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Using optogenetics to assess neuroendocrine modulation of heart rate in Drosophila melanogaster larvae

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Abstract The Drosophila melanogaster heart has become a principal model in which to study cardiac physiology and development. While the morphology of the heart in Drosophila and mammals is different, many of the molecular mechanisms that underlie heart development and function are similar and function can be assessed by similar physiological measurements, such as cardiac output, rate, and time in systole or diastole. Here, we have utilized an intact, optogenetic approach to assess the neural influence on heart rate in the third instar larvae. To simulate the release of modulators from the nervous system in response to environmental influences, we have directed expression of channel-rhodopsin variants to targeted neuronal populations to assess the role of these neural ensembles in directing release of modulators that may affect heart rate in vivo. Our observations show that the activation of targeted neurons, including cholinergic, dopaminergic, and serotonergic neurons, stimulate the release of cardioactive substances that increase heart rate after the initial activation at both room temperature and in a cold environment. This parallels previous studies suggesting these modulators play a crucial role in altering heart rate when applied to exposed hearts and adds to our understanding of chemical modulation of heart rate in intact Drosophila larvae.

Keywords Cardiac · Optogenetics · Drosophila melanogaster · Heart rate · Modulators

Abbreviations
5-HT Serotonin
ATR All-trans-retinal
Ach Acetylcholine
Cha-Gal4 GAL4 driver targeting expression to cholinergic neurons (choline acetyltransferase)
ChR2 Channel-rhodopsin-2
ChR2–XXL Channel-rhodopsin-2–XXL
ChR2-H134RII-mcherry Less sensitive channel-rhodopsin-2
CNS Central nervous system
DA Dopamine
HR Heart rate
Ple-Gal4 GAL4 driver targeting expression to dopaminergic neurons (pale)
Ppk-Gal4 GAL4 driver targeting expression to class IV dendritic arborization sensory neurons (pickpocket)
Trh-Gal4 GAL4 driver targeting expression to serotonergic neurons (Tryptophan hydroxylase)

Introduction

The Drosophila melanogaster heart has rapidly become a principal model in which to study cardiac physiology and development. While the morphology of the heart in Drosophila and mammals differs, many of the molecular
mechanisms that underlie heart development and function are similar (Bodmer and Venkatesh 1998). In addition, the hearts are functionally assessed by comparable physiological measurements, such as cardiac output, rate, and time in systole or diastole (Choma et al. 2011). A number of studies have used the Drosophila heart to identify proteins that are crucial in regulating cardiac muscle contraction and ion transport (Bier and Bodmer 2004; Wolf et al. 2006; Ocorr et al. 2007; Cammarato et al. 2011). These proteins are known to share similar functions in mammals. In addition, recent analyses have begun to shed light on endogenous modulators and hormones that directly influence heart rate (HR) and rhythmicity. It has been shown that abundant neuromodulators active in Drosophila and other insects, including acetylcholine (Zornik et al. 1999; Malloy et al. 2016), serotonin (Majeed et al. 2014; Hillyer et al. 2015), dopamine (Collins and Miller 1977; Zornik et al. 1999; Titlow et al. 2013), glutamate (Hillyer et al. 2015), octopamine (Johnson et al. 1997; Zornik et al. 1999), and melatonin (VanKirk et al. 2016), display modulatory effects on the cardiac pacemaker. Furthermore, many of the receptors mediating the chronotropic and inotropic action of these modulators have been identified in the aforementioned studies. All of these compounds are systemic in humans and many of these receptors that are targeted in these studies share human homologues. Thus, the release of modulators from the central nervous system (CNS) that may alter cardiac function in humans through the activation of their receptors on the heart directly or through the modulation of release of cardioactive substances from the nervous system into the blood may display similar actions in the fruit fly model. These studies highlight important features that make the Drosophila heart a potentially significant model in providing insight into the molecular mechanisms fundamental to human heart function. Advancing our understanding of the role of endogenous compounds and their receptors in influencing cardiac function will help to foster investigation into potential pharmaceutical and genetic therapies for human cardiac pathologies. Although it is becoming well known that the Drosophila heart is quite sensitive to changes in circulating modulators/hormones as well as hemolymph pH (Badre et al. 2005; Desai-Shah et al. 2010; de Castro et al. 2014), it is important to continue to address the role of these hormones in regulating cardiac function.

The Drosophila circulatory system is an open system that consists of a simple dorsal vessel with a posterior heart and anterior aorta. The dorsal vessel is a tube that spans the rostral–caudal axis of the animal and is made up of multiple types of cardiomyocytes (Gu and Singh 1995; Lehner et al. 2012). Hemolymph is drawn into the heart through ostia in the posterior pump and circulated through an aorta back into the visceral lumen (Molina and Cripps 2001). The pacemaker of the larval heart is located caudally and, like in the human heart, is completely myogenic (Rizki 1978; Dowse et al. 1995; Gu and Singh 1995; Johnson et al. 1998). During the majority of the larval stage, the heart is completely devoid of neural innervation; however, in the late third instar, there appears to be neurons innervating the rostral tissue of the aorta, but the function of this innervation has not been addressed (Johnstone and Cooper 2006). Neural innervation persists into the adult stage.

