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RESEARCH ARTICLE

Drosophila as a model system for cardiology: The case of melatonin and heartbeat regularity.

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ABSTRACT

Our objective in this review is to summarize evidence of the strong cardiac rhythmicity-enhancing power of melatonin in the *Drosophila melanogaster* model system and discuss the implications of these findings in the context of fundamental cardiac pacemaker function and potential clinical applications. *Drosophila* has proven itself as an exceptional research organism given the far-reaching genetic and molecular tools it offers. We consider details of the fly's myogenic, ion-channel-based pacemaker and summarize aspects of its neurohomonal control. Melatonin, in the context of cardiology, has predominately been associated with its antioxidant properties in the prevention of reperfusion damage after infarct, but we have strongly confirmed the few reports of its effect strengthening rhythmicity. We discuss our clear results showing that melatonin is capable of converting normal noisy heartbeat to an extremely regular oscillator. It rescues the very uneven beat of the hearts of flies bearing a serious mutation in a gene encoding one of its core pacemaker ion channels. Possible mechanisms for these effects are considered.

Keywords: *Drosophila* pacemaker, ion channels, heartbeat regularity, melatonin

Introduction

The common fruit fly, *Drosophila melanogaster*, was instrumental in delineating the fundamental science of genetics[1]. It has contributed to the understanding of a wide array of processes ranging from development to circadian rhythms[1]. Notably, with regard to the latter example, in 2017, the Nobel Prize for physiology or medicine was awarded to scientists Jeffrey Hall, Michael Rosbash, and Michael Young for their fly work on 24-h rhythmicity[1]. The full range of genetic and molecular manipulation tools has been key, and the fly genome has been fully sequenced[2]. 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[3]. 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 system[4].

Because of the simplicity in its structure and availability of these powerful tools, the *Drosophila* heart has emerged as a model system for unraveling the genetic and molecular mechanisms of cardiac development, function, and aging[5,6]. 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[3]. Although heart structure in *Drosophila* is very different from that of vertebrates, many of the basic elements for cardiac specification and differentiation are conserved[3]. The findings even extend to helping to unravel the relationship between diet and cardiac disease, as it has even been found that a high-fat diet can lead to obesity and cardiac dysfunction in the fly, and the pathway involved has been unraveled[7,8].

The Fly Heart and its Pacemaker

Hearts existed in animals before the split between insects and mammals. This is borne out by developmental evidence[9] and despite radically different adult morphology, the homologies in physiology remain compelling[10]. The *Drosophila* heart, or dorsal vessel, is located medially and dorsally in the hemocoel and transports hemolymph through the larval and adult body cavity[11,12,13]. 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[12,13,14].

The first systematic investigation of heart function in *Drosophila* began with Rizki in 1978[12]. He reported that heartbeat is triphasic namely contraction (systole), relaxation (diastole), and a pause (diastasis). Rate is affected by temperature, and these result from alteration in the duration of diastasis[12]. Pacemakers of insects were once considered to be neurogenic[15,16], but this has been shown not to be the case[16]. The most conclusive evidence of the myogenicity of the *Drosophila* heart comes from studies with tetrodotoxin, which interdicts sodium-dependent ion channel currents and has no effect on the heart[17,18]. Add to this the report that a temperature-sensitive mutation that encodes an important sodium channel, *paralytic*$^{temperature\ sensitive}$ (*para*^{ts}) also shows no effect on the heart at restrictive temperatures[18].

Work on the fly heart languished until a 1992 report on the effects of Deuterium Oxide on heart rate and function[19]. After this, efforts began to elucidate the mechanisms of fly heart function beginning with the hypothesis that the pacemaker is myogenic and consists of a population of interacting ion channels and a combination of genetic and pharmacological approaches was the key[18,20]. As part of this general approach, it was taken as a given that across phyla, true pacemaker cells 1) must have spontaneous activity; 2) they must lack an inward rectifying K^+ current; 3) must be insensitive to tetrodotoxin; and 4) must be sensitive to Ca^{2+} channel blockers[21]. The pacemaker must be connected to the myocardium with by an excitation/contraction (EC) coupling mechanism which elicits power contraction[22].

An essential part of fly heart research required the development of appropriate measurement and analytic techniques. In our lab, it was decided to concentrate on the early pupa, just as the transition from third stage larvae to the pupa begins. The animal is transparent at this point and the heart in the intact animal is easily isolated in the viewer of a microscope, eliminating the complicating movements of other organs[18,19]. The pupa becomes immobile at this point as well. Optical recording by phototransistor with illumination provided by a DC power source is straightforward and noninvasive. Individuals tested in this manner can be raised to adulthood and bred after phenotypic establishment even after injection of pharmacological agents, greatly enhancing genetic and molecular

approaches. Analytical techniques for this system were needed and we adapted several time series analyses to the task (review[23]). In our work a 30 s sample of the heartbeat signal was chosen as the standard. Simple plotting of the time series is done to visualize the beat and the segments are subjected to further numerical analysis. Next, autocorrelation is applied[24] and to augment this, we measure the height of the third peak (with peak one at t=0) as the "Rhythmicity Index" (RI) as a useful statistic[23,24,25]. In addition, we use our adaptation of Maximum Entropy Spectral Analysis[23,26,27] to provide spectra of the heart's rhythm. Software was developed to measure Inter Beat Intervals (IBI) based on successive peaks in optical density[28]. These techniques can be seen in the figures provided below. Injections of pharmacological agents were done throughout our work with a World Precision Instruments (WPI, Sarasota Florida) nanoliter injection system in 46 nL boluses[20].

Employing pharmacology, the L-Type Ca^{2+} channel blockers verapamil, and diltiazem[29] were shown to slow heart rate[17]. Tetraethylammonium (TEA) is a broad spectrum K^+ channel blocker[30] and also slows the heart[17,20], hence establishing K^+ channels as potential pacemaker components, but this only reveals the general picture, as it inhibits all known potassium currents[30]. Further genetic work was needed and initiated; three mutations were reported early on to slow heart rate and produce cardiac arrhythmia[18,20]: *no action potential* *temperature sensitive* (*nap^{ts}*)[31] is an allele of the gene *maleless* (*mle*)[32]. This gene interacts with at least one gene, *para^{ts}*,[33] in an unknown manner[34,35]. N*ap^{ts}* causes a reduction in heart rate and prevents the heart from reacting normally to changes in temperature[18]. *Dopa decarboxylase* *temperature sensitive* (*Ddc^{ts}*) blocks the synthesis of a number of monoamine neurotransmitters[36,37]. The final one was *amnesiac* (*amn*) a learning and memory mutant[38,39]. None of these were unequivocally revealing with regard to the structure of the underlying ion channel oscillator.

