anti-fibrillating and defibrillating potential of atorvastatin, melatonin, eicosapentaenoic acid and docosahexaenoic acid demonstrated in isolated heart model. J. Physiol. Pharmacol. 66:83–89.

Benova T, Viczenczova C, Radosinska J, Bacova B, Knezl V, Dosenko V, Weismann P, Zeman M, Navarova J, Tribulova N. 2013. Melatonin attenuates hypertension-related proarrhythmic myocardial maladaptation of connexin-43 and propensity of the heart to lethal arrhythmias. Can J Physiol Pharmacol 91:633–639.

Berridge MJ, Bootman MD, Lipp P. 1998. Calcium--a life and death signal. Nature 395:645–8.

Bertuglia S, Reiter RJ. 2007. Melatonin reduces ventricular arrhythmias and preserves capillary perfusion during ischemia-reperfusion events in cardiomyopathic hamsters. J. Pineal Res. 42:55–63.

Bier E, Bodmer R. 2004. Drosophila, an emerging model for cardiac disease. Gene 342:1–11.

Blask DE, Sauer LA, Dauchy RT. 2002. Melatonin as a chronobiotic/anticancer agent: cellular, biochemical, and molecular mechanisms of action and their implications for circadian-based cancer therapy. Curr. Top. Med. Chem. 2:113–32.

Bodmer R. 1993. The gene tinman is required for specification of the heart and visceral muscles in Drosophila. Development 118:719–29.

Bodmer R. 1995. Heart development in Drosophila and its relationship to vertebrates. Trends Cardiovasc. Med. 5:21–28.

Bodmer R, Venkatesh T V. 1998. Heart Development in Drosophila and Vertebrates : Conservation of Molecular Mechanisms. Dev. Genet. 186:181–186.

Bodmer R, Wessels RJ, Johnson E DH. 2005. Heart development and function. In: L.I. Gilbert, K. Iatrou SG, editor. Comprehensive Molecular Insect Science. London: Elsevier.

Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, Ferguson K. 1993. A family of human phosphodiesterases homologous to the dunce learning and memory gene product of Drosophila melanogaster are potential targets for antidepressant drugs. Mol. Cell. Biol. 13:6558–6571.

Bonilla E, Medina-Leendertz S, Díaz S. 2002. Extension of life span and stress resistance of Drosophila melanogaster by long-term supplementation with melatonin. Exp. Gerontol. 37:629–38.

Bonilla E, Medina-Leendertz S, Villalobos V, Molero L, Bohórquez A. 2006. Paraquat-induced oxidative stress in Drosophila melanogaster: Effects of melatonin, glutathione, serotonin, minocycline, lipoic acid and ascorbic acid. Neurochem. Res. 31:1425–1432.

Brand a H, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Brautigan DL, Pinault FM. 1991. Activation of membrane protein-tyrosine phosphatase involving cAMP- and Ca2+/phospholipid-dependent protein kinases. Proc. Natl. Acad. Sci. U. S. A. 88:6696–700.

Brodbeck D, Amherd R, Callaerts P, Hintermann E, Meyer UA, Affolter M. 1998. Molecular and biochemical characterization of the aaNAT1 (Dat) locus in Drosophila melanogaster: differential expression of two gene products. DNA Cell Biol. 17:621–33.

Brody T, Cravchik A. 2000. Drosophila melanogaster G Protein – coupled Receptors. J. Cell Biol. 150:83–88.

Broeckel U, Schork NJ. 2004. Identifying genes and genetic variation underlying human diseases and complex phenotypes via recombination mapping. J. Physiol. 554:40–5.

Brüggemann a, Pardo L a, Stühmer W, Pongs O. 1993. Ether-à-go-go encodes a voltage-gated channel permeable to K+ and Ca2+ and modulated by cAMP. Nature 365:445–448.

Bubenik GA, Brown GM, Grota LJ. 1976. Immunohistochemical localization of melatonin in the rat Harderian gland. J. Histochem. Cytochem. 24:1173–7.

Bullock TH. 1955. Compensation for temperature in the metabolism and activity of poikilotherms. Biol. Rev. 30:311–342.

Caers J, Verlinden H, Zels S, Vandersmissen HP, Vuerinckx K, Schoofs L. 2012. More than two decades of research on insect neuropeptide GPCRs: An overview. Front. Endocrinol. (Lausanne). 3:1–30.

Celinski K, Konturek SJ, Konturek PC, Brzozowski T, Cichoz-Lach H, Slomka M, Malgorzata P, Bielanski W, Reiter RJ. 2011. Melatonin or L-tryptophan accelerates healing of gastroduodenal ulcers in patients treated with omeprazole. J. Pineal Res. 50:389–94.

Chan MY, Pang SF, Tang PL, Brown GM. 1984. Studies on the kinetics of melatonin and N-acetylserotonin in the rat at mid-light and mid-dark. J. Pineal Res. 1:227–36.

Chatfield C. 1980. The Analysis of Time Series.

Chen C-Q, Fichna J, Bashashati M, Li Y-Y, Storr M. 2011. Distribution, function and physiological role of melatonin in the lower gut. World J. Gastroenterol. 17:3888–98.

Cho KO, Wall JB, Pugh PC, Ito M, Mueller SA, Kennedy MB. 1991. The alpha subunit of type II Ca2+/calmodulin-dependent protein kinase is highly conserved in Drosophila. Neuron 7:439–50.

Cho S, Joh TH, Baik HH, Dibinis C, Volpe BT. 1997. Melatonin administration protects CA1 hippocampal neurons after transient forebrain ischemia in rats. Brain Res. 755:335–8.

Colas JF, Launay JM, Kellermann O, Rosay P, Maroteaux L. 1995. Drosophila 5-HT2 serotonin receptor: coexpression with fushi-tarazu during segmentation. Proc. Natl. Acad. Sci. U. S. A. 92:5441–5.

Conti A, Conconi S, Hertens E, Skwarlo-Sonta K, Markowska M, Maestroni JM. 2000. Evidence for melatonin synthesis in mouse and human bone marrow cells. J. Pineal Res. 28:193–202.

Coto-Montes a, Hardeland R. 1999. Antioxidative effects of melatonin in Drosophila melanogaster: antagonization of damage induced by the inhibition of catalase. J. Pineal Res. 27:154–158.

Coulom H, Birman S. 2004. Chronic exposure to rotenone models sporadic Parkinson's disease in Drosophila melanogaster. J. Neurosci. 24:10993–8.

Cripps RM, Olson EN. 2002. Control of cardiac development by an evolutionarily conserved transcriptional network. Dev. Biol. 246:14–28.

Crozier WJ, Federighi H. 1925. TEMPERATURE CHARACTERISTIC FOR HEART RHYTHM OF THE SILKWORM. J. Gen. Physiol. 7:565–70.

Curtis NJ, Ringo JM, Dowse HB. 1999. Morphology of the pupal heart, adult heart, and associated tissues in the fruit fly, Drosophila melanogaster. J. Morphol. 240:225–35.

Dascal N, Snutch TP, Lübbert H, Davidson N, Lester HA. 1986. Expression and modulation of voltage-gated calcium channels after RNA injection in Xenopus oocytes. Science 231:1147–50.

Delisle BP, Anson BD, Rajamani S, January CT. 2004. Biology of cardiac arrhythmias: Ion channel protein trafficking. Circ. Res. 94:1418–1428.

Di W, Kadva A, Johnston A. 1997. Variable bioavailability of oral melatonin. New Engl. J.:1021–1030.

Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, et al. 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448:151–156.

Diez ER, Prados LV, Carrión A, Ponce ZAZ, Miatello RM. 2009. A novel electrophysiologic effect of melatonin on ischemia/reperfusion-induced arrhythmias in isolated rat hearts. J. Pineal Res. 46:155–160.

Ding CN1, Cao YX, Zhou L, Zhu DN, Shen ZY, Fei MY YP. 2001. Effects of microinjection of melatonin and its receptor antagonists into anterior hypothalamic area on blood pressure and heart rate in rats. Acta Pharmacol. Sin.22: 997-1002.

Dominguez-Rodriguez A, Abreu-Gonzalez P. 2010. Myocardial ischemia-reperfusion injury: Possible role of melatonin. World J. Cardiol. 2:233–236.

Dowse H, Ringo J, Power J, Johnson E, Kinney K, White L. 1995. A congenital heart defect in Drosophila caused by an action-potential mutation. J. Neurogenet. 10:153–68.

Dowse HB. 2009. Chapter 6 Analyses for Physiological and Behavioral Rhythmicity. In: Methods in Enzymology. p. 141–174.

Dowse HB. 1989. The search for hidden periodicities in biological time series revisited. J. Theor. Biol. 139:487–515.

Drysdale R, Warmke J, Kreber R, Ganetzky B. 1991. Molecular characterization of eag: a gene affecting potassium channels in Drosophila melanogaster. Genetics 127:497–505.

Dubocovich ML. 1983. Melatonin is a potent modulator of dopamine release in the retina. Nature 306:782–4.

Dubocovich ML. 1988. Luzindole (N-0774): a novel melatonin receptor antagonist. J. Pharmacol. Exp. Ther. 246:902–10.

Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J. 2010. International Union of Basic and Clinical Pharmacology . LXXV . Nomenclature , Classification , and Pharmacology of G Protein-Coupled Melatonin Receptors. 62:343–380.

Dubocovich ML, Hudson RL, Sumaya IC, Masana MI, Manna E. 2005. Effect of MT1 melatonin receptor deletion on melatonin-mediated phase shift of circadian rhythms in the C57BL/6 mouse. J. Pineal Res. 39:113–20.

Dubocovich ML, Markowska M. 2005. Functional MT1 and MT2 melatonin receptors in mammals. Endocrine 27:101–10.

Dubocovich ML, Masana MI, Benloucif S. 1999. Molecular pharmacology and function of melatonin receptor subtypes. Adv. Exp. Med. Biol. 460:181–90.

Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ, Masana MI. 2003. Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front. Biosci. 8:d1093–108.

Dubocovich ML, Takahashi JS. 1987. Use of 2-[125I]iodomelatonin to characterize melatonin binding sites in chicken retina. Proc. Natl. Acad. Sci. U. S. A. 84:3916–3920.