While the pupal stage is commonly used for examining cardiac function due to the fact that it is immobile for injection and inspection, this stage in Drosophila development is highly dynamic. Vast neural circuit rewiring, muscle breakdown, and reassembly of internal and external structures occur during this stage, making analysis of specific neural circuit and/or hormonal influence on HR somewhat challenging. In addition, as mentioned previously, the adult heart is modulated by neuronal inputs, which complicates addressing the function of hormones directly on the intact heart (Dulcis and Levine 2003, 2005). Therefore, the larval stage in Drosophila development serves as an ideal model for the observation of direct systemic modulation of cardiac function. In previous analyses performed by our lab utilizing larval Drosophila, a semi-intact method, in which the larvae were dissected and the heart exposed directly to solutions, was utilized. A distinct advantage exists in using such a technique as one can assess the direct actions of controlled concentrations of modulators on the HR without the influence of additional modulators or hormones that may circulate the hemolymph as a result of stress from injections or other alternative approaches. Analysis using this method has led to the discovery of direct modulation of HR of a number of modulators as well as the receptors through which they act in cardiac tissue in larval Drosophila. The pharmacological approach on the semi-intact preparation allows for the use of agonists and antagonists for the identification of these important receptor subtypes without the need to rely on low-level mRNA expression profiling. While this approach serves useful purposes and has provided necessary insights, it fails to simulate the role of neuroendocrine released modulators in regulating cardiac function. The use of an intact larval preparation allows one to investigate the role of specific neural populations, and the modulators/hormones they release, in pacing the heart. In addition, it has been noted that the HR is much higher in intact larvae than in a dissected preparation bathed in a physiological saline. It is likely that the saline often used in such analyses and the composition of the hemolymph in an intact, closed system is not equivalent, as saline lacks endogenous combinations of peptides and modulators that influence the heart. Thus, an intact approach more closely mimics changes in cardiac function in vivo as a result of variations in neural circuit activity in response to environmental stressors. To date, there are no studies, to our
knowledge, that have been performed in larval *Drosophila*, that address the role of neural-derived modulators that may influence heart function while circulating the hemolymph in vivo.

Since the larval HRs are fairly high at room temperature (22 °C), the more subtle effects of modulators within the hemolymph might not be as pronounced. We have noted in a previous study that intact as well as exposed hearts in dissected preparations are substantially slowed at 10 °C (Zhu et al. 2016a, b). The exposed larval hearts respond well to modulators (5-HT, OA, DA, Ach, and tyramine) at this temperature. Thus, we examined the possibility of detecting the effects of exciting the specific neurons containing modulators as well as defined sensory neurons on intact larvae conditioned to 10 °C to determine if the HR is altered. Even at 10 °C, the larval heart does beat well (50–100 beats per min), so any modulators released into the hemolymph can be readily circulated within the whole body cavity. In addition, it has been shown in previous analyses that channel rhodopsin (ChR2) is functional at 10 °C in acute conditions (Zhu et al. 2016b).

The advent of optogenetics has revolutionized the ability to temporally control the activity of excitable cells. While the majority of its use has centered on driving activity changes in neural populations to deduce the neural basis of behavior, optogenetic drive of cardiac muscle has recently been introduced in model organisms (Alex et al. 2015; Zhu et al. 2016a, b). However, the use of optogenetics in assessing indirect regulation of cardiac pace making has yet to be addressed. Here, we have chosen to utilize the light sensitive cation channel ChR2–XXL (Dawydow et al. 2014) to drive the activation of specific neural populations. Specifically, we have targeted the activation of cholinergic neurons (Cha-Gal4), dopaminergic neurons (ple-Gal4), and serotonergic (5-HT) neurons (Trh-Gal4) through tissue-specific expression of ChR2–XXL to assess how systemic release of the modulators synthesized by these neurons alters HR in vivo. In addition, we activated a subset of class IV dendritic arborization (da) sensory neurons (ppk-Gal4) known to be critical in mediating response to noxious stimuli (Hwang et al. 2007; Xiang et al. 2010; Johnson and Carder 2012; Kim et al. 2013; Kim and Johnson 2014) to examine if they could lead indirectly to alterations in HR.

The channel-rhodopsin–2–XXL variant was recently created, placed under the control of a UAS activation sequence, and cloned into *Drosophila* (Dawydow et al. 2014). It has been shown to produce more robust and longer photocurrents due in large part to its increased expression, enhanced affinity for a cofactor, all-trans-retinal (ATR), and potential increased single channel conductance (Dawydow et al. 2014). Expression of this rhodopsin allows for low-light applications as to prevent off-target effects and could be of use in targeting deep neural and muscle tissue in other model organisms as well as in humans. We have chosen to use this variant to ensure robust neuromodulator release, so that influence on the heart can be evaluated.

### Materials and methods

#### Fly rearing

All flies used for HR analyses were held for a few days at 22 °C in a 12 h light/dark incubator before being tested. All animals were maintained in vials partially filled with a cornmeal–agar–dextrose–yeast medium. The general maintenance is described in Campos-Ortega (1974).