A precisely directed genetic screen subsequently yielded three K^+ channels as candidate pacemaker components[20]. The most effective mutation in this category affecting the heart is *slowppoke* (*slo*)[40] which encodes the pore-forming subunit of the channel[41]. This mutation, when homozygous, almost eliminates heartbeat, and eliminates temperatures sensitivity[20]. There are two known Ca^{++} activated K^+ currents (CaKs), a fast one (I_{CF}) and a slow one (I_{CS})[42] and *slo* interferes with I_{CF}[40,41]. In vertebrates, CaKs have been implicated in cardiac repolarization[21]. Injection of charybdotoxin (CTX) was shown to yield results in the fly similar to those produced by *slo*[20]. *slo* plays a central role in the melatonin work summarized below.

In summary, our lab has characterized four genes encoding ion channels as being necessary for normal heartbeat through analysis of mutant heartbeat and pharmacology[20,43]. We briefly summarize these and describe the resulting model system. Three of the channels allow the passage of K^+ and the other carries a Ca^{2+} current[43]. Johnson et al.[20,44] proposed a model for the *Drosophila* cardiac pacemaker based on these genetic and pharmacological findings: A delayed-rectifier potassium channel current (I_{Kr}) containing an α subunit encoded by *ether à go-go* (*eag*)[45,46,47,48] carries a hyperpolarizing leak comparable to the known mammalian "funny current" (I_f)[49] . The K^+ efflux has the effect of depolarizing sino-atrial cells so that a voltage-gated calcium channel, the α_1 subunit of which we believe to be encoded by *cacophony* (*cac*)[50,51,52], opens to allow Ca^{2+} to enter the cell and is central to the cycle[53]. ω-conotoxin (ω-CgTx) MVIIC, an antagonist of N- and P/Q-type calcium channels blocks this current[54,55], and it is effective in disrupting *Drosophila* heartbeat[56]. This Ca^{2+} influx opens the CAK encoded by *slo*. It is blocked by CTX[57]. A fast voltage-gated potassium channel (K_V, A-type) encoded by *Shaker* (*Sh*)[58,59] and blocked by 4-aminopyridine (4-AP)[47,57,60] also opens. This efflux of K^+ repolarizes the membrane[21,60,61,62,63,64]. The *eag* channel also activated by the Ca^{2+} depolarization, but in a delayed fashion and The K^+ current, I_f, begins hyperpolarization and the cycle restarts. This model has been tested by mathematical modeling by Crook[10] which shows the components hypothesized are capable of generating sustained oscillations.

Understanding how the pacemaker is controlled by the organism is essential. Work on the synthesis, receptors and actions of small molecule transmitters in the fly is ongoing. For a few salient examples, pupal heart rate in the fly has been shown to be sensitive to serotonin, dopamine octopamine, norepinephrine and acetylcholine (in that order), which increase heart rate[65]. The gene DOPA *Decarboxylase$^{temperture\ sensitive}$* (*DDC^{ts}*)[36] is responsible for encoding a gene essential for production of dopamine, serotonin and likely norepinphrine[63] reduces heart rate[44]. *Drosophila* peptides Dromyosuppressin, DPKQDFRMRFamde, and PDNFMRFamide are cardio inhibitory[66]. In this

context, it is notable that transmitter levels must themselves be regulated and this would be under the control of endocytosis[67]. We found that the mutations in the gene *shibire* (*shi*), *shi^{ts}* and *shi^{ts2}* [68] which encodes dynamin[69,70], essential for endocytosis[71] and which result in a temperature-dependant paralysis[68] also results in cardiac dysfunction[66]. Full coverage of the wide range of cellular mechanisms is well beyond the range of this paper, however the most relevant areas relevant to the action of melatonin will be covered in detail below. An in-depth review is available in Bodmer et al.[10].

Melatonin

The relatively simple molecule melatonin was extracted from pineal glands in 1958[72]. This hormone lightens the skin color of tadpoles, frogs, toads, and fish[73,74]. Melatonin exhibits a range of biological activities across species from bacteria to mammals[75]. Melatonin effects in vertebrates range across regulating circadian rhythms, acting as a neuromodulator, a hormone, a cytokine and a biological response modifier[76,77]. It also affects brain, immune, gastrointestinal, cardiovascular, renal, bone and endocrine functions, and acts as an oncostatic and anti-aging molecule[78,79,80,81]. Some of these activities are receptor-mediated, including via melatonin membrane receptors and nuclear receptors[83,84,85,86,87]. Melatonin may also act in a receptor-independent fashion[88,89] including, critical to cardiology, interactions with reactive oxygen species (ROS) and those mediated by its bioactive metabolites[90,91,92,93,94,95].