Dubocovich ML, Yun K, Al-Ghoul WM, Benloucif S, Masana MI. 1998. Selective MT2 melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. FASEB J. 12:1211–20.

Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S. 1976. Dunce, a mutant of Drosophila deficient in learning. Proc. Natl. Acad. Sci. U. S. A. 73:1684–1688.

Dulcis D, Levine RB. 2003. Innervation of the heart of the adult fruit fly, Drosophila melanogaster. J. Comp. Neurol. 465:560–78.

Elkins T, Ganetzky B, Wu CF. 1986. A Drosophila mutation that eliminates a calcium-dependent potassium current. Proc. Natl. Acad. Sci. U. S. A. 83:8415–9.

Engel JE, Wu CF. 1992. Interactions of membrane excitability mutations affecting potassium and sodium currents in the flight and giant fiber escape systems of Drosophila. J. Comp. Physiol. A. 171:93–104.

Eslami M, Badkoubeh RS, Mousavi M, Radmehr H, Salehi M, Tavakoli N, Avadi MR. 2007. Oral ascorbic acid in combination with beta-blockers is more effective than beta-blockers alone in the prevention of atrial fibrillation after coronary artery bypass grafting. Tex. Heart Inst. J. 34:268–274.

Falconer DS. 1960. Introduction to Quantitative Genetics. England: Longman Group Ltd.

Fischer TW, Slominski A, Zmijewski MA, Reiter RJ, Paus R. 2008. Melatonin as a major skin protectant: from free radical scavenging to DNA damage repair. Exp. Dermatol. 17:713–30.

Fozzard HA. 1986. The Heart and cardiovascular system: scientific foundations. Raven Press.

Frasch M. 1995. Induction of visceral and cardiac mesoderm. Nature 374:464–467.

Fries EF. 1926. TEMPERATURE AND FREQUENCY OF HEART BEAT IN THE COCKROACH. J. Gen. Physiol. 10:227–37.

Ganetzky B, Wu CF. 1982. Indirect suppression involving behavioral mutants with altered nerve excitability in Drosophila melanogaster. Genetics 100:597–614.

Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler C a, Rothrock CR, Eapen RS, Hirayama-Yamada K, Joo K, et al. 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424:443–447.

Gaus G, Casaretto M, Stieve H. 1994. Cardioinhibitory peptides from Limulus polyphemus: modulation of the neurogenic heart. J. Comp. Physiol. B. 164:191–4.

Gibbs FP, Vriend J. 1981. The half-life of melatonin elimination from rat plasma. Endocrinology 109:1796–8.

Girouard H, Denault C, Chulak C, de Champlain J. 2004. Treatment by n-acetylcysteine and melatonin increases cardiac baroreflex and improves antioxidant reserve. Am. J. Hypertens. 17:947–54.

Gitto E, Tan DX, Reiter RJ, Karbownik M, Manchester LC, Cuzzocrea S, Fulia F, Barberi I. 2001. Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrioxamine (desferoxamine) in rat liver homogenates. J. Pharm. Pharmacol. 53:1393–1401.

Griffith LC, Verselis LM, Aitken KM, Kyriacou CP, Danho W, Greenspan RJ. 1993. Inhibition of calcium/calmodulin-dependent protein kinase in Drosophila disrupts behavioral plasticity. Neuron 10:501–9.

Gu GG, Singh S. 1995. Pharmacological analysis of heartbeat in Drosophila. J. Neurobiol. 28:269–80.

Gülçin I. 2008 Dec 2. Measurement of antioxidant ability of melatonin and serotonin by the DMPD and CUPRAC methods as trolox equivalent.

Haley B, Hendrix D, Trang V, Levine M. 2009. A simplified miRNA-based gene silencing method for Drosophila melanogaster. Dev. Biol. 321:482–490.

Hanegan JL. 1973. Control of Heart Rate in Cecropia Moths: Response to Thermal Stimulation. J. Exp. Biol. 59:67–76.

Hardeland R, Cardinali DP, Srinivasan V, Spence DW, Brown GM, Pandi-Perumal SR. 2011. Melatonin--a pleiotropic, orchestrating regulator molecule. Prog. Neurobiol. 93:350–84.

Hardeland R, Tan D-X, Reiter RJ. 2009. Kynuramines, metabolites of melatonin and other indoles: the resurrection of an almost forgotten class of biogenic amines. J. Pineal Res. 47:109–26.

Hartzell HC. 1988. Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. Prog. Biophys. Mol. Biol. 52:165–247.

Harvey RP. 1996. NK-2 homeobox genes and heart development. Dev. Biol. 178:203–216.

Hauser F, Williamson M, Cazzamali G, Grimmelikhuijzen CJP. 2006. Identifying neuropeptide and protein hormone receptors in Drosophila melanogaster by exploiting genomic data. Briefings Funct. Genomics Proteomics 4:321–330.

Head KA. 2005. Melatonin. Altern. Med. Rev. 10:326–336.

Heinrich B. 1970. Nervous control of the heart during thoracic temperature regulation in a sphinx moth. Science 169:606–7.

Heron M. 2013. Deaths: leading causes for 2010. Natl. Vital Stat. Rep. 62:1–97.

Hewes RS, Taghert PH. 2001. Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. Genome Res. 11:1126–1142.

Hickman AB, Klein DC, Dyda F. 1999. Melatonin Biosynthesis. Mol. Cell 3:23–32.

Hille B. 2001. Ionic Channels of Excitable Membranes. 3rd ed. Sinauer, Sunderland, editors. Mass.

Hintermann E, Jeno P, Meyer U a. 1995. Isolation and characterization of an arylalkylamine N-acetyltransferase from Drosophila melanogaster. FEBS Lett. 375:148–150.

Hirata F, Hayaishi O, Tokuyama T. 1974. In vitro and in vivo formation of two new metabolites of melatonin. J. Biol. 249:1311–1313.

Hochman B. 1971. Drosophila melanogaster. 11: Genetics 67:235–252.

Holland ND, Venkatesh T V, Holland LZ, Jacobs DK, Bodmer R. 2003. AmphiNk2-tin, an amphioxus homeobox gene expressed in myocardial progenitors: insights into evolution of the vertebrate heart. Dev. Biol. 255:128–37.

Iguichi H, Kato KI, Ibayashi H. 1982. Age-dependent reduction in serum melatonin concentrations in healthy human subjects. J. Clin. Endocrinol. Metab. 55:27–9.

Imbesi M, Arslan AD, Yildiz S, Sharma R, Gavin D, Tun N, Manev H, Uz T. 2009. The melatonin receptor MT1 is required for the differential regulatory actions of melatonin on neuronal "clock" gene expression in striatal neurons in vitro. J. Pineal Res. 46:87–94.

Irisawa H, Brown HF, Giles W. 1993. Cardiac pacemaking in the sinoatrial node. Physiol. Rev. 73:197–227.

Iuvone PM, Brown AD, Haque R, Weller J, Zawilska JB, Chaurasia SS, Ma M, Klein DC. 2002. Retinal melatonin production: role of proteasomal proteolysis in circadian and photic control of arylalkylamine N-acetyltransferase. Invest. Ophthalmol. Vis. Sci. 43:564–72.

Jennings T, Ringo J, Dowse H. 2009. The relationship of heart function to temperature in Drosophila melanogaster and its heritability. J. Exp. Zool. A. Ecol. Genet. Physiol. 311:689–96.

Johnson E, Ringo J, Bray N, Dowse H. 1998. Genetic and pharmacological identification of ion channels central to the Drosophila cardiac pacemaker. J. Neurogenet. 12:1–24.

Johnson E, Ringo J, Dowse H. 1997. Modulation of Drosophila heartbeat by neurotransmitters. J. Comp. Physiol. B. 167:89–97.

Johnson E, Ringo J, Dowse H. 2000. Native and heterologous neuropeptides are cardioactive in Drosophila melanogaster. J. Insect Physiol. 46:1229–1236.

Johnson E, Sherry T, Ringo J DH. 2002. Modulation of the cardiac pacemaker of Drosophila : cellular mechanisms. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 172:227–236.

Jones J. 1977. The circulatory system of insects. Springfield Ill.: Thomas.

Kaplan WD, Trout WE. 1969. The behavior of four neurological mutants of Drosophila. Genetics 61:399–409.

Karasek M, Woldanska-Okonska M, Czernicki J, Zylinska K, Swietoslawski J. 1998. Chronic exposure to 2.9 mT, 40 Hz magnetic field reduces melatonin concentrations in humans. J. Pineal Res. 25:240–4.

Keating MT, Sanguinetti MC. 1996. Molecular genetic insights into cardiovascular disease. Science 272:681–685.

Kernan MJ, Kuroda MI, Kreber R, Baker BS, Ganetzky B. 1991. napts, a mutation affecting sodium channel activity in Drosophila, is an allele of mle, a regulator of X chromosome transcription. Cell 66:949–959.

Kim Y. 1989. Drosophila NK-homeobox genes. Natl. Acad. Sci. 86:7716–7720.

Kitay JI, Altschule MD. 1954. The pineal gland: a review of the physiologic literature. Published for the Commonwealth Fund by Harvard University Press.

Klinedinst SL, Bodmer R. 2003. Gata factor Pannier is required to establish competence for heart progenitor formation. Development 130:3027–3038.

Knollmann BC, Roden DM. 2008. A genetic framework for improving arrhythmia therapy. Nature 451:929–936.

Kraft P, de Andrade M. 2003. Group 6: Pleiotropy and multivariate analysis. Genet. Epidemiol. 25 Suppl 1:S50–6.

Kveder S. 1961. The metabolism of melatonin (N-acetyl-5-methoxytryptamine) and 5-methoxytryptamine. J. Biol. Chem. 236:3214–3220.

Laidler KJ. 1972. Unconventional applications of the Arrhenius law. J. Chem. Educ. 49:343.

Lappano R, Maggiolini M. 2011. G protein-coupled receptors: novel targets for drug discovery in cancer. Nat. Rev. Drug Discov. 10:47–60.