#### *Drosophila* lines

The filial 1 (F1) generations were obtained by crossing virgin females of the recently created ChR2 line (which is very sensitive to light) called y¹ w¹¹¹8; PBac{UAS-ChR2. XXL}VK00018 (BDSC stock # 58374) (Dawydow et al. 2014) with males from each driver line targeting specific neural populations. The driver lines used in this study include: *Trh-Gal4* (BDSC stock # 38389), *Cha-Gal4; UAS-GFP* (BDSC stock # 6793), *ple-Gal4* (BDSC stock # 8848), and *ppk-Gal4* (BDSC stock # 32078). These lines were all purchased from Bloomington *Drosophila* Stock Center (BDSC) in Bloomington, Indiana, USA. In addition, we also used *UAS-ChR2-H134RII-mcherry; Trh-Gal4* (III) homozygous line, which was kindly provided by Dr. Schoofs et al. (2014). This line expresses a less sensitive Chr2 variant in 5-HT neurons. Adult flies from the driver (Gal4) lines and the UAS-Chr2–XXL line were crossed on the standard fly food. Flies from a parental line, y¹ w¹¹¹8; PBac{UAS-ChR2.XXL}VK00018 (BDSC stock # 58374), were used as controls in assessing the influence of neural-based modulators on HR. Progeny from these adults was not crossed with a Gal4 line; therefore, the expression of ChR2–XXL is absent in these larvae. The *Trh-Gal4* line (UAS-Chr2–H134R-mcherry; Trh-Gal4 (III) homozygous) was crossed with the UAS-Chr2–XXL line and, therefore, carries two different UAS constructs. In the text from this point on, the tested F1 generation will be referred to simply as “Trh-Gal4 X UAS-Chr2–XXL” for simplicity.

#### Preparation of food supplemented with all-trans-retinal and flies prior to testing

All-trans-retinal (ATR; Sigma-Aldrich, St. Louis, MO, USA) was diluted in the standard fly food to a final
Blue-light influence on heart rate of parental control lines.

Column 1 Raw average heart rates were calculated at five timepoints: (1) under white light (2) 1 min following exposure to blue light (3–5) subsequent 10-min intervals following exchange to blue light in room (22 °C) and cold temperatures (10 °C) (a1, a2–b1, b2 and c1, c2–d1, d2, respectively). Column 2 The average percent changes in HR and individual percent changes for each preparation were calculated at four timepoints: (1) 1 min following blue-light exposure and subsequent 10-min intervals following the initial change to examine the change in HR with continued blue-light exposure (2–5) for room temperature (22 °C) (a1, a2–b1, b2) and cold temperature (10 °C) (c1, c2–d1, d2). The influence of blue light alone on a control line was minimal concentration of 100 µM or 1 mM or/and protected from light with aluminum foil. For control experiments, larvae were cultured in food that only contained the solvent (absolute ethanol in the same volume used for the ATR mixtures in the fly food). The ATR or ethanol food mixtures were left alone for 48 h prior to adding larvae to allow some evaporation of the alcohol solvent from the mixture. It has been noted that larval development slows in the presence of ethanol, so this evaporation precaution was taken to limit its developmental influence. Adult flies from the driver (Gal4) lines and the UAS-Chr2–XXL line were crossed on the standard fly food. Approximately 3 days following the cross, the second instar larvae were removed from the standard food vials and placed in 1 mM ATR-food mixtures and left for 48 h prior to testing.

Monitoring heart rate in the intact larva

The movement of the trachea is commonly used to monitor Drosophila larval HR because of the clear contrast of the structures (White et al. 1992; Dasari and Cooper 2006). Early third instar larvae were stuck ventrally on a glass slip using double stick tape in such a way that mouth hooks are free to move. Care was taken not to stick the spiracles to the tape. The glass slip was placed on top of a dark surface to maximize contrast between the background and the translucent larval body wall. The HR was measured for 1 min in white light, followed by 1 min in blue light (470 nm wavelength, LED supply, LXLM-PB01-0040, 70 lm @ 700 mA) from a high intensity LED that was focused on the specimen through a 10× ocular objective, while the HR was counted (Titlow et al. 2014). The photon flux (number of photons per second per unit area) was measured with an LI-COR (model Li-1000 data Logger, LDL 3774), which produced around 550 µmol s-1m-2 per uA on the surface of the larvae. Following initial 1-min counts, HR was counted again every 10 min, while larvae were exposed to blue light to detect changes over a longer period of time. The heartbeats were counted by an observer’s eye with the use of a dissecting microscope.

Statistical analysis

All data are expressed as raw values or as a mean (±SEM). The Mann–Whitney Rank Sum Test was used to assess, within line at each timepoint measured, the difference in HR elicited in response to a + all-trans-retinal (ATR) diet versus a −ATR diet to evaluate the efficacy of the addition of ATR on altering HR as a result of activating select neural populations. In addition, a Mann–Whitney Rank Sum Test was used to test differences in percent changes in HR for experimental lines vs. a control line (y1 w1118; PBac{UAS-Chr2.XXL}VK00018parental line). The groups were separated based on their prior feeding (+ATR or −ATR) and the percent change in HR at each indicated timepoint was compared. Since larvae often displayed variation in baseline HR, raw data are presented and are provided as beats per minute (BPM) and also as percent changes in HR. Comparisons between the +ATR and −ATR-fed larvae within lines as well as from the control line vs. experimental lines were made to assess the efficacy ATR supplementation, as well as the role of modulator release, on HR. This analysis was performed with the Sigma Stat software. P of ≤0.05 is considered as statistically significant. The number of asterisks is considered as P ≤ 0.05 (*), P ≤ 0.02 (**), and P ≤ 0.001 (***)