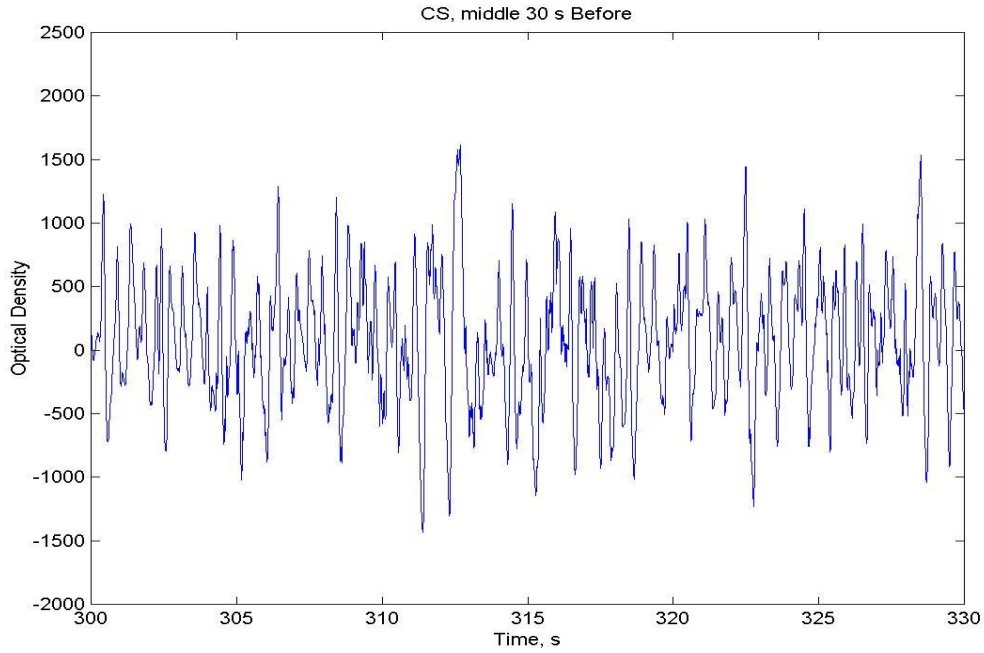
Melatonin is an indolamine synthesized from the amino acid L-Tryptophan and serotonin is a precursor[96]. Arylalkylamine N-acetyltransferases (AANATs) enzymes catalyze the rate limiting step in melatonin synthesis in the pineal gland in vertebrates[96]. More specifically, conversion of serotonin (5-hydroxytryptomine; 5-HT) to N-acetylserotonin, a precursor of melatonin, is catalyzed by serotonin N-acetyltransferase (AANAT) in a reaction requiring acetyl coenzyme A (AcCoA)[96]. Two different AANATs (AANAT1 and AANAT2) have been identified and characterized in *Drosophila melanogaster*[97,98,99]. Hydroxy indole orthomethyl transferase, (HIOMT), which is needed for O-methylating N-acetylserotonin in the final step of the pathway, has not yet been discovered in *Drosophila*. It has been argued that melatonin is totally absent in the fly[100].

Melatonin is a powerful antioxidant[101] and is commonly used to prevent reperfusion injury occurring when cardiac tissue, deprived of oxygen during infarction, is re-oxygenated; this is the actual cause of much of the damage associated with infarcts[102]. Another far lesser-known effect of melatonin is to lessen the arrhythmicity associated with infarcts, but it has been reported[102,103,104,105]. This is a central finding in the context of the fly work summarized here. As part of a general screening for cardioactive agents, we tested melatonin for effects on fly heart function[28]. The remainder of this paper will consider the results of this work and put it in general context. The reader is referred to the original paper for full details of materials, methods, tabulated and graphical results, and statistical analyses[28].

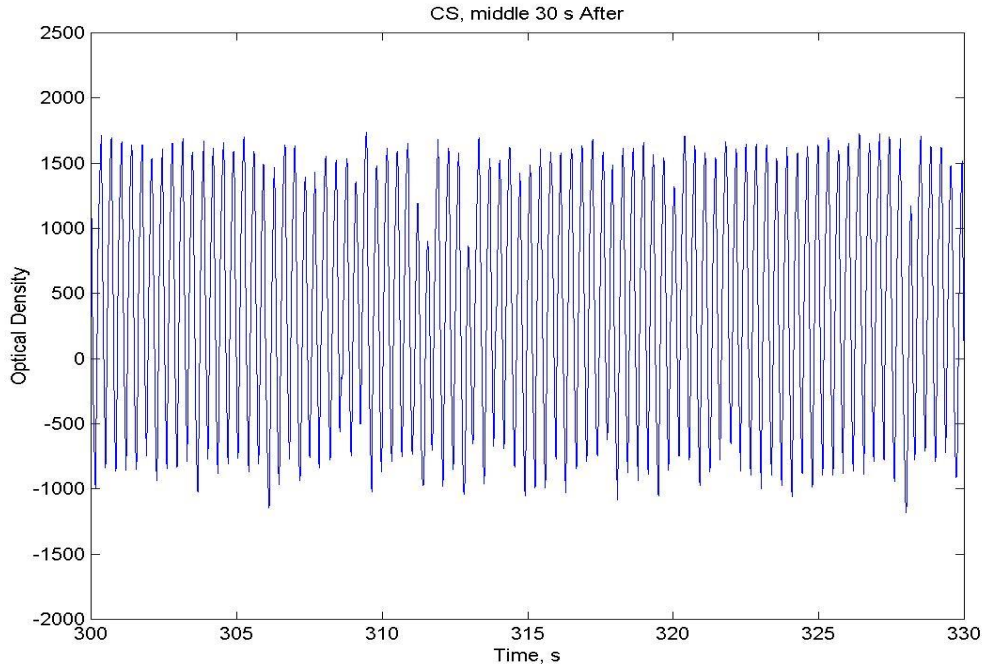
We addressed five issues in this work[28]: 1) Does melatonin have any effects on cardiac function in *Drosophila*, and if so, what are they? 2) Having found a profound effect on rhythmicity, is that effect related in any way to any change in heart rate? 3) Is the effect on rhythmicity related in any way to the antioxidant properties of the hormone? 4) Are the effects mediated by a receptor, and if so, what could that receptor be? 5) Can melatonin rescue rhythmicity in flies bearing mutations that produce core defects in the pacemaker system? 6) What are possible mechanisms for the change in rhythmicity?

The undisturbed heartbeat of Wild-Type *Drosophila* is not a highly regular noise-free oscillation as has been previously shown[18,19]. Fig. 1a (reprinted with permission from Springer) is fairly typical of results we get in our pupal preparations and illustrates the moderate irregularity in beating. In the experiment depicted here, the heart was recorded optically, as described above, for 10 min at 25° C, then injected with 1 mM melatonin and recorded for a second 10-min interval. 30-s segments taken at the midpoint of these recordings were used in analysis. One such 30-s segment of a pre-injection recording is shown. Following through the suite of analyses to see normal wild-type heartbeat, Fig 1c shows an autocorrelation (see above) taken from the 30 s of data shown, from which we obtain our RI statistic described above, roughly 0.3 in this instance. Fig 1e displays the MESA spectrum, as described above, from the same 30s segment. There are multiple peaks, corresponding with the fairly irregular autocorrelation function. This is a result of the variation in the IBI shown in Fig 1g for the full 600s and again in Fig. 1i for the 30s that were analyzed as described, showing the irregularities in higher resolution.