Lee CG, Chang K a., Kuroda MI, Hurwitz J. 1997. The NTPase/helicase activities of Drosophila maleless, an essential factor in dosage compensation. EMBO J. 16:2671–2681.

Lee PP, Pang SF. 1993. Melatonin and its receptors in the gastrointestinal tract. Biol. Signals 2:181–93.

Leja-Szpak A, Jaworek J, Pierzchalski P, Reiter RJ. 2010. Melatonin induces pro-apoptotic signaling pathway in human pancreatic carcinoma cells (PANC-1). J. Pineal Res. 49:248–255.

Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. 1958. Isolation Of Melatonin, The Pineal Gland Factor That Lightens Melanocytes. J. Am. Chem. Soc. 80:2587–2587.

Levine JD, Funes P, Dowse HB, Hall JC. 2002. Signal analysis of behavioral and molecular cycles. BMC Neurosci. 3:1.

Li JC, Xu F. 1997. Influences of light-dark shifting on the immune system, tumor growth and life span of rats, mice and fruit flies as well as on the counteraction of melatonin. Biol. Signals 6:77–89.

Lipsius SL, Hüser J, Blatter L a. 2001. Intracellular Ca2+ release sparks atrial pacemaker activity. News Physiol. Sci. 16:101–106.

Lissoni P, Ardizzoia A, Tisi E, Rossini F, Barni S, Tancini G, Conti A, Maestroni GJ. 1993. Amplification of eosinophilia by melatonin during the immunotherapy of cancer with interleukin-2. J. Biol. Regul. Homeost. Agents 7:34–6.

Littleton JT, Ganetzky B. 2000. Ion channels and synaptic organization: analysis of the Drosophila genome. Neuron 26:35–43.

Livingstone MS. 1981. Two mutations in Drosophila differentially affect the synthesis of octopamine, dopamine, and serotonin by altering the activities of two different amino acid decarboxylases. Soc. Neurosci. Abstr. 7:351.

Lochner A. 2006. Short and Long Term Effects of Melatonin on Myocardial Post-Ischemic Recovery. J. Pineal Res. 40:56–63.

Lockwood WK, Bodmer R. 2002. The patterns of wingless, decapentaplegic, and tinman position the Drosophila heart. Mech. Dev. 114:13–26.

Lopez AD, Murray CC. 1998. The global burden of disease, 1990-2020. Nat. Med. 4:1241–3.

Lusis AJ, Mar R, Pajukanta P. 2004. Genetics of atherosclerosis. Annu. Rev. Genomics Hum. Genet. 5:189–218.

MacLennan DH. 2000. Ca2+ signalling and muscle disease. Eur. J. Biochem. 267:5291–5297.

Majeed ZR, Stacy A, Cooper RL. 2014. Pharmacological and genetic identification of serotonin receptor subtypes on Drosophila larval heart and aorta. :205–219.

Man GC-W, Wong JH, Wang WW-J, Sun G-Q, Yeung BH-Y, Ng T-B, Lee SK-M, Ng BK-W, Qiu Y, Cheng JC-Y. 2011. Abnormal melatonin receptor 1B expression in osteoblasts from girls with adolescent idiopathic scoliosis. J. Pineal Res. 50:395–402.

Manda K, Ueno M, Anzai K. 2007. AFMK, a melatonin metabolite, attenuates X-ray-induced oxidative damage to DNA, proteins and lipids in mice. J. Pineal Res. 42:386–393.

Manda K, Ueno M, Anzai K. 2008. Space radiation-induced inhibition of neurogenesis in the hippocampal dentate gyrus and memory impairment in mice: ameliorative potential of the melatonin metabolite, AFMK. J. Pineal Res. 45:430–8.

Markou T, Theophilidis G. 2000. The pacemaker activity generating the intrinsic myogenic contraction of the dorsal vessel of Tenebrio molitor (Coleoptera). J. Exp. Biol. 203:3471–3483.

Mayo JC, Sainz RM, Tan D-X, Hardeland R, Leon J, Rodriguez C, Reiter RJ. 2005. Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages. J. Neuroimmunol. 165:139–49.

McCann F. 1969. The insect heart as a model of electrophysiological studies. In: Kerkut GA, editor. Experiments in Physiology and Biochemistry, vol. 2. Volume 2. New York: Academic Press. p. 60–88.

McCann F, Sanger JW. 1969. Ultrastructure and function in an insect heart. Experientia. Suppl. 15:29–46.

McCann F V. 1963. Electrophysiology of an insect heart. J. Gen. Physiol. 46:803–821.

McCord CP, Allen FP. 1917. Evidences associating pineal gland function with alterations in pigmentation. J. Exp. Zool. 23:207–224.

Mhatre MC, van Jaarsveld AS, Reiter RJ. 1988. Melatonin in the lacrimal gland: first demonstration and experimental manipulation. Biochem. Biophys. Res. Commun. 153:1186–92.

Miller A. 1950. The internal anatomy and histology of the imago of Drosophila melanogaster. In: Demerec M, editor. Biology of Drosophila. New York and London: John Wiley & Sons. p. 420–534.

Miller TA. 1985. Heart and diaphragms. In: GA Kerkut LG, editor. Comprehensive Insect Physiology, Biochemistry, and Pharmacology. New York: Permagon. p. 119–130.

Moczydlowski E, Lucchesi K, Ravindran A. 1988. An emerging pharmacology of peptide toxins targeted against potassium channels. J. Membr. Biol. 105:95–111.

Morady F. 1999. Radio-frequency Ablation as Treatment for Cardiac Arrhythmias. N. Engl. J. Med. 340:534–544.

Morgan KR. Shelley TE. 1988. Body temperature regulation in desert robber flies (Diptera: Asilidae). Ecol. Entomol. 13:419–428.

Morin D, Simon N, Deprés-Brummer P, Lévi F, Tillement JP, Urien S. 1997. Melatonin high-affinity binding to alpha-1-acid glycoprotein in human serum. Pharmacology 54:271–5.

Neely GG, Kuba K, Cammarato A, Isobe K, Zhang L, Murata M, Elmén L, Gupta V, Sarangi R, Dan D, et al. 2010. A Global In Vivo Drosophila RNAi Screen Identifies NOT3 as a Conserved Regulator of Heart Function. Cell 141:142–153.

Noda Y, Mori A, Liburdy R, Packer L. 1999. Melatonin and its precursors scavenge nitric oxide. J. Pineal Res. 27:159–63.

Ohsako S, Nishida Y, Ryo H, Yamauchi T. 1993. Molecular characterization and expression of the Drosophila Ca2+/calmodulin-dependent protein kinase II gene. Identification of four forms of the enzyme generated from a single gene by alternative splicing. J. Biol. Chem. 268:2052–62.

De Oliveira Silva S, Ximenes VF, Livramento JA, Catalani LH, Campa A. 2005. High concentrations of the melatonin metabolite, N1-acetyl-N 2-formyl-5-methoxykynuramine, in cerebrospinal fluid of patients with meningitis: a possible immunomodulatory mechanism. J. Pineal Res. 39:302–306.

Olivera BM, Miljanich GP, Ramachandran J, Adams ME. 1994. Calcium channel diversity and neurotransmitter release: the omega-conotoxins and omega-agatoxins. Annu. Rev. Biochem. 63:823–67.

Osborne KA, Robichon A, Burgess E, Butland S, Shaw RA, Coulthard A, Pereira HS, Greenspan RJ, Sokolowski MB. 1997. Natural behavior polymorphism due to a cGMP-dependent protein kinase of Drosophila. Science 277:834–6.

Oxenkrug G. 2005. Antioxidant effects of N-acetylserotonin: Possible mechanisms and clinical implications. Ann. N. Y. Acad. Sci. 1053:334–347.

Ozaki Y, Lynch HJ, Wurtman RJ. 1976. Melatonin in rat pineal, plasma, and urine: 24-hour rhythmicity and effect of chlorpromazine. Endocrinology 98:1418–24.

Pallanck L, Ganetzky B. 1994. Cloning and characterization of human and mouse homologs of the Drosophila calcium-activated potassium channel gene, slowpoke. Hum. Mol. Genet. 3:1239–1243.

Pandi-Perumal SR, Trakht I, Srinivasan V, Spence DW, Maestroni GJM, Zisapel N, Cardinali DP. 2008. Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. Prog. Neurobiol. 85:335–53.

Pang SF, Dubocovich ML BG. 1993. Melatonin receptors in peripheral tissues: a new area of melatonin research. Biol. Signals 2:1477–180.

Park M, Venkatesh T V, Bodmer R. 1998. Dual role for the zeste-white3/shaggy-encoded kinase in mesoderm and heart development of Drosophila. Dev. Genet. 22:201–11.

Park M, Wu X, Golden K, Axelrod JD, Bodmer R. 1996. The wingless signaling pathway is directly involved in Drosophila heart development. Dev. Biol. 177:104–116.

Paulis L, Simko F. 2007. Blood pressure modulation and cardiovascular protection by melatonin: potential mechanisms behind. Physiol. Res. 56:671–84.

Periasamy M, Huke S. 2001. SERCA pump level is a critical determinant of Ca(2+)homeostasis and cardiac contractility. J. Mol. Cell. Cardiol. 33:1053–63.

Pieri C, Marra M, Moroni F, Recchioni R, Marcheselli F. 1994. Melatonin: a peroxyl radical scavenger more effective than vitamin E. Life Sci. 55:PL271–6.

Pieri C, Marra M, Moroni F, Recchioni R, Marcheselli F. 1994. Melatonin: a Peroxyl Radical Scavenger More Effective than Vitamin E. Science 55:271–276.

Pilling MJ, Seakins PW. 1996. Reaction Kinetics. Oxford University Press.

Pittendrigh CS. 1954. ON TEMPERATURE INDEPENDENCE IN THE CLOCK SYSTEM CONTROLLING EMERGENCE TIME IN DROSOPHILA. Proc. Natl. Acad. Sci. U. S. A. 40:1018–29.

Poeggeler B. 2005. Melatonin , Aging , and Age-Related Diseases: perspectives for prevention, intervention, and therapy. Endocrine 27:201–212.

Qian L, Liu J, Bodmer R. 2007. Cardiovascular Development. Adv. Dev. Biol. 18:1–29.