Results

Blue-light influence on heart rate

It has long been known that larval Drosophila display a negative phototaxis behavior. Upon exposure to light, larvae swing their anterior in avoidance (Jennings 1904; Mast 1911; Grossfield 1978; Sawin et al. 1994). Larvae display photoavoidance even in the absence of Bolwig’s Organ (Xiang et al. 2010). Thus, it is assumed that blue light is significant in influencing neural circuit activity within the CNS. Because of this, we tested the potential influence of blue light in stimulating the release of cardioactive modulators. In an effort to control for this influence alone, avoiding any targeted neural populations, we utilized the UAS-Chr2–XXL parental line as a control. In addition, to avoid confounding variables, these larvae were separated into two groups based on the presence of ATR (Fig. 1a1, a2) or the absence of ATR (Fig. 1b1, b2) in their food prior to testing. Due to the fact that there is a high degree of variability in baseline HR in larvae (generally between 160 and 200 beats per minute (BPM) at room temperature (22 °C) and 80–100 BPM at 10 °C), the data are presented as raw changes in BPM (Fig. 1a1, b1) at each timepoint indicated as well as percent change from one timepoint to another (Fig. 1a2, b2). The percent changes indicate percent change.
from the previous timepoint (i.e., continued negative percent changes indicate continued drop in HR from original baseline in white light counts and a percent change close to zero represents an HR that has stabilized over time). All the succeeding analyses were performed in a similar fashion; however, for the sake of simplicity, and due to the fact that baseline rates change from preparation to preparation, only percent changes are indicated in the subsequent figures. It is noted that, at 22 °C, the alteration in HR upon initial exposure to blue light, as well as after continuous (10, 20, and 30 min) exposure, produced highly varied results. In the ATR-fed larvae, the initial exposure to blue light induced a negative percent change in five out of six preparations from baseline (white light), representing an average percent decrease for the six preparations of −1.48% (Fig. 1a2). After 10-min continuous exposure, the HR further decreased by an average of 2.90%; however, three out of six preparations displayed a positive percent change following this 10-min period. The average HR for this group continued to decrease on average to −0.53% after a 20-min exposure before rebounding after 30 min (Fig. 1a1, a2). The final timepoint measured represented a slight positive percent change from the previous point as two out of the six preparations displayed a positive percent change in HR, which was not statistically significant. Therefore, in +ATR-fed larvae at 22 °C, blue light does not induce a significant percent change in HR at any timepoint measured (Rank Sum Test p > 0.05 at all timepoints). Likewise, larvae fed a diet without ATR supplementation exhibit similarly varied responses to the initial exposure to blue light as well as longer (10–30 min) exposure to blue light (Fig. 1b1, b2). The initial exposure to blue light induces an average negative percent change from baseline of −1.24%, with only two of the six preparations exhibiting a positive percent change (Fig. 1b2). The HR rebounds in five out of six preparations, representing an non-significant positive percent change of 1.24%, before reducing an average of 0.15% after 20 min of continuous blue-light exposure (Fig. 1b2). After 30 min, four out of six preparations exhibit a positive percent change; however, this does not represent a statistically significant increase. As in the +ATR group, blue light does not induce any significant percent change in HR at any timepoint measured in the −ATR group (Rank Sum Test p > 0.05).

As previously mentioned, because it was noted that the HR in intact larvae at 22 °C was quite high, we considered the idea that any further increases by light exposure would be difficult to deduce. To observe if slowing baseline rates allowed for easier observation of changes upon blue-light exposure, we assayed control groups of larvae (±ATR) at 10 °C. The same experimental paradigm was utilized at 10 °C as described previously. It is noted that the variability that existed in the preparations at room temperature is shared at 10 °C. The baseline rates dramatically decreased (between 80 and 100 BPM) (Fig. 1c1, d1) compared to 22 °C; however, there was little difference in observed degree of change following blue-light exposure. In both the +ATR and −ATR groups, a positive percent change was exhibited upon initial blue-light exposure (Fig. 1c2, d2). In particular, five out of six preparations in the +ATR group displayed a slight increase in HR, while four out of six preparations in the −ATR group showed an increase in HR, representing positive percent changes of 4.8 and 4.23%, respectively. After 10 min of continuous blue-light exposure, the HR decreased two out of the six preparations in the +ATR group and three out of six preparations in the −ATR group (Fig. 1c2, d2). At the 20- and 30-min time periods, the +ATR preparations displayed high variability in their changes in HR, with four out six preparations exhibiting a reduction in HR at the 20-min timepoint and two out of six preparations exhibiting a negative percent change in HR from 20 to 30 min (Fig. 1c2). The −ATR group also showed variability over time; however, at the 30-min timepoint, four out of six preparations displayed a negative percent change, representing an average decrease of 10.1% (Fig. 1d2). Just as was observed in the room temperature environment, there was no significant change in percent measures of HR at any timepoint measured in both the +ATR and −ATR groups (Rank Sum Test p > 0.05). Thus, the role of blue light, alone in modulating HR in both environments, is minimal.