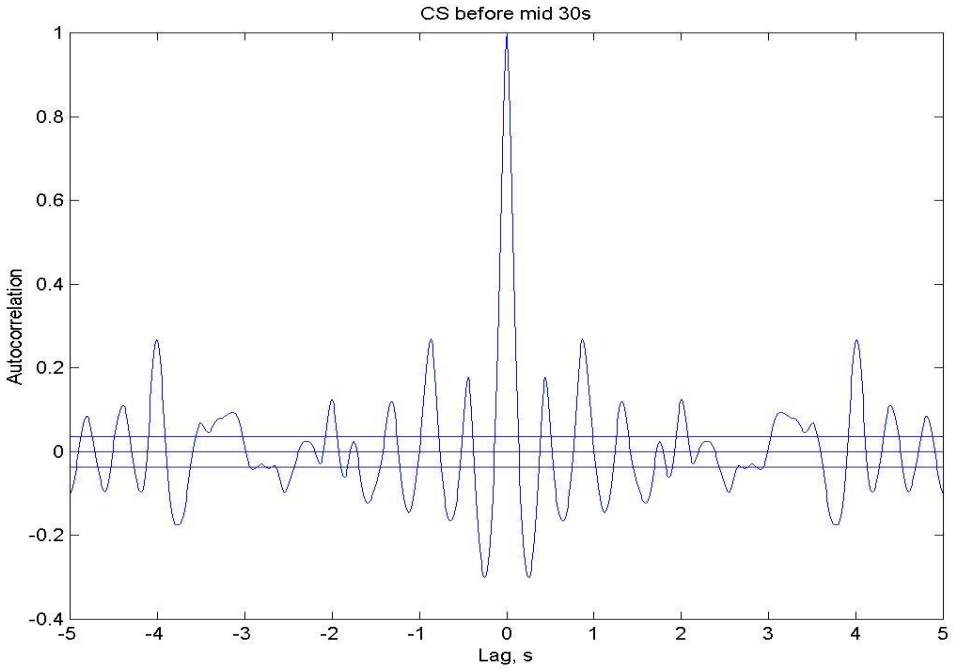
a


CS, middle 30 s Before
Optical Density
Time, s
2500
2000
1500
1000
500
0
-500
-1000
-1500
-2000
300
305
310
315
320
325
330

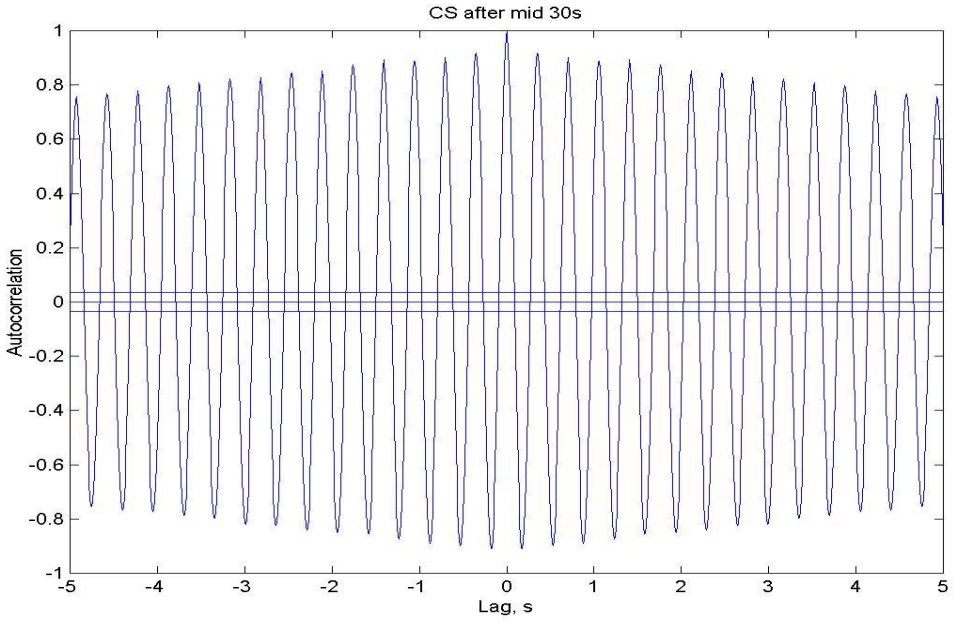
b


CS, middle 30 s After
Optical Density
Time, s
2500
2000
1500
1000
500
0
-500
-1000
-1500
-2000
300
305
310
315
320
325
330