Ranade K, Hinds D, Hsiung CA, Chuang L-M, Chang M-S, Chen Y-T, Pesich R, Hebert J, Chen Y-DI, Dzau V, et al. 2003. A genome scan for hypertension susceptibility loci in populations of Chinese and Japanese origins. Am. J. Hypertens. 16:158–62.

Ray VM, Dowse HB. 2005. Mutations in and deletions of the Ca2+ channel-encoding gene cacophony, which affect courtship song in Drosophila, have novel effects on heartbeating. J. Neurogenet. 19:39–56.

Reiter LT, Potocki L, Chien S, Gribskov M, Bier E. 2001. A Systematic Analysis of Human Disease-Associated Gene Sequences In. Genome Res.:1114–1125.

Reiter RJ. 1991. Melatonin: the chemical expression of darkness. Mol. Cell. Endocrinol. 79:C153–8.

Reiter RJ. 1996. Functional aspects of the pineal hormone melatonin in combating cell and tissue damage induced by free radicals. Eur. J. Endocrinol. 134:412–20.

Reiter RJ, Tan D-X. 2003. Melatonin: a novel protective agent against oxidative injury of the ischemic/reperfused heart. Cardiovasc. Res. 58:10–9.

Reiter RJ, Tan D-X, Mayo JC, Sainz RM, Lopez-Burillo S. 2002. Melatonin, longevity and health in the aged: an assessment. Free Radic. Res. 36:1323–9.

Reiter RJ, Tan DX, Qi WB. 1998. Suppression of oxygen toxicity by melatonin. Zhongguo Yao Li Xue Bao 19:575–581.

Reiter RJ, Tan DX, Manchester LC, Pilar Terron M, Flores LJ KS. 2007. Medical implications of melatonin: receptor-mediated and receptor-independent actions. Adv. Med. Sci. 52:11–28.

Reppert SM. 1997. Melatonin Receptors: Molecular Biology of a New Family of G Protein-Coupled Receptors. J. Biol. Rhythms 12:528–531.

Reppert SM, Godson C, Mahle CD, Weaver DR, Slaugenhaupt SA, Gusella JF. 1995. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. Proc. Natl. Acad. Sci. U. S. A. 92:8734–8.

Reppert SM, Perlow MJ, Tamarkin L, Klein DC. 1979. A diurnal melatonin rhythm in primate cerebrospinal fluid. Endocrinology 104:295–301.

Reppert SM, Weaver DR, Godson C. 1996. Melatonin receptors step into the light: cloning and classification of subtypes. Trends Pharmacol. Sci. 17:100–2.

Rezzani R, Rodella LF, Bonomini F, Tengattini S, Bianchi R, Reiter RJ. 2006. Beneficial effects of melatonin in protecting against cyclosporine A-induced cardiotoxicity are receptor mediated. J. Pineal Res. 41:288–95.

Rizki TM. 1978. The circulatory system and associated cells and tissues. In: Ashburner M; WT, editor. The Genetics and Biology of Drosophila. London: Academic Press. p. 1839–1845.

Robbins J, Aggarwal R, Nichols R, Gibson G. 1999. Genetic variation affecting heart rate in Drosophila melanogaster. Genet. Res. 74:121–8.

Roberts WC. 1990. Sudden cardiac death: a diversity of causes with focus on atherosclerotic coronary artery disease. Am. J. Cardiol. 65:13B–19B.

Roca AL, Godson C, Weaver DR, Reppert SM. 1996. Structure, characterization, and expression of the gene encoding the mouse Mel1a melatonin receptor. Endocrinology 137:3469–77.

Roote J, Russell S. 2012. Toward a complete Drosophila deficiency kit. Genome Biol. 13:149.

Roth JA, Kim BG, Lin WL, Cho MI. 1999. Melatonin promotes osteoblast differentiation and bone formation. J. Biol. Chem. 274:22041–7.

Sahna E, Deniz E, Aksulu HE. 2006. Myocardial ischemia-reperfusion injury and melatonin-Review. Anadolu Kardiyol. Derg. 6:163–8.

Sahna E, Parlakpinar H, Turkoz Y, Acet a. 2005. Protective effects of melatonin on myocardial ischemia/reperfusion induced infarct size and oxidative changes. Physiol. Res. 54:491–5.

Sanguinetri MC, Currant ME, Spectort PS. 1996. Spectrum of HERG K + -channel dysfunction in cardiac arrhythmia inherited. Physiology 93:2208–2212.

Sanyal S, Consoulas C, Kuromi H, Basole a., Mukai L, Kidokoro Y, Krishnan KS, Ramaswami M. 2005. Analysis of conditional paralytic mutants in Drosophila sarco-endoplasmic reticulum calcium ATPase reveals novel mechanisms for regulating membrane excitability. Genetics 169:737–750.

Sanyal S, Jennings T, Dowse H, Ramaswami M. 2006. Conditional mutations in SERCA, the Sarco-endoplasmic reticulum Ca2+-ATPase, alter heart rate and rhythmicity in Drosophila. J. Comp. Physiol. B. 176:253–63.

Saudou F, Boschert U, Amlaiky N, Plassat J, Hen R, Biologique IDC, Medecine F De, Tcc AAT, Ttt TTT, Gtt TTG, et al. 1992. A family of Drosophila serotonin receptors with distinct intracellular signalling properties and expression patterns of receptors that interact with G proteins and include the. EMBO J. 1:7–17.

Schaefer M, Hardeland R. 2009. The melatonin metabolite N-acetyl-5-methoxykynuramine is a potent singlet oxygen scavenger. J. Pineal Res. 46:49–52.

Schaeffer HJ, Sirotkin A V. 1997. Melatonin and serotonin regulate the release of insulin-like growth factor-I, oxytocin and progesterone by cultured human granulosa cells. Exp. Clin. Endocrinol. Diabetes 105:109–12.

Schefler WC. 1969. Statistics for the Biological Sciences. 1st ed. Addison-Wesley Publishing Company.

Von Schilcher F. 1976. The behavior of cacophony, a courtship song mutant in Drosophila melanogaster. Behav. Biol. 17:187–96.

Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, Maron BJ, Seidman CE, Seidman JG. 1998. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281:108–111.

Schultheiss TM, Burch JBE, Lassar AB. 1997. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev. 11:451–462.

Sewerynek E, Reiter RJ, Melchiorri D, Ortiz GG, Lewinski A. 1996. Oxidative damage in the liver induced by ischemia-reperfusion: protection by melatonin. Hepatogastroenterology. 43:898–905.

Shiu SYW, Pang B, Tam CW, Yao K-M. 2010. Signal transduction of receptor-mediated antiproliferative action of melatonin on human prostate epithelial cells involves dual activation of Gα(s) and Gα(q) proteins. J. Pineal Res. 49:301–11.

Silva B, Goles NI, Varas R, Campusano JM. 2014. Serotonin receptors expressed in Drosophila mushroom bodies differentially modulate larval locomotion. PLoS One 9: e89641.

Silva SO, Rodrigues MR, Ximenes VF, Bueno-da-Silva AEB, Amarante-Mendes GP, Campa A. 2004. Neutrophils as a specific target for melatonin and kynuramines: effects on cytokine release. J. Neuroimmunol. 156:146–52.

Silverman WR, Roux B, Papazian DM. 2003. Structural basis of two-stage voltage-dependent activation in K+ channels. Proc. Natl. Acad. Sci. U. S. A. 100:2935–40.

Slominski A, Fischer TW, Zmijewski MA, Wortsman J, Semak I, Zbytek B, Slominski RM, Tobin DJ. 2005. On the role of melatonin in skin physiology and pathology. Endocrine 27:137–48.

Slominski A, Tobin DJ, Zmijewski M a., Wortsman J, Paus R. 2008. Melatonin in the skin: synthesis, metabolism and functions. Trends Endocrinol. Metab. 19:17–24.

Slominski RM, Reiter RJ, Schlabritz-Loutsevitch N, Ostrom RS, Slominski AT. 2012. Melatonin membrane receptors in peripheral tissues: Distribution and functions. Mol. Cell. Endocrinol. 351:152–166.

Smith L a, Wang X, Peixoto a a, Neumann EK, Hall LM, Hall JC. 1996. A Drosophila calcium channel alpha1 subunit gene maps to a genetic locus associated with behavioral and visual defects. J. Neurosci. 16:7868–7879.

Smith L a., Peixoto A a., Kramer EM, Villella A, Hall JC. 1998. Courtship and visual defects of cacophony mutants reveal functional complexity of a calcium-channel alpha1 subunit in Drosophila. Genetics 149:1407–1426.

Sokolowski MB. 1980. Foraging strategies of Drosophila melanogaster: a chromosomal analysis. Behav. Genet. 10:291–302.

Sweeny B. Hasting J. 1960. Effects of temperature upon diurnal rhythms. Cold Spring Harb. Symp. Quant. Biol. 25:87–104.

Taghli-Lamallem O1, Akasaka T, Hogg G, Nudel U, Yaffe D, Chamberlain JS, Ocorr K BR. 2008. Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. Aging Cell 7:237–249.

Tamura H, Nakamura Y, Korkmaz A, Manchester LC, Tan D-X, Sugino N, Reiter RJ. 2009. Melatonin and the ovary: physiological and pathophysiological implications. Fertil. Steril. 92:328–43.

Tamura H, Nakamura Y, Terron MP, Flores LJ, Manchester LC, Tan D-X, Sugino N, Reiter RJ. 2008. Melatonin and pregnancy in the human. Reprod. Toxicol. 25:291–303.

Tan D, Reiter RJ, Manchester LC, Yan M, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M, Hardeland R. 2002. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. Curr. Top. Med. Chem. 2:181–97.

Tan D-X, Manchester LC, Hardeland R, Lopez-Burillo S, Mayo JC, Sainz RM, Reiter RJ. 2003. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. J. Pineal Res. 34:75–8.

Tan DX, Manchester LC, Reiter RJ, Qi W, Kim SJ, El-Sokkary GH. 1998. Ischemia/reperfusion-induced arrhythmias in the isolated rat heart: prevention by melatonin. J. Pineal Res. 25:184–91.