Efficacy of all-trans-retinal supplementation and neural-based influence on heart rate

We next assessed the efficacy of ATR supplementation in producing differences in responses. It has previously been reported that the photocurrent produced in cells expressing the ChR2–XXL variant is much greater compared to the less sensitive variants (Dawydow et al. 2014). In addition, it has been noted in larval behavioral analysis in experiments performed in our lab that the ChR2–XXL-mediated response to blue light is extremely robust. Even in the absence of ATR and when exposed to a white light stimulus, larvae expressing Chr2 in motor neurons exhibit strong contractions of their body wall muscles. This led us to test the efficacy of responses in HR in larvae exposed to 1 mM ATR supplementation compared with no ATR supplementation within each line. The average percent changes in HR at each timepoint tested (1–5) were recorded for the groups and compared, and the differences between the +ATR and −ATR groups were recorded. It is noted that the difference in HR within the lines for the +ATR group and −ATR group was minimal. At 22 °C, out of 20 total timepoints tested among line (four timepoints per line × 5 lines), a significant difference between the groups was only observed twice (Fig. 2).
The initial change from white light to blue light in the Cha-Gal4 X UAS-Chr2–XXL line with ATR supplementation exhibited an increase in HR that represented a significant difference compared to the −ATR group (p < 0.05; Rank Sum Test) (Fig. 2a1). In addition, the line expressing the less sensitive Chr2 variant (H134RII-mcherry) in 5-HT neurons displayed a significant change in HR after the −ATR group (p < 0.05; Rank Sum Test) (Fig. 2d1). Within these lines, no significant difference was observed between the + and −ATR groups at any subsequent timepoints (Fig. 2a1, d1). The additional lines displayed no significant difference in HR at all timepoints tested (Fig. 2).

Likewise, the influence of added ATR on HR was minimal at cold temperature in each of the tested lines. Again, among all tested timepoints within each line, only two timepoints displayed a significant difference in the percent change in HR between groups. As at room temperature, the initial exchange from white light to blue light induced an increase in HR in the Cha-Gal4 X UAS-Chr2–XXL (+ATR) line that represented a significant difference in comparison with the −ATR group (Fig. 2a2). The subsequent timepoints following timepoint 1 displayed no significant difference between + and −ATR groups. In addition, the percent change in HR at timepoint 1 in the ple-Gal4 X UAS-Chr2–XXL line displayed a significant difference between the groups (Fig. 2c2); however, in this case, the +ATR group displayed a less robust increase in HR compared to the −ATR group. Consistent with the room temperature data, no significant differences were observed in the ppk-Gal4 X UAS-Chr2–XXL or Trh-Gal4 X UAS-Chr2–XXL lines, and, unlike at room temperature, no significant difference arose within the Trh-Gal4 X UAS-Chr2–H134RII-mcherry line (Fig. 2).

Chemical modulation of heart rate

Given that ATR supplementation was shown to produce a minimal difference in the responses when compared to the −ATR groups, we assessed the role of neural-based chemical modulation in flies fed a diet supplemented with ATR (1 mM) to remove the additional dietary variables. As noted, we targeted several populations of neurons that release modulators and/or hormones that have previously been shown to influence HR in a semi-intact larval preparation. The average percent changes in HR upon exchange from white light to blue light, followed by a 1-min waiting period (1-Fig. 3) and at the succeeding 10-min timepoints following the initial exchange (2–5-Fig. 3) were calculated for each fly line and compared to a control line (Fig. 3). This analysis was performed at 22 °C (Fig. 3a) and in a room with a constant temperature of 10 °C (Fig. 3b).

At 22 °C, upon optic stimulation, release of acetylcholine and activation of target populations of cholinergic neuronal signaling resulted in an average positive percent change of 4.74%, which represented a significant difference compared to the control line, which displayed a negative percent change of −1.48% (Fig. 3a; Rank Sum Test p < 0.05) from baseline. Likewise, the initial activation of UAS-Chr2–XXL in dopaminergic and serotonergic (5-HT) neurons induced significant increases in HR, with average percent changes from a white light to blue-light stimulus of 3.87 and 7.95%, respectively (Rank Sum Test p < 0.05; p < 0.03, respectively). Because it has been shown that 5-HT exhibits a strong influence on HR in both room temperature and acute cold settings in situ (Majeed et al. 2014; Zhu et al. 2016b), the expression of the less sensitive ChR-2 variant (H134RII-mcherry) was also driven in 5-HT neurons. At 22 °C, the activation of this variant induced a significant positive percent change in HR following a 1-min waiting period as well (Fig. 3a) (Rank Sum Test p < 0.05). Only one line exhibited an inhibitory influence on HR upon activation. Blue-light activation of class IV da neurons induced a slight negative percent change of −0.47% in HR, which does not represent a significant difference when compared with the change in the control line (Fig. 3a).

Therefore, after the initial stimulus and subsequent release of neuromodulators/hormones into the hemolymph, with the exception of the ppk line, the activation of all targeted neuronal populations elicited a positive influence on HR.