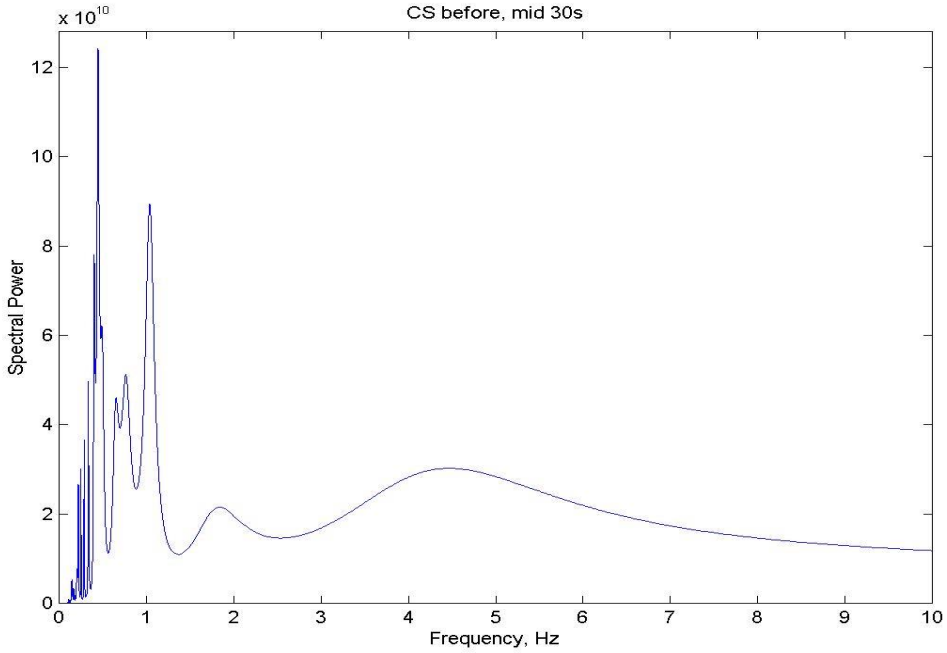
c


CS before mid 30s
Autocorrelation
Lag, s

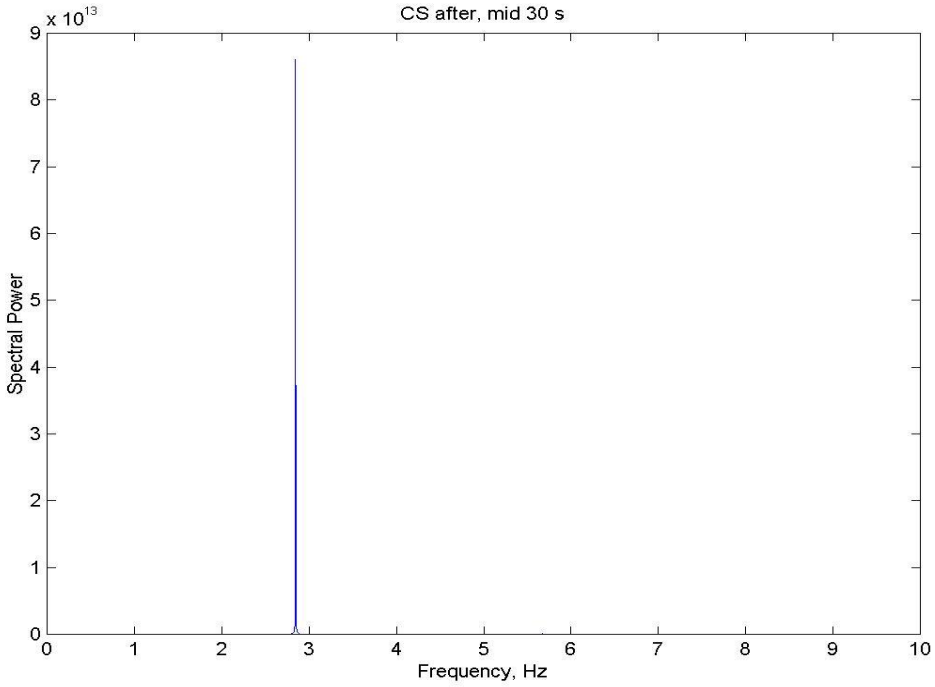
d


CS after mid 30s
Autocorrelation
Lag, s

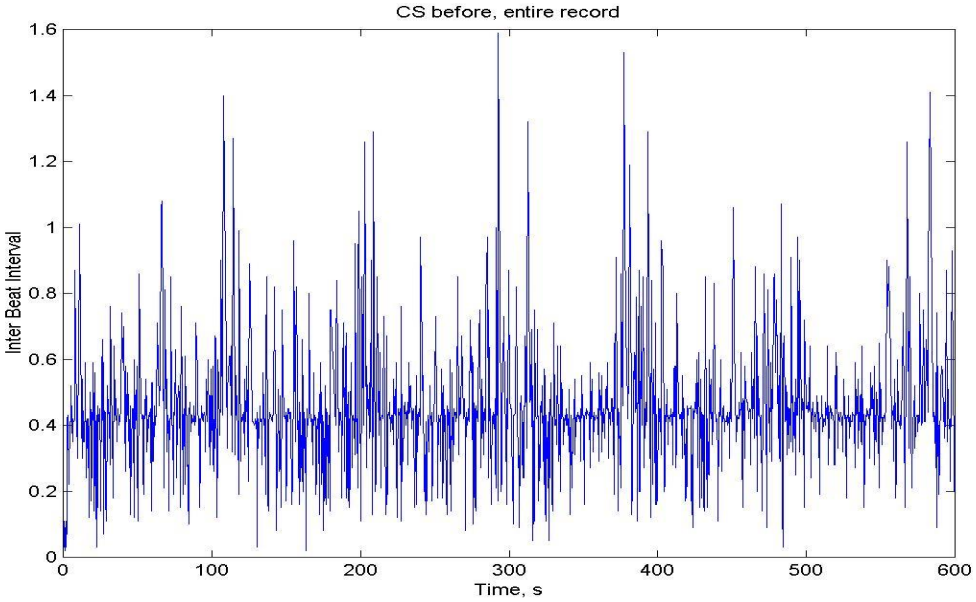
e


CS before, mid 30s
x 10^10
Spectral Power
Frequency, Hz
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
9
10

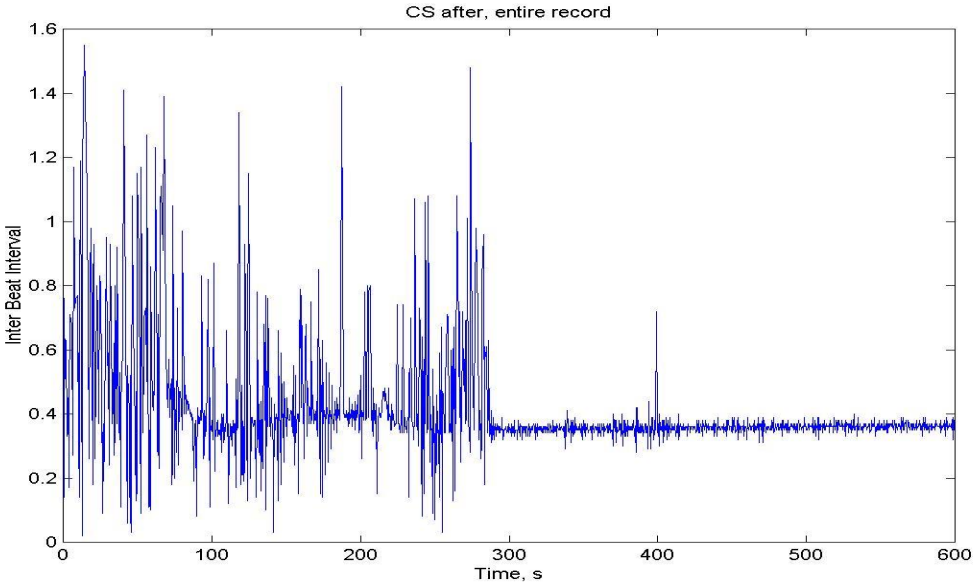
f


CS after, mid 30 s
x 10^13
Spectral Power
Frequency, Hz
0
1
2
3
4
5
6
7
8
9
0
1
2
3
4
5
6
7
8
9
10