Tang X, Wang Y, Li D, Luo J, Liu M. 2012. Orphan G protein-coupled receptors (GPCRs): biological functions and potential drug targets. Acta Pharmacol. Sin. 33:363–371.

Tengattini S, Reiter RJ, Tan D-X, Terron MP, Rodella LF, Rezzani R. 2008. Cardiovascular diseases: protective effects of melatonin. J. Pineal Res. 44:16–25.

Terán R, Bonilla E, Medina-Leendertz S, Mora M, Villalobos V, Paz M, Arcaya JL. 2012. The life span of Drosophila melanogaster is affected by melatonin and thioctic acid. Invest. Clin. 53:250–61.

Thompson SH. 1977. Three pharmacologically distinct potassium channels in molluscan neurones. J. Physiol. 265:465–488.

Trautwein W, Hescheler J. 1990. Regulation of cardiac L-type calcium current by phosphorylation and G proteins. Annu. Rev. Physiol. 52:257–74.

Turjanski AG, Estrin DA, Rosenstein RE, McCormick JE, Martin SR, Pastore A, Biekofsky RR, Martorana V. 2004. NMR and molecular dynamics studies of the interaction of melatonin with calmodulin. Protein Sci. 13:2925–38.

Ulrych T, Bishop T. 1975. Maximum entropy spectral analysis and autoregressive decomposition. Rev. Geophys. Sp. Phys.

Valles AM, White K. 1988. Serotonin-containing neurons inDrosophila melanogaster: Development and distribution. J. Comp. Neurol. 268:414–428.

Vaudry H, Seong JY, editors. 2014. Neuropeptide GPCRs in neuroendocrinology. Frontiers E-books.

Vaughan GM, Mason AD, Reiter RJ. 1986. Serum melatonin after a single aqueous subcutaneous injection in Syrian hamsters. Neuroendocrinology 42:124–7.

Vazan R, Pancza D, Béder I, Styk J. 2005. Ischemia-reperfusion injury--antiarrhythmic effect of melatonin associated with reduced recovering of contractility. Gen. Physiol. Biophys. 24:355–9.

Vazan R, Styk J, Béder I, Pancza D. 2003. Effect of melatonin on the isolated heart in the standard perfusion conditions and in the conditions of calcium paradox. Gen. Physiol. Biophys. 22:41–50.

Venkatesh T V, Park M, Ocorr K, Nemaceck J, Golden K, Wemple M, Bodmer R. 2000. Cardiac enhancer activity of the homeobox gene tinman depends on CREB consensus binding sites in Drosophila. Genesis 26:55–66.

De Vreede-Swagemakers J, Gorgels a P, Dubois-Arbouw WI, Van Ree JW, Al E. 1997. Out-of-hospital cardiac arrest in the 1990s: a population-based study in the Maatrich area on incidence, characteristics and survival. J. Am. Coll. Cardiol. 30:1500–1505.

Waldhauser F, Waldhauser M, Lieberman HR, Deng MH, Lynch HJ, Wurtman RJ. 1984. Bioavailability of oral melatonin in humans. Neuroendocrinology 39:307–13.

Walter M, Kiger J. 1984. The Dunce of Drosophila : Roles of Ca2 + and Specific Phosphodiesterase Activity. J. Neurosci. 4:495–501.

Warmke J, Drysdale R, Ganetzky B. 1991. A distinct potassium channel polypeptide encoded by the Drosophila eag locus. Science 252:1560–1562.

Warmke JW, Ganetzky B. 1994. A family of potassium channel genes related to eag in Drosophila and mammals. Proc. Natl. Acad. Sci. U. S. A. 91:3438–42.

Wasserthal LT. 2007. Drosophila flies combine periodic heartbeat reversal with a circulation in the anterior body mediated by a newly discovered anterior pair of ostial valves and “venous” channels. J. Exp. Biol. 210:3707–19.

Wessells RJ, Bodmer R. 2004. Screening assays for heart function mutants in Drosophila. Biotechniques 37:58–60, 62, 64 passim.

White LA, Ringo JM, Dowse HB. 1992. Effects of deuterium oxide and temperature on heart rate in Drosophila melanogaster. J. Comp. Physiol. B. 162:278–83.

Wilson SH, Olden K. 2004. The Environmental Genome Project: phase I and beyond. Mol. Interv. 4:147–56.

Winfree AT. 1980. The Geometry of Biological Time. Springer Science & Business Media.

Witt-Enderby PA, Bennett J, Jarzynka MJ, Firestine S, Melan MA. 2003. Melatonin receptors and their regulation: biochemical and structural mechanisms. Life Sci. 72:2183–98.

Wolk R. 2000. Arrhythmogenic mechanisms in left ventricular hypertrophy. Europace 2:216–223.

Wu CF, Ganetzky B, Haugland FN, Liu AX. 1983. Potassium currents in Drosophila: different components affected by mutations of two genes. Science 220:1076–8.

Wu CF, Ganetzky B, Jan LY, Jan YN, Benzer S. 1978. A Drosophila mutant with a temperature-sensitive block in nerve conduction. Proc. Natl. Acad. Sci. U. S. A. 75:4047–4051.

Yu HS, Hernandez V, Haywood M, Wong CG. 1993. Melatonin inhibits the proliferation of retinal pigment epithelial (RPE) cells in vitro. In Vitro Cell. Dev. Biol. Anim. 29A:415–418.

Yu L, Sun Y, Cheng L, Jin Z, Yang Y, Zhai M, Pei H, Wang X, Zhang H, Meng Q, et al. 2014. Melatonin receptor-mediated protection against myocardial ischemia/reperfusion injury: Role of SIRT1. J. Pineal Res.:228–238.

Zaffran S, Frasch M. 2002. Early signals in cardiac development. Circ. Res. 91:457–469.

Zeriouh M, Sabashnikov A, Choi Y-H, Fatullayev J, Reuter H, Popov A-F, Langebartels G, Kimmig L, Rahmanian PB, Wittwer T, et al. 2014. A novel treatment strategy of new onset atrial fibrillation after cardiac surgery: an observational prospective study. J. Cardiothorac. Surg. 9:83.

Zhang, P-Y, Xu X, Li X. 2014. Cardiovascular diseases : oxidative damage and antioxidant protection. Eur. Rev. Med. Pharmacol. Sci. 18:3091–3096.

Zheng Z, Croft JB, Giles WH, Mensah G a. 2001. Clinical Investigation and Reports Sudden Cardiac Death in the United States , 1989 to 1998. Circulation.:2158–2163.

APPENDIX

THE RELATIONSHIP OF HEART FUNCTION TO TEMPERATURE IN *Drosophila melanogaster* AND ITS HERITABILITY

Abstract

We measured heart rate and rhythmicity (regularity) of heartbeat in *Drosophila melanogaster* at five different temperatures (20°, 25°, 30°, 35°, and 37° C.), for a Florida population and estimated the narrow-sense heritability of both traits. Heritability of heart rate ranged from 0.16 to 0.24, but was statistically significant only at 20° (h^2 = 0.24) and at 30° (h^2 = 0.23). The heritability of heartbeat rhythmicity ranged from –0.034 to 0.11, and was not significant at any temperature. Heart rate increased linearly with increasing temperature; the temperature-dependence of heart rate was itself heritable (h^2 = 0.29). Heart rhythmicity varied curvilineanearly and was well-represented by a parabolic function, peaking at about 27° suggesting a temperature optimum. The regularity of the heartbeat did not covary with heart rate except at 20°. Neither heart rate nor regularity covaried with the change in heart rate with temperature. For this population of *D. melanogaster*, we conclude that there is substantial genetic variation for the mechanism whereby the cardiac pacemaker reacts to changes in temperature, but not for the cardiac pacemaker's rhythmicity. The small values of h^2 for temperature-specific heart rate and heartbeat rhythmicity suggest that these traits are adaptive.

Introduction

The *Drosophila* heart, or dorsal vessel, is located medially and dorsally in the hemocoel and transports hemolymph through the larval and adult body cavity (Miller, 1950; Rizki, 1978; Curtis et al., 1999). The anterior third of the dorsal vessel forms the aorta. The posterior segment, or heart proper, contains three pairs of openings called ostia to admit hemolymph

(Rizki, 1978; Curtis et al., 1999; Wasserthal, 2007). Heart pacemaking in *Drosophila* is myogenic (Dowse et al., 1995; Gu & Singh, 1995; Bodmer et al., 2005) , and originates from a caudal pacemaker (Rizki, 1978), an assembly of ion channels, including those passing Ca^{2+} and K^{+}, interacting in an oscillatory manner (Dowse et al., 1995; Johnson et al., 1998; Bodmer et al., 2005). The beat consists of a contraction phase (systole) and a relaxation phase (diastole) (Rizki, 1978; Curtis et al., 1999; Wasserthal, 2007). Heartbeat may reverse requiring nervous control of a second anterior pacemaker (Dulcis & Levine, 2003; Wasserthal, 2007).

Increasing temperature raises heart rate in the fly (Rizki, 1978; White et al., 1992; Dowse et al., 1995; Ashton et al., 2001; Ray & Dowse, 2005). The precise mechanism of this change is not understood. It is of considerable interest to know how the heart reacts to temperature changes and what factors influence any such reactions. That there may be a strong genetic component is suggested by tests of flies bearing the mutations *no action potential*^{TS} (*nap*^{TS}) and *slowpoke* (*slo*), phenotypes of which include attenuation or elimination of the response of the heart to temperature (Dowse et al., 1995; Johnson et al., 1998). Using a deficiency screen QTL approach,

Ashton et al., (2001) identified nine areas of the genome that either increased or decreased P1 heart rate.

There appears to be one report of narrow-sense heritability of heart rate for *D. melanogaster* (Robbins et al., 1999). They measured heart rate in inbred lines from many geographic locations, and therefore this work gives an estimate that includes variation between as well as within a population. Temperature in this work was likely ambient. Because both the rate and the regularity of the heartbeat in *Drosophila* are temperature-sensitive, we monitored heart function in a single population at five temperatures across the wide range of 20° to 37°, and determined narrow-sense heritabilities of these traits within and across temperatures.