While the influence of blue-light stimulation induced a positive percent change in HR in four of the five lines tested at room temperature, the subsequent changes in HR at the succeeding timepoints were less predictable across each line. Ten minutes following the count after the preparations were exposed to blue light, and four out of five lines displayed a negative percent change. These changes mirrored closely the change in the control line, which exhibited a −2.94% reduction in HR from the previous timepoint (an additive drop of approximately 4.4% from baseline) (Fig. 3a). The only line that displayed a continued increase in HR from the previous count was the 5-HT line expressing Chr2-H134RII, which showed a positive percent change of approximately 3.2% (Fig. 3a). Therefore, it is apparent that the excitatory influence from the modulators or hormones released from the targeted neural populations in four out of the five experimental lines tested was diminished after 10 min. However, upon continued observation after an additional 10-min period, an increase in HR was observed in three of the five experimental lines. Specifically, the line expressing Chr2–XXL in cholinergic neurons (Cha-Gal4) and the line expressing Chr2–XXL in dopaminergic neurons (ple-Gal4) displayed positive percent changes from the previous timepoint measured. The increase in HR continued following an additional 10-min
Fig. 2  Efficacy of all-trans-retinal supplementation. The average percent changes in HR were calculated at four timepoints: (1) 1 min following blue-light exposure and subsequent 10-min intervals following the initial change to examine the change in HR with continued blue-light exposure (2→5) in room temperature (22 °C) (column 1) and in cold temperature (10 °C) (column 2). The average percent changes at each timepoint Mann–Whitney Rank Sum Test was used for analysis, (**p < 0.01; *p < 0.05). The influence of ATR was shown to be minimal in inducing significant differences between the groups.
period in the *ple-Gal* X *UAS-ChR2–XXL* line, as the HR increased from the previous timepoint measured approximately 6.8% on average (Fig. 3a). In addition, the *Trh-Gal4* X *UAS-ChR2-H134RII–mcherry* (serotonergic) line displayed a positive percent change of 3.5% from the previous timepoint (from 4 to 5) (Fig. 3a). Unlike the positive percent changes in HR observed upon the initial change from white light to blue light, the positive percent changes displayed by these lines at the subsequent timepoints did not represent statistically significant increases relative to control (Rank Sum Test; \( p > 0.05 \)). Likewise, negative percent changes observed at timepoints beyond initial change (timepoints 2 through 5) did not exhibit statistically significant reductions relative to the control line, with one exception: the *ppk-Gal4* X *UAS-ChR2–XXL* (class IV da sensory neurons) line displayed negative percent change of −29.1%, representing a significant change (Rank Sum Test; \( p > 0.05 \)) (Fig. 3a). Therefore, it is apparent that the change in HR after a 10-min exposure to blue light was minimal across all lines; however, the initial enhanced rate observed following the initial exposure to blue light was sustained in four out of six lines tested, with the exceptions being the control line and the *ppk-Gal4* X *UAS-ChR2–XXL* (class IV da sensory neurons) line. Only these two lines displayed HRs that dropped below the initial HR observed under a white light stimulus, signifying that, although we did not observe a continued increase in HR upon constant blue-light exposure, the release of the targeted modulators elevated rates throughout the entire experimental time period from the initial baseline counts.

As mentioned previously, the change in HR in response to activation of select neural populations was observed at 10 °C. Upon the initial change to blue light, a positive percent change in HR was observed in all six lines tested, including the control (Fig. 3b). Specifically, four out five of the experimental lines displayed a higher percent change relative to the control, with the *Trh-Gal4*
X UAS-ChR2-H134RII-mcherry line displaying a significant positive percent change of 7.5% (Fig. 3b) (Rank Sum Test; \( p < 0.05 \)). While at 22 °C, the ppk-Gal4 X UAS-ChR2–XXL line exhibited a consistent negative percent change in HR throughout the experimental time course, there was a positive percent increase in HR in this line in the cold environment. Consistent with what was observed at room temperature, the initial increase in HR observed after exchange to blue light was abolished after a 10-min period of constant blue-light exposure in four out of six lines tested, with only the Cha-Gal4 X UAS-ChR2–XXL (2.3%) and Trh-Gal4 X UAS-ChR2–XXL (1.4%) lines displaying continued increases in HR (Fig. 3b). Neither of these increases, however, were statistically significant relative to the control line, which exhibited an average negative percent change of −8.4% (Fig. 3b) (Rank Sum Test; \( p < 0.05 \)). In addition, following 20 min of constant exposure, from timepoints 3 to 4, the HR in all lines remained relatively constant, with three out of six lines displaying an average positive percent change in rates and two, Cha-Gal4 X UAS-ChR2–XXL and Trh-Gal4 X UAS-ChR2–XXL, displaying average negative percent changes of 4.2 and −2.1%, respectively, which represents a stabilization from the previous timepoint. Likewise, the percent change from timepoints 4 to 5 also displayed minimal changes in HR in all lines tested, with no significant differences in rate changes relative to the control (Fig. 3b).

In both environments, the initial exchange from white light to blue-light-induced positive percent changes in HR in all experimental lines tested with one notable exception being the ppk-Gal4 X UAS-ChR2–XXL at 22 °C. The control lines exhibited slight changes in HR upon exposure to blue light, with a negative percent change observed at room temperature and a small positive percent change observed at 10 °C. The significant changes observed upon initial exchange to blue light were diminished in each line, and a continued significant increase was not observed; however, at both temperatures, the rates that increased in the experimental lines never fell back below baseline. Therefore, it
was noted that the HRs stabilized after 10 min of constant blue-light exposure (Fig. 3).