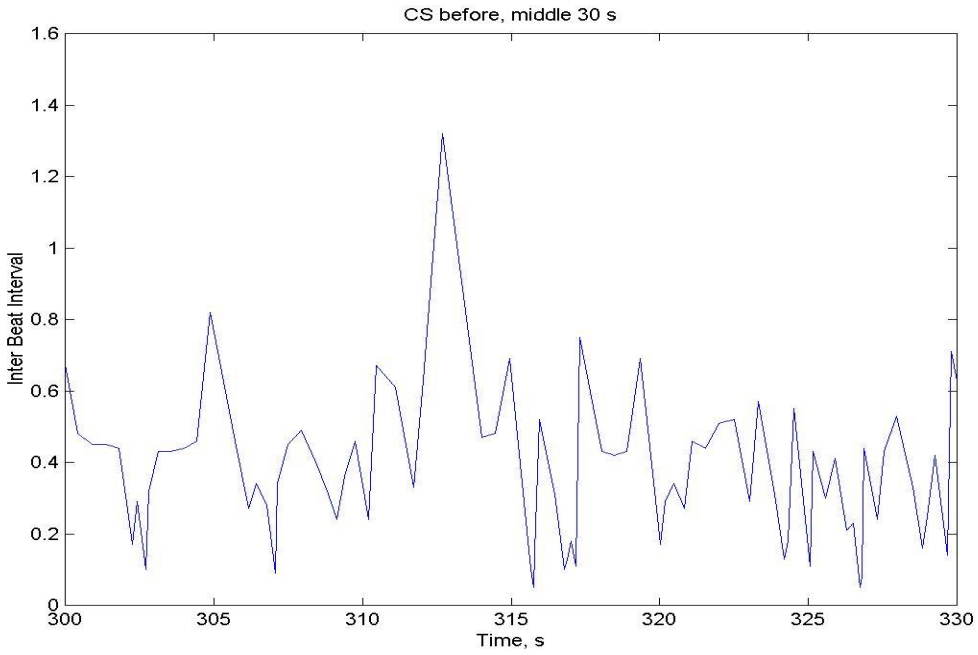
g


CS before, entire record
Inter Beat Interval
Time, s
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
100
200
300
400
500
600

h


CS after, entire record
Inter Beat Interval
Time, s
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
100
200
300
400
500
600

i


CS before, middle 30 s
Inter Beat Interval
Time, s
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
300
305
310
315
320
325
330

i

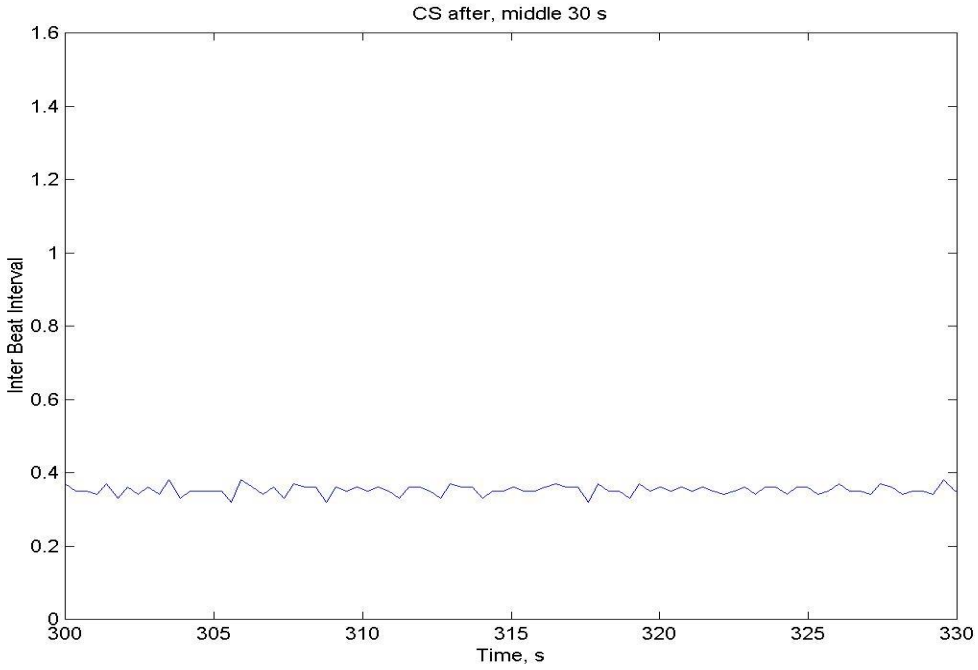

CS after, middle 30 s
Inter Beat Interval
Time, s
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
300
305
310
315
320
325
330

Figure 1. 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 and after f); g) Interbeat Interval (IBI) analysis of entire 10 minute recording before melatonin injection and after, h); 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, a 30-s segment starting at the midpoint after melatonin injection.

This amount of irregularity, found in all wild type animals, must be considered normal. One must presume that this is considered optimum and necessary as it is a result of natural selection. Human hearts also show this fundamental mildly chaotic beat, and extreme regularity is seen as a sign of pathology[106].

The right hand column of figures depicts the full suite of analyses for the same fly after injection of 1mM melatonin, as described above. Again, recording went on for 10 m and 30 s segments starting at the midpoint were used for analyses. The changes are unequivocal and striking. Simple inspection of Fig. 1b shows a change from a fairly noisy, moderately irregular signal to extreme regularity. This is borne out by the extraordinary change in the autocorrelogram in Fig. 1d. The MESA plot in Fig. 1f is similarly changed to what appears to be a line spectrum. The highly irregular plot of IBI in Fig. 1g shows the profound change when the melatonin is injected at the midpoint in Fig. 1h. IBI for the 30s segments that were used on the other analyses are depicted in high resolution in Figs. 1i (before) and 1j (after) injection. It can be concluded that melatonin increased cardiac rhythmicity unequivocally. The original paper tabulates and shows statistics to bear this out.

One possible explanation for these results might be that heart rate is increased by melatonin. Just speeding up an oscillator could simply explain any apparent subjective change in regularity. Serotonin is known to accelerate FR in *Drosophila*[44], so we used this reagent to test the hypothesis. Injection of serotonin predictably sped up the hearts in all our preparations, but RI was unaffected. Melatonin, conversely, did not speed up the heart and actually reduced FR slightly while substantially increasing RI[28].