Materials and Methods

Fly culture and the experimental population

A laboratory population was established by mass-mating the progeny of 35 wild, inseminated *D. melanogaster* females collected in Tampa, Florida. Thereafter, the population was maintained in twelve 250-ml culture bottles containing malt-molasses-yeast-cornmeal-agar medium (0.5% propionic acid added to the medium to control mold growth). A Tomac® tissue was added to each culture bottle to promote pupation out of the culture medium. Flies were cultured at 25° C in a 12:12 Light:Dark cycle in a humidified incubator. Approximately 200 adults were transferred to fresh medium in each bottle when subculturing.

As necessary, adults were sexed under CO_2 anesthesia. Adults were transferred either by tapping groups from one culture vessel to another or by gentle aspiration of individuals.

Individual crosses were carried out in 35-ml vials, using the same culture conditions as described above, but without a tissue added to the medium.

Measurement of heartbeat

All test subjects were collected at the third-instar larval stage just as they were entering the early pupal (P1) stage. At this point, they are translucent, making any heart movements transmitted to the surrounding organs easily visible. Each pupa was placed on a glass slide in a drop of distilled water and set in the light beam of an Olympus binocular microscope. The light source was powered by a DC supply to eliminate 60 Hz variation. The water served to increase the brilliance of the illumination passing through the pupa, and also helped in heat transfer from the slide as the temperature was varied. The pupa was adjusted so that one clear portion of the abdomen filled the field of view with just the surrounding fat bodies visible. The movement of these organs results in a variation in illumination that can be detected by a phototransistor. This signal is amplified and recorded in a microcomputer through a DAS8 AD converter. Temperature of the slide was maintained with a Sensortek TS-4 unit, however it was not possible to record temperatures within the animals. Recording was done at five different temperatures (20°, 25, ° 30°, 35°, 37°). For each pupa, 30 seconds of recording was performed at each temperature. In between measurements 1.5 minutes of acclimation time was allowed.

Analysis of heartbeat

Data from individual flies taken at each temperature were analyzed by autocorrelation (Chatfield, 1980) and Maximum Entropy Spectral Analysis (MESA) (Ulrych & Bishop, 1975; Dowse & Ringo, 1989; Levine et al., 2002; Dowse, 2009). Estimates of heart rate were based on the spectral analysis, autocorrelation, and inspection of the raw data plots.

Rhythmicity of heartbeat results in recurring positive and negative peaks in the autocorrelogram, and the decay envelope reflects regularity in the signal (Chatfield, 1980). The value of the correlation coefficient at the second peak is the rhythmicity index. The value at lag zero is always one (Dowse et al., 1995; Levine et al., 2002; Dowse, 2009). Empirically, we observe that as rhythmicity index decreases, there are more skipped beats, irregular intervals between beats, periods of heartbeat cessation, and rate variations with time (Johnson et al., 2000; Levine et al., 2002; Dowse, 2009).

The temperature-dependences of heart rate and rhythmicity index were estimated for each individual from least-squares regression (temperature on heart rate or rhythmicity index); the resulting slopes were our measures of each phenotype. We also did this regression for all individuals pooled together across families to get values for the entire population.

We tested whether there was a relationship between frequency of heartbeat and the rhythmicity index at each of the test temperatures. We also queried whether either frequency or rhythmicity of heartbeat were related to the slope of the regression line of temperature on frequency at the temperature at which the hearts were most rhythmic,

25°. We computed correlation coefficients (r) individually in EXCEL® for each of the above interactions across all test animals and asked if $r > 0$ using a two-tailed t-test (Schefler, 1969) ($N = 317$, $df = 315$, $t = 1.968$).

Crosses and heritability

The heartbeat of white pupae randomly selected from the general stock population was measured without knowing sex. Each pupa was reared in isolation in a 35-ml culture vial. Families were established from these individuals. When these flies eclosed, individual virgin pairs were isolated as parents of families to be tested further. Each such family was reared in a 35-ml culture vial. Heartbeat was monitored in four randomly selected offspring of each family when those offspring reached the white pupa stage. Fifty families were so studied.

At each temperature, narrow-sense heritability (h^2) was calculated for heart rate and rhythmicity by least squares regression of the mean parental (mid-parent) values on the means of the four offspring within a family. The slope of this regression line is the estimate of h^2 (Falconer, 1960). Further, we estimated the heritability of the *response* to temperature by calculating mid-parent and mean offspring values for each family from the slopes derived from the individual regressions of temperature on rate and rhythmicity for each individual. We then regressed mean mid-parent values on mean offspring values. In all tests of significance, $\alpha = 0.05$.

Results

Effects of temperature on heart rate frequency and rhythmicity

Across the range tested for all subjects pooled, heart rate increases monotonically with temperature and is well-represented by a straight line (slope = 0.09; intercept = 0.23; $r^2 = 0.67$) (Fig. A.1a). Given the strength of this linear relationship, we tested the fit of the data according to the method of Arrhenius wherein $\ln(\text{rate}) = \ln A - E_a/RT$, where A is the "pre-exponential", or intercept of the plot, E_a is the activation energy of the system in $\text{Kcal}\cdot\text{mol}^{-1}$, T is the temperature in °K, and R is the gas constant (1.985877 $\text{cal}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$) (Pilling & Seakins, 1996). Arrhenius plots have been shown to be of use in other than studies of simple chemical reaction, having been applied to a wide range of processes ranging from cricket chirping to the rate of heartbeat in the terrapin (Laidler, 1972). The regression of 1/T on ln(rate) is a good fit ($r^2 = 0.64$). This yields an activation energy of ~6000 $\text{Kcal}\cdot\text{mol}^{-1}$. The Q_{10} for this relationship across the common physiological range of 20° to 30° is 1.44. (Q_{10} = heart rate at 30°/heart rate at 20°.)

We did a similar analysis for all animals across temperature for the rhythmicity index. This statistic has a nonlinear relationship to temperature, highest in the mid-range between 25º and 30º C, falling off at the extreme temperatures. We did linear regression, and find there is a weakly significant linear relationship ($r^2 = 0.03$), but clearly this is not a good description of the data. We next fit a parabola to the data which offers an exceptionally good representation ($r^2 = 0.88$). The estimated peak occurs at ~27º (Fig A.1b).

Correlations among heart rate, rhythmicity index and response to changing temperature

There was a significant correlation between heart frequency and rhythmicity index only at 20º ($r = 0.252$, $t = 4.62$) (See materials and Methods above for details of test). Relationships at all other temperatures were not significant (25º: $r = 0.056$, $t = 0.99$; 30º: $r = 0.024$, $t = 0.4366$: 35º, $r = -0.029$: $t = -0.515$; 37º: $r = -0.077$, $t = -1.37$). At 25º, at which temperature the rhythmicity index was near maximum, frequency of heartbeat is not correlated with the slope of the regression line ($r = 0.135$, $t = 0.135$), nor is rhythmicity index ($r = -0.08$, $t = -0.08$) (see Materials and Methods above for details of test).

Heritability of heart rate, rhythmicity, and temperature-dependence of heart rate

Heritability of heart rate calculated at the five test temperatures ranged from 0.16 to 0.24, and the heritability of the heartbeat rhythmicity index ranged from –0.034 to 0.11; Table A.2. Heritability of heart rate was statistically significant (based on significance of the regression analysis itself) at 20° ($h^2 = 0.24$) and at 30° ($h^2 = 0.23$); Table A.1. The estimates of heritability of rhythmicity index was not significant at any temperature (Table A.2).

In the test of heritability of *response* to change in temperature, in which we regressed mean filial slopes for each family on mid parent values, we found a clear result. A significant portion of the variance among slopes was genetic: $h^2 = 0.29$, $r^2 = 0.08$, $t = 2.398$, $df = 50$, $P = 0.02$ (Figure A.3)

Table A.1. Heritabilities for heart rate.

Temperature	Heritability (h^2)	r^2	t value	Degrees of freedom (df)	P value
20ºC	**0.239***	**0.18**	**3.414**	**50**	**0.001**
25ºC	**0.170**	**0.03**	**1.759**	**50**	**0.085**
30ºC	**0.230***	**0.08**	**2.308**	**50**	**0.025**
35ºC	**0.165**	**0.03**	**1.586**	**50**	**0.119**
37ºC	**0.178**	**0.05**	**1.875**	**50**	**0.067**

Significant

Table A.2 Heritabilities for rhythmicity indices.

Temperature	Heritability (h^2)	r^2	t value	Degrees of freedom (df)	P value
20ºC	**-0.034**	**-0.02**	**-0.311**	**50**	**0.757**
25ºC	**0.019**	**-0.02**	**0.231**	**50**	**0.818**
30ºC	**0.110**	**-0.01**	**0.854**	**50**	**0.397**
35ºC	**0.057**	**-0.01**	**0.599**	**50**	**0.551**
37ºC	**0.083**	**0.00**	**0.884**	**50**	**0.381**