Discussion

Here, we present the first study on the role of targeted neural-based hormones/modulators on modulation of heart rate (HR) in *Drosophila melanogaster* larvae. We have illustrated that the utilization of optogenetics is useful in assessing the neural-based influence of modulators on cardiac function. In adding to the ever-increasing literature regarding the chemical and mechanical modulation of HR, we have further enhanced understanding of cardiac function in *Drosophila* and progressed its use as a tractable model for the translational studies. In doing so, we have also progressed understanding of the efficacy with which one can utilize optogenetics in studies related to physiological processes not directly pertaining to the neural basis of behavior.

Influence of blue-light stimulation alone is minimal in inducing changes in heart rate

We have shown that the influence of blue light by itself on the activation of endogenously released cardioactive modulators on cardiac function is minimal. In each setting utilized (i.e., cold and room temperature; ±ATR), the role of blue light alone in stimulating release of modulators/hormones that may influence heart function was not significant. While it may appear disingenuous, recent analysis has shown the influence of blue light on *Drosophila* larval behavior to be robust, even in the absence of the important visual organ (Xiang et al. 2010). Therefore, it is important to understand the potential impacts of utilizing optogenetics on cardiac function, particularly as it relates to long-term, developmental studies. In performing this analysis, we noted an interesting characteristic. As can be seen in the preceding figures, there is a high degree of variation in HR even within an individual preparation. We noted that when intact larvae are stuck to tape as was performed in this analysis, they still maintain their ability to initiate body wall contractions. The body wall contractions cause brief, periodic pauses in heart contraction, therefore, modulating HR for a given time period. Others who have performed similar techniques have noted this occurrence. A study by Sénatore et al. (2010) identified a crucial mechanoreceptor, *Painless*, that is essential in mediating response to the body wall contraction-induced mechanical perturbation of cardiac tissue. Although we did not directly correlate contraction occurrence with altered HR, the variation within preparations could very likely be explained by this phenomenon.

Retinal supplementation effect is minimal in neural-based influence on heart rate

In addition, we have shown that the supplementation of all-trans-retinal (ATR) at a concentration of 1 mM is minimal in its influence in significantly changing the cardiac response to release of targeted modulators. Out of the total 20 timepoints tested in the two environments, a significant difference between the +ATR groups and −ATR groups was observed in the *Cha-Gal4 UAS-ChR2–XXL* line (at both room and cold temp) and in the *Trh-Gal4 UAS-ChR2-H134RII-mcherry* line (at room only) upon the initial exchange from white light to blue light. In these cases, the ATR group displayed a significantly greater positive percent change relative to the −ATR group. In addition, the +ATR group displayed a less robust response in the *ple-Gal4 UAS-ChR2–XXL* line at 10 °C relative to the −ATR group, which represented a statistically significant difference. While we have noticed in our behavioral analyses, using both larval and adult *Drosophila*, that ATR supplementation is significant in enhancing responses to a blue-light stimulus, the results here suggest that the addition of this cofactor does not induce significantly varied responses between the majority of treatment groups expressing the ChR2–XXL variant in the targeted neurons. The difference in efficacy of ATR supplementation in regard to the functioning and expression of ChR2 variants, including ChR2–XXL, has been detailed previously (Dawydow et al. 2014). It has been shown that the supplementation of ATR enhances ChR2 photocurrent amplitude in response to blue light when expressed in host cells and it is suggested that this is due in large part to the reduced degradation of the translated protein when associated with the ATR cofactor; however, retinal supplementation is not required for functioning of the ChR2–XXL variant (Dawydow et al. 2014). Therefore, the supplementation of ATR is assumed here, and has been shown previously, to increase the abundance of the channels in the cell membrane, likely the primary factor underlying the enhanced photocurrent in relation to a −ATR-treated preparation (Dawydow et al. 2014). Thus, we predicted to see a significant difference between our +ATR and −ATR groups as we suspected an enhanced release of targeted modulators in the lines expressing ChR2–XXL, even though it has been shown to function without retinal. Moreover, we predicted to see a significant difference between groups in the line expressing the less sensitive channel-rhodopsin variant (ChR2-H134RIImcherry) due to the idea that this variant has shown to be significantly less responsive without ATR supplementation (Dawydow et al. 2014). It is important to note that we did indeed notice a significant difference upon the initial blue-light stimulation in this line at room temperature, as there was a negative percent change in the −ATR group. This is
likely due to the significantly reduced responsiveness and sensitivity of this variant in the absence of ATR. As for the ChR2–XXL expressing lines, it is of interest that the differences across the lines were minimal between the groups. We suspect the need for an abundant release of modulators/hormones into the hemolymph to observe an effect on cardiac function. While the difference in quantity of neurotransmitter/modulator release at synapses in the nervous system as a result of ATR presence may induce obvious changes in neural circuit function, the action of enzymes in breaking down the released substances or their re-uptake by neurons or glia may dampen their influence on tissue distant from the source of release. High release of these neurotransmitters may even desensitize target receptors within the CNS, which then affects activity properties of a targeted neuroendocrine cell. It is not known if the neurons stimulated directly raise the transmitter, they release into the hemolymph as entire neural circuits are also modulated by these compounds. Thus, the relative difference in efficacy of responses in a non-neuronal tissue as a result of ATR-mediated enhancement of neuromodulator release is likely less pronounced. We suspect this to be the case in this situation, as the larval heart is not innervated directly by nerves, and therefore, the modulator/hormonal action on the heart requires transport through the hemolymph. It is assumed that the difference in modulator release in the presence of ATR versus in the absence of ATR is insufficient in producing a significant alteration in HR. Future experiments, including high performance liquid chromatography (HPLC), can be performed to test the relative concentration of modulators released into the hemolymph following blue-light activation of various neural populations via different ChR2 variants in the presence and absence of ATR to follow up on these questions as they pertain to neuroendocrine influence on physiological functions.