Yet another hypothesis needing to be tested makes the assumption that the strong antioxidant effect of melatonin might play some role. To assess this, we tested ascorbic acid, also a powerful antioxidant[101] for any action on RI and found none[28]. There has been other work on ROS effects on heart function in the fly. One line of work considered the possibility that ROS may act in a paracrine manner to stabilize the pacemaker in cardiomyocytes[107]. It was shown that ROS are exceptionally low in normal fly cardiomyocytes, but are found in moderate concentrations in the paracardial cells[107]. By various manipulations, these concentrations were altered, and when ROS were reduced, the effect was to lowering cardiac rhythmicity, arguably via a paracrine pathway[107]. The fact that the universal effect of melatonin is to increase rhythmicity substantially in our hands, this is further evidence that alterations of ROS are not the mode of operation of melatonin.

There is a strong likelihood that the effect of melatonin on heart pacemaking is mediated via a receptor. To evaluate this possibility, we tested the effects of luzindole which is a known antagonist for MT_1 and MT_2 melatonin receptors in humans[84]. In this protocol, we employed a double injection technique using receptor agonists and antagonists. The time frame was 10 minutes of initial recording, followed by a second ten minutes of recording after injection of both agents just as before. To validate the technique, we also ran tests with serotonin, known to accelerate heart rate[44] co-injected with a known serotonin antagonist, ketanserin[65,108]. In addition to melatonin, we also tested 2-[^{125}I] iodomelatonin, which is a selective, high-affinity ligand commonly employed in characterizing melatonin receptor sites[109].

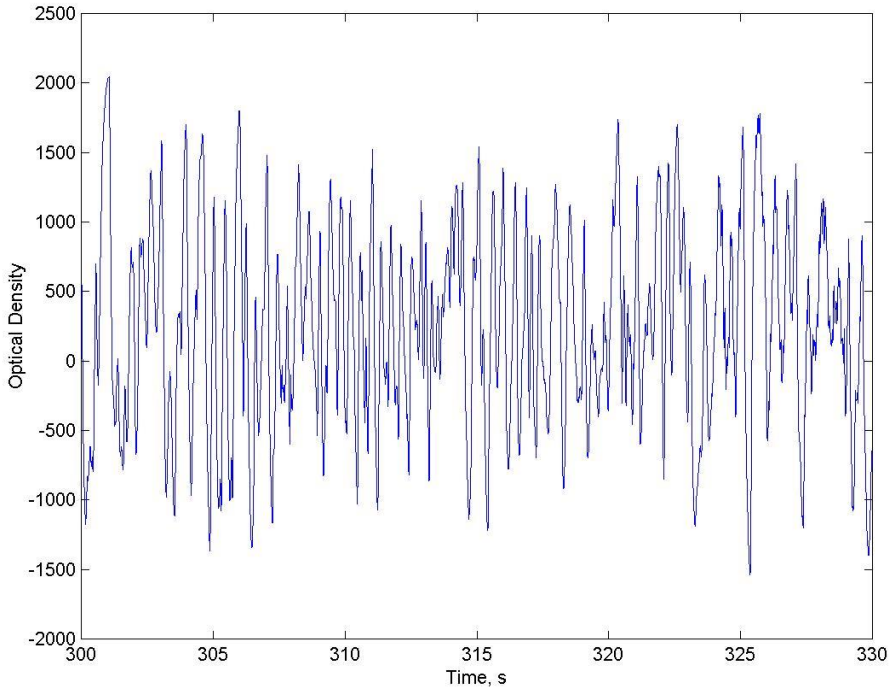
Injection of luzindole alone resulted in a significant drop in RI in CS and in other mutants tested in the genetic part of our approach; FR dropped slightly, but significantly in CS, but not in the other mutants[28]. This, by itself, argues for the existence of melatonin in the fly, or at least some similar cross-reacting compound. Luzindole pre-injection resulted in a total block of melatonin action in CS wild-type flies, with an actual significant drop

in RI. Injection of the melatonin agonist 2-[^{125}I] iodomelatonin showed an RI increase of 53% [28].

Given the clear result that there is the involvement of a receptor-mediated process in the fly, the next question concerns the identity of that receptor. This was done via an RNAi knockout protocol[110]. We employed orphan receptor genes to do this[28]. This required a heart-specific driver, GMH5-Gal4, a fragment from the *tinman* gene[111,112] cloned into the P{GaWB} vector upstream of the Gal4 sequences, and multiple copies of the UAS-Gal4 elements drove strong expression of the orphan receptors at the stage of our test animals[28,113]. We tested 5 orphans with particular interest in CG *4313*, which has a close homology with known human MT receptors[114]. Indeed, we found that RNAi blockage of this receptor gene completely eliminated the effect of melatonin on RI, and a BLAST search (NCBI 2016) comparing CG4313 to the human genome picked out human melatonin receptor MT 1A with high significance. (See[28] for further details and discussion of molecular and genetic details). It is worth considering that the cardioprotective role of melatonin in humans may actually be receptor-mediated. In the rat, cyclosporine-A-induced cardiotoxicity is reduced by melatonin and this protection is blocked by luzindole[115]. Also in the rat model, where melatonin is known to be protective against myocardial ischemia, luzindole blocks melatonin protection[116,117]. Luzindole also blocks melatonin's role in reducing blood pressure by binding to ML1 receptors in the anterior hypothalamic area[118].

Given the considerable power of studying mutants in fly work, we used this tool extensively in this program. Of particular interest was the possibility that melatonin can rescue wild-type rhythmicity in flies bearing mutations that reduce it in the heart. For full coverage, please see[28]. In covering the work leading to our current understanding of the structure of the fly pacemaker, concentrating on alterations in the core ion channels in the plasma membrane, discussed above, the mutation *slo* is the most disruptive[20]. Many flies bearing this mutation often have little discernable heartbeat at all[20]. Figure 2 (reprinted with permission from Springer) depicts the results of melatonin injection on the raw heartbeat signal in parallel with the wild-type results shown in Fig. 1a and b. Analysis shows that wild-type heart function is not only rescued, but the above normal levels of rhythmicity seen in wild-type are equaled[28].

a)



b)