FIGURE LEGENDS

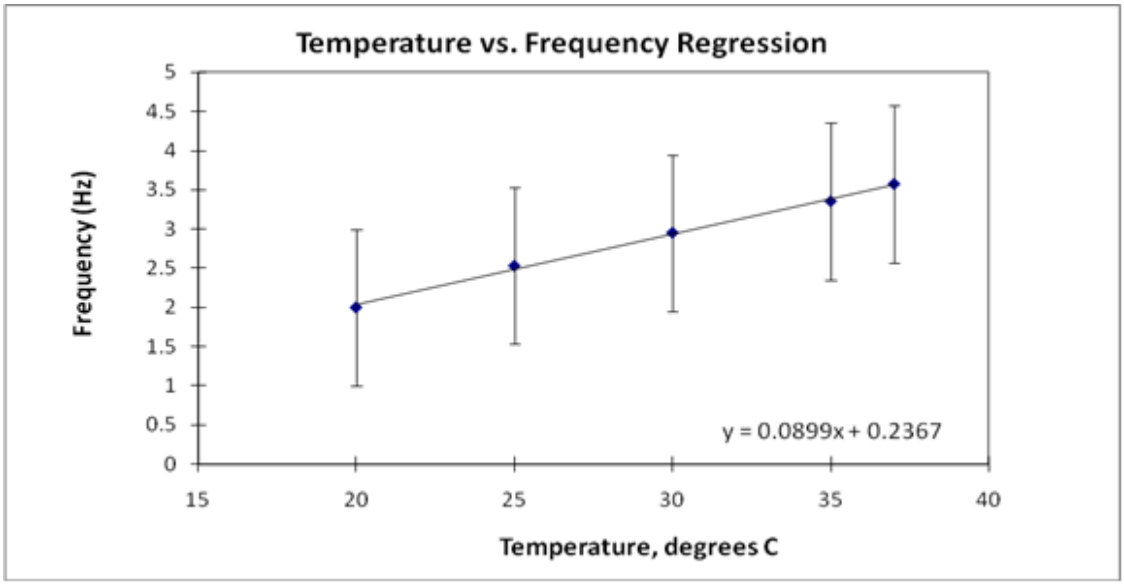
Figure A.1. Response of heart rate and rhythmicity to change in temperature. (a) Regression of temperature on frequency for all animals (N=300, $r^2 = 0.67$). Parameters of regression equation shown bottom right. Means indicated by diamond symbols, ± S.D. Regression equation: $y = 0.09x + 0.024$. b) Results of nonlinear regression, fitting a parabola to the rhythmicity index as a function of temperature for all flies (N = 300, $r^2 = 0.88$). Means indicated by diamond symbols, ± S.D. Regression equation: $y = -0.0018(x - 26.94)^2 + 0.7$.

Figure A.2. Regression analyses of mid parent heart rate on mean offspring heart rate. This analysis yields narrow-sense heritability of the trait, h^2. (a) 20ºC (Equation: $y = 0.24x + 1.48$). (b) 30ºC (Equation: $y = 0.23x + 2.2$). Regressions done at 25ºC, 35ºC and 37ºC were not significant (data not shown).

Fig A.3. Regression of mid-parent on filial mean slopes derived from individual regressions of temperature on heart rate for all flies. The narrow-sense heritability of the response to temperature was significant. Parameters of the regression equation are shown bottom right. $h^2 = 0.29$. Regression equation: $y = 0.29x + 0.07$.

Figure A.1 Response of heart rate and rhythmicity to change in temperature

a) Regression of temperature on frequency for all animals



b) Results of nonlinear regression, fitting a parabola to the rhythmicity index as a function of temperature for all flies

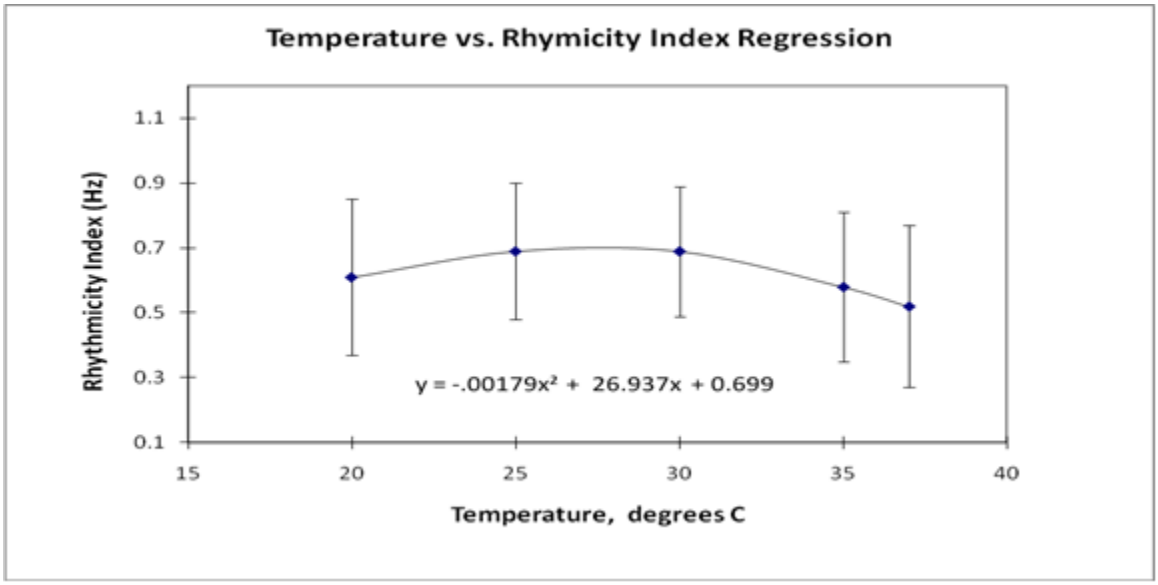
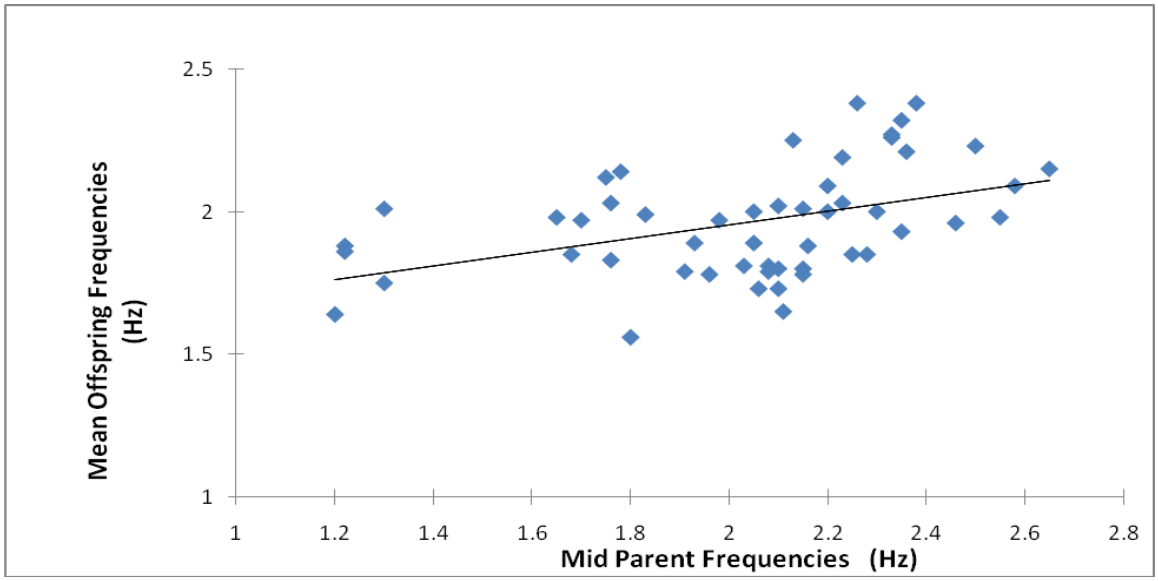


Figure A.2 Regression analyses of mid parent heart rate on mean offspring heart rate

a) At 20˚C



b) At 30˚C

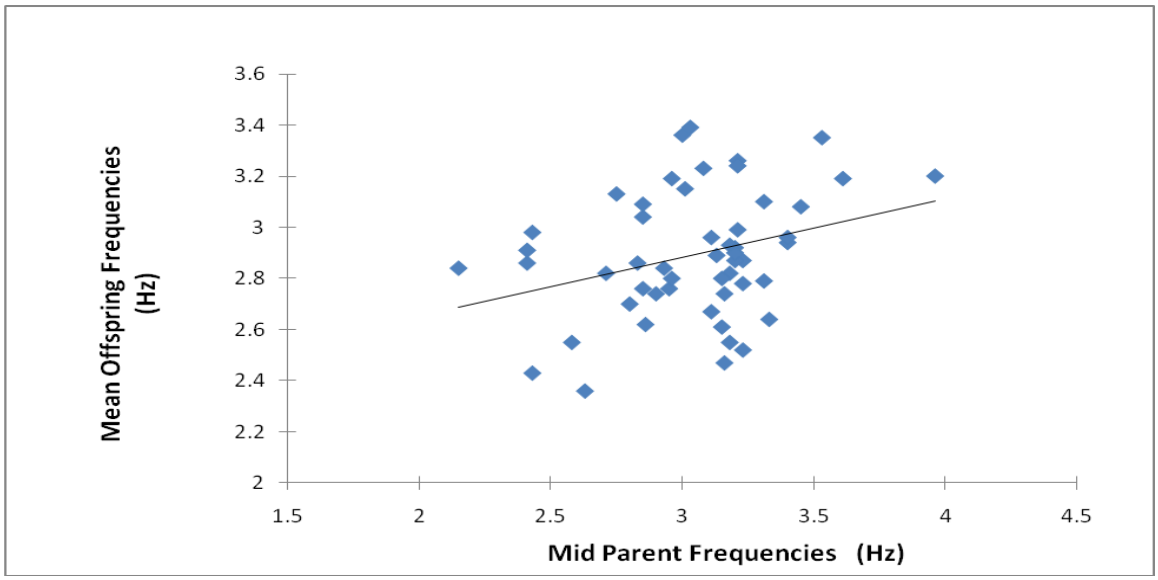
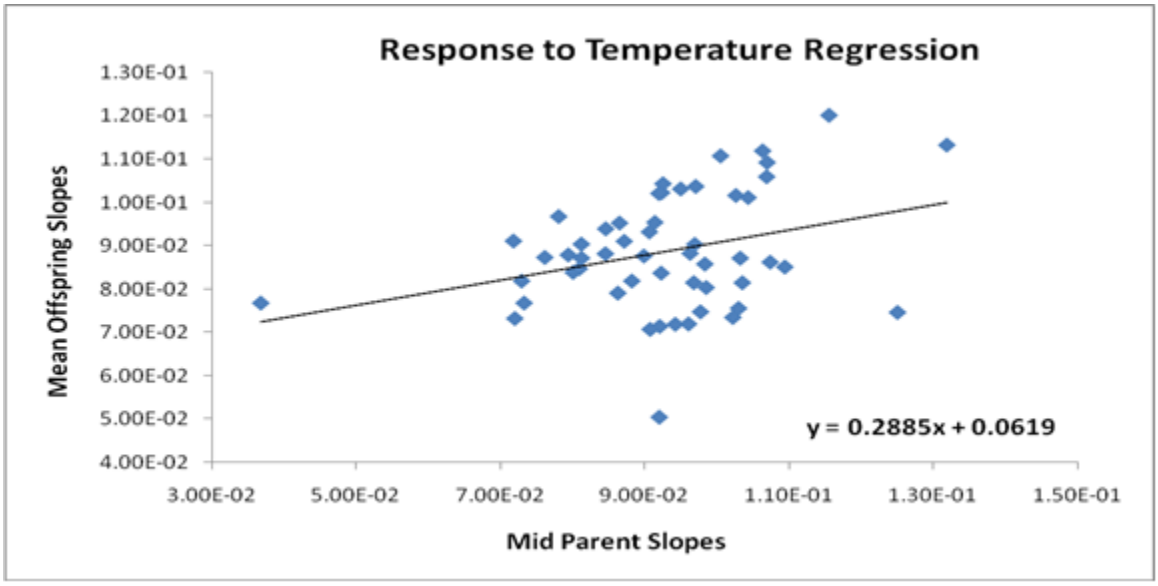