Release of targeted modulators enhance heart rate upon initial stimulation

The importance of investigating the neural basis on influence of vital organs including the heart in Drosophila is highlighted by the fact that an autonomic nervous system in invertebrates, including insect, is known to play a crucial role in regulating the function of vital organs. Anatomical and behavioral studies of a potential autonomic system in invertebrates were started back in the 1920s and 1940s by J. U. Orlov and A. A. Zavarzin (Orlov 1926, 1927, 1929; Zavarzin 1941; Nozdračhev 1983, 1996; Nozdračhev and Bagaev 1983; Shuranova et al. 2006). Just as for higher organisms, invertebrates require behaviors that allow for escape from predation or danger. Drosophila larvae show a nocifensive response with a characteristic “corkscrew-like roll” behavior when confronted with a parasitic wasp (Hwang et al. 2007; Sulkowski et al. 2011; Robertson et al. 2013) or strong aversive stimuli (Titlow et al. 2014). A rapid and robust movements of a larva, which does not possess neural stimulation of the cardiac tube, may require humoral factors to increase HR for distribution of endocrine factors and nutrient supply to activate the skeletal muscles to maintain active escape responses. In addition, environmental factors such as cold may require the cardiac system to remain functional, so that response to stimuli is maintained and appropriate nutrient dispersal for regulation through transitional stages, such as with cold hardening or conditioning for longer term cold survival, is conserved. Cold conditioning in some insects involves osmolarity changes, antifreeze proteins, or compounds to be distributed throughout the organism (Ring 1982; MacMillan et al. 2015). It is possible that neuroendocrine hormones help to maintain cardiac function during an environmental transition (Zhu et al. 2016b). Previous analysis in a semi-intact system has shown that the heart is stable at 10 °C; however, the exposure to modulators had varying effects on the heart at this temperature, suggesting unique roles of modulation of the heart at low temperature. In addition, the average HR in the exposed heart is much lower at this temperature, compared to the HR in intact larvae. We have begun to address these questions by targeting subsets of sensory and interneuron populations that may be important in regulating larval heart rate in response to environmental changes. While previous analysis has implicated modulators important in regulating HR, whether these modulators affect cardiac function in a similar manner through release from the nervous system in response to changes in the state of the animal has not yet been addressed.

As stated above, it has been shown using semi-intact preparations that the application of acetylcholine (Ach) (Malloy et al. 2016), dopamine (DA) (Titlow et al. 2013), and serotonin (5-HT) (Majeed et al. 2014) each induced increases in HR in a dose-dependent manner. Additional analysis has identified octopamine as an important modulator in regulating HR as well, as it has been shown to decrease HR in cold environment (Zhu et al. 2016a, b). In these studies, semi-intact preparations were utilized for analysis, which enabled control of the concentration of the modulator that was exposed directly to the heart. While the concentrations of circulating modulators as a result of activation of our targeted neuronal populations are not clear here, we presume that the concentrations of the different modulators are greater than what has been identified in vivo. It has been shown that DA modulates peripheral organs through circulation at the micromolar range (Matsumoto et al. 2003). The circulating concentration of 5-HT and Ach is unknown under normal conditions, but given the abundance of cholinergic affenter sensory neurons and Ach and 5-HT interneurons, we anticipate the release of...
Ach, DA, and 5-HT through targeting a host of cells augments circulating concentrations. Regardless, our findings here correspond remarkably similarly with the semi-intact analyses at room temperature, in that activation of neurons that release the modulators tested in these previous studies showed positive influences on HR. Specifically, the initial increase in HR upon release of Ach, DA, and 5-HT at room temperature represented significant increases relative to the control line. Of note, the only line that displayed a negative percent change after the initial blue-light exposure and upon subsequent activation was the ppk-Gal4 X UAS-Chr2-XXL line. While it is assumed that the activation of all cholinergic neurons through use of the Cha-Gal4 driver likely causes a substantial increase in hemolymph Ach concentration, use of the ppk-Gal4 driver targets only a subset of dendritic arborization sensory neurons (class IV) and the corresponding increase in hemolymph Ach concentration is lower. Activation of this subset of neurons is known to be both required and sufficient in regulation of response to nociceptive stimuli (Hwang et al. 2007; Xiang et al. 2010; Johnson and Carder 2012; Kim et al. 2013; Kim and Johnson 2014). The subsequent behavior initiated, including the strong ‘corkscrew’ like roll described previously, comes at an energetic cost. We, therefore, thought that we may observe changes in HR as a result of activating circuits that may release cardioactive substances that could enhance HR to provide necessary endocrine factors assisting in skeletal muscle activation. It was surprising that we detected a decrease in HR that continued throughout the experimental