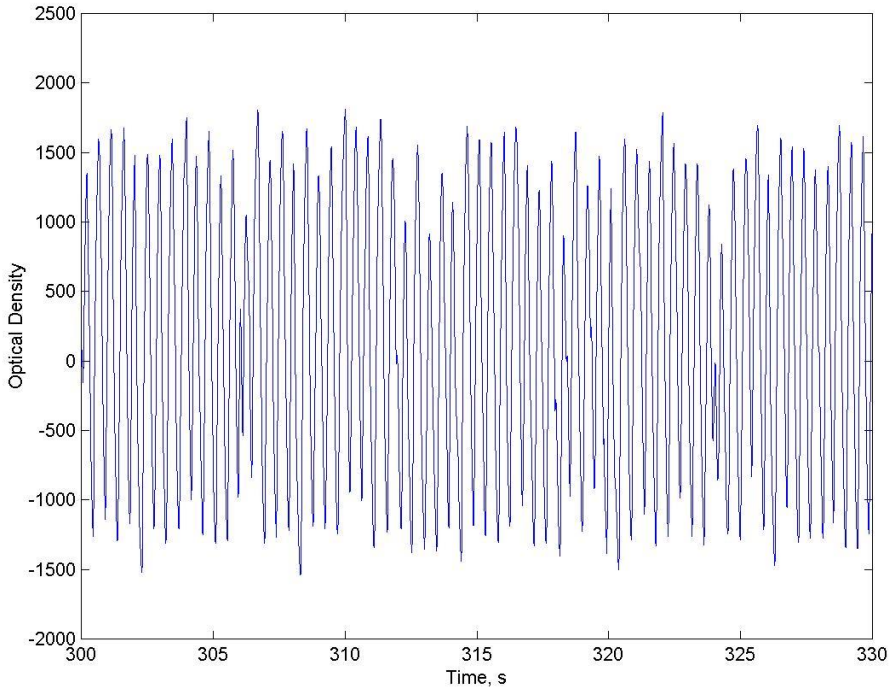


Fig. 2 Heartbeat of a pupa bearing the heart ion channel mutation *slowpoke (slo)* before (a) and after (b) injection with 1000μM melatonin. (Reprinted with permission from Springer).

The final matter to be considered is the mechanism by which melatonin acts to produce the observed results. Evidence presented above implicates a receptor mediated process. But the nature of what exactly results from this trigger is not clear.

Among the results summarized above, the most difficult to incorporate into any hypothesis is how is it possible for a heart pacemaker that has a core component of its physical mechanism damaged by mutation to beat normally when treated with a pharmaceutical agent. This is clearly the case with flies bearing the *slo* mutation. A second observation that must be dealt with is the extraordinary rapidity of the change to a much greater than normal regularity, both in mutant animals and wild-type. Perusal of Fig. 1h, depicting IBI, shows that the switchover occurs seemingly from one beat to the next. This is typical of what we recorded, and subjectively it looks as though some sort of switch is being thrown.

To lay the groundwork for this discussion, it is necessary to consider the constitutive irregularity of normal, wild-type heartbeat. As has been noted, insect hearts are not alone in this, as mammalian hearts are seen to have an irregular beat[106]. The nature of this has been under considerable study[119]. Of particular note is that certain cardiac pathologies, including congestive heart failure, display more regular beating[106]. Theorists have argued from two essential assumptions. First, that the normal irregularity is a result of *1/f* "noise" in a determinate system; the other is that this is a result of the cardiac oscillator being fundamentally chaotic[119,120]. It is far beyond the scope of this paper to discuss this in any detail, but it is essential to bring it up in the light of the remarkable results we obtained with melatonin to emphasize that "normal heartbeat" is anything but regular. In perusing Fig 1, it is essential to reemphasize that the signal seen after melatonin injection is being produced by the same heart, and that the change takes place literally over the time scale of a single beat. Given the

considerable evidence we uncovered that this is receptor mediated, it is difficult not to conclude that some element in the oscillating system is responsible for introducing either the noise, or is responsible for chaos, and that this element is altered through the action of melatonin. Yet the rate remains unchanged, hence is difficult to think one of the core ion channel components of the oscillator is responsible. The results here can potentially shed light on the question of chaos vs. noise.

With this last question firmly in mind, the next concern is how it is possible to explain the results shown in Fig. 2 for a fly bearing the *slo* mutation. As noted, we have evidence of the Ca^{2+} activated K^+ channel it encodes being one such essential component of the pacemaker10,[20,]. These flies show noise/chaos-free oscillatory behavior identical to wild-type under the influence of melatonin. The analogy would be having a button on the dashboard that could make a vehicle with a broken crankshaft suddenly begin to run flawlessly.

One intriguing possibility that penetrates to the very mechanism of pacemaking is that there is a possible "backup" system present. Over some time work has been ongoing investigating a second, distinct Ca^{2+} oscillator being an essential part of the cardiac pacemaker[121,122,123,124]. This has been dubbed the "Calcium Clock" and is in addition to the ensemble of ion channels on the on the cell membrane of pacemaker cells, termed the "M" oscillator in this context[121,122,123,124]. The underlying mechanism involves Local Calcium Release (LCR) from the sarcoplasmic reticulum by ryanodine receptors, followed by its reuptake by the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA)[121,122,123,124]. This oscillator would interact with the M clock to form a cooperative system. There is considerable experimental evidence supporting this hypothesis[123]. Clinically, 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 SERCA activity[125].

Of particular significance to the current discussion of a "backup", it was shown that pacemaker cells can continue to oscillate even when the plasma membrane is voltage clamped[121,124]. Melatonin might be hypothesized to trigger the jump to the cytosolic system, or perhaps an altered cooperative relationship. This would explain the finding that even fly heart pacemaker cells in *slo* mutants that bear a missing or damaged core Ca^{2+}-gated K^+ channel can beat with the same unnaturally regular rhythmicity as wild-type[28].

Ca-P60A^{Kum170} is an EMS-induced heat sensitive mutation in the fly gene that encodes SERCA[126]. This *Drosophila* SERCA mutant shows a severe decrease in heart rhythmicity after being subjected to a 2.5 minute heat shock at 41°C [127,128]. Of importance in this discussion we found that heat-shocked flies bearing the above mutation in SERCA showed a return to near normal heart function after our melatonin injection procedure[129]. In addition it has been shown that a mutation in the fly ryanodine receptor also results in severe cardiac dysfunction in the fly[130].

Conclusion

The results summarized here provide adequate justification for continued work on understanding the underlying physiological effect of melatonin on heart function not just in the fly, but in mammalian models. This work may reveal basic underlying principles of the physics of the oscillation in the pacemaker, likely shedding further light on the interactions of the membrane and cytoplasmic ion channels. As important, is the possible employment of melatonin as a pharmaceutical agent to reduce cardiac arrhythmicity resulting from a number of pathological conditions. In the latter instance, translational work is warranted.

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