Figure A.3 Regression of mid-parent on filial mean slopes derived from individual regressions of temperature on heart rate for all flies



Discussion

In *D. melanogaster*, heart rate increased with increasing temperature, a result consistent with previous fly studies (Rizki, 1978; Dowse et al., 1995; Ashton et al., 2001; Ray & Dowse, 2005) and studies in other insects (Crozier & Federighi, 1925; Fries, 1926). This is to be expected for poikilotherms, in which the rate of most physiological processes vary positively with temperature (Sweeny & Hasting, 1960; Winfree, 1980). In contrast, heart rhythmicity varied with temperature in a non-linear way: it was lower at extreme temperatures than at intermediate temperatures, the latter being close to the optimum for the survival and reproduction of this species (Ashburner, 1989). It is provocative that two important characteristics of heart function, rate and regularity, have very different relationships to changes in temperature as shown in Fig. A.2.

Narrow-sense heritability of heart rate in the Florida population ranged between 0.16 and 0.24 and was temperature-dependent. The two largest heritabilities for this trait were for heartbeat measured at 20° ($h^2 = 0.24$) and 30° C ($h^2 = 0.23$). For the other temperatures, heritability was not statistically significant, though that may reflect the low statistical power of the experimental design. In contrast, narrow-sense heritability of the rhythmicity of heartbeat was much lower (range: X to Y) and it was not significant at any temperature. A perhaps surprising and provocative finding was the discovery that the response of heart rate to temperature was itself heritable ($h^2 = 0.29$).

Robbins et al. (1999), reported a heritability of heart rate of 0.30. In that study the estimate was based on variation among inbred lines, some derived from a Michigan population and others based on strains collected at other, widely separated geographic

locations. Consequently, the earlier study gave an estimate of heritability which, if not species-wide, represents inter-population variability in addition to variation within a population. Also, if there is substantial epistatic variance for heart rate in this species, a heritability estimate based on variance among inbred strains would have included this variance as well as additive variance. Regression of offspring mean on parental mean contains a fraction (*viz*. ¼) of additive X additive interaction variance. It remains to be determined how much genetic variance is due to epistasis. Another difference between this report and that of Robbins et al. (1999), relates to temperature. In both studies, flies were cultured at 25° C, which is thought to be close to the optimal rearing temperature for this species (Ashburner, 1989). However, in the present study, heart rate was measured at five different temperatures, and each temperature was precisely maintained, while Robbins et al. (1999), presumably recorded at ambient. Despite the methodological differences between the two studies, the heritabilities are reasonably similar.

Robbins et al. (1999), pointed out that the ability of a heart to beat regularly at a certain rate is a remarkable phenotype, and one that reflects genetic regulation. The heritabilities reported here bear out this statement, especially the very low values measured for heartbeat rhythmicity. Even the largest heritability we measured, for temperature-dependence of heart rate, the size of h^2 was modest (0.29). Natural selection within a population reduces the amount of additive genetic variation for the trait under selection. While a paucity of additive genetic variation for a trait does not imply that the trait has been subjected to natural selection, the converse is true: a large

amount of additive genetic variation for a trait implies that it has not been under strong directional selection.

Ultimately, changes in heart rate must, perforce, be mediated through modulation of transmembrane potentials in the pacemaker (Bodmer et al., 2005). This may be a direct effect of temperature. In insects, the heart itself is heated or cooled according to ambient temperature and metabolic activity. It may be that varying temperatures alter state variables of the oscillating ensemble of ion channels comprising the pacemaker. The activation energy estimated from the Arrhenius plot is of interest in this regard, as it suggests that covalent bonds are not being broken (Laidler, 1972). The Q_{10} is also fairly low.

Most metabolic reactions experience a rate increase when exposed to increasing temperatures (Sweeny & Hasting, 1960). However, a surprising range of metabolic processes are seen to be unresponsive to temperature change, i.e. must be compensated in some way (Bullock, 1955; Winfree, 1980). For example, circadian clock oscillators in *Drosophila*, are seen to have a nearly flat temperature response (Pittendrigh, 1954). If this be the case for the heart, the pacemaker might, at least in part, be *indirectly* affected by temperature, perhaps through neurotransmitter control. Previous work in other insects supports this interpretation. In order for an insect to respond to variations in both internal and external conditions, its heart rate must be variable and under control. In the moth, circulation of haemolymph may be thermoregulatory, and increasing heart rate in response to raised temperatures is a result of involvement of the nervous system in sensing heat and controlling rate (Miller, 1985). Heinrich (1970a,b), has demonstrated such thermoregulation in *Manduca sexta*. When the thorax overheats, the heart responds

by increased pumping of hemolymph into the abdomen, a response eliminated by sectioning of the ventral nerve cord. The site of the thermoregulation was traced to the pterothoracic ganglia in *Cecropia* moths by artificial thermal clamping (Hanegan, 1973). Desert robber flies, *Promachus gigantius* and *Efferia texana* were shown to thermoregulate, but with differences related to body mass (Morgan & Shelley, 1988).

Such putative indirect effects would likely be mediated via diffusible transmitters acting upon ionotropic receptors, or through networks of intracellular signaling pathways leading to biochemical modifications of pacemaker components (Bodmer et al., 2005). The neurotransmitters norepinephrine, serotonin, dopamine, and acetylcholine are cardioacceleratory (Johnson et al., 1997). *Limulus* GHSLLHFamide (Gaus et al., 1994; Johnson et al., 2000; Bodmer et al., 2005). The catecholamines may act through G-Protein coupled receptors, which is the case in vertebrates (Hartzell, 1988; Trautwein & Hescheler, 1990). cGMP is implicated in at least one control pathway as non-hydrolyzable forms (Brautigan & Pinault, 1991) increase heart rate in the fly (Johnson et al., 2002). *Drosophila* homozygous for the *sitter* allele of the *foraging* gene (Sokolowski, 2001; de Belle et al., 1989), which encodes a cGMP-dependent kinase (Osborne et al., 1997), have accelerated heart rate (Johnson et al., 2002). Heart Rhythmicity Index was affected by pertussis toxin and by agonists and antagonists of both α adrenergic and 5-HT_2 receptors; this suggests involvement of two different types of G proteins (Johnson et al., 2002). Pacemaker sensitivity to serotonin was eliminated in animals heterozygous for the homozygous-lethal mutation *l(4)16*, an allele of the gene that encodes CaM-kinase-II (Hochman, 1971; Cho et al., 1991; Ohsako et al., 1993; Griffith et al., 1993). Norepinephrine sensitivity was unaffected (Johnson et al., 2002).

Identical results were obtained when flies were treated with the CaM-kinase-II inhibitor KN-93 (Johnson et al., 2002).

White et al. (1992) tested for effects of heavy water consumption (water containing deuterium, $_1H^2$, or D_2O) and temperature change on heart rate. D_2O reduced heart rate at all temperatures tested. Heart rate rose linearly between 18° and 33° in flies drinking protonated water. This held for D_2O concentrations of 20% and 40%, but the D_2O reduced the sensitivity to the temperature changes as the slopes flattened proportionally at each concentration indicating an interaction between the two treatments of an unknown nature.

Human cardiovascular function is influenced by multiple genetic factors and knowing about the underlying genetic variation in these traits can be useful in medicine and health care (Broeckel & Schork, 2004; Lusis et al., 2004; Wilson & Olden, 2004). Findings in the fly are directly applicable to the human heart as a growing number of genes have been identified with homologous function in both organisms (Bodmer et al., 2005).

BIOGRAPHY OF THE AUTHOR

Tricia L. VanKirk was born in Skowhegan, Maine on February 12th, 1972. She was raised in Skowhegan and graduated from Skowhegan Area High School in 1990. She attended the University of Maine where she earned a Bachelor's degree in Zoology in 1994. Beginning in the summer of 1994, she traveled for one year with the musical group, Up With People, as a vocal soloist. With this group she also participated in over 300 hours of community service and stayed with a multitude of host families all over the world. Her musical adventures led her to Switzerland, where she lived and worked in the health care field for 5 years. She returned to the University of Maine in 2000 and began to work on her Master's degree in Zoology in Dr. Harold B. Dowse's lab. While working as a teaching assistant for Dr. Dowse's comparative anatomy course, she discovered her love for teaching. Starting in 2002, she worked as adjunct faculty for Kennebec Valley Community College, Eastern Maine Community College, University of Maine at Augusta and Husson University. She was offered and accepted a full time faculty position at Husson University in 2008 and continues to teach in the human sciences there. In 2014, she was appointed the director of the new Health Sciences Program at Husson. She met and fell in love with her husband, Dr. James VanKirk, and his three children in 2009. They were married in December of 2011 and presently live in Veazie, Maine where they enjoy skiing, boating, traveling, their three dogs and making music together. Tricia plans to continue teaching and expanding the Health Sciences Program at Husson, while participating in future *Drosophila* research with Dr. Dowse at the University of Maine. Tricia is a candidate for the Doctor of Philosophy degree in Biological Sciences from the University of Maine in December 2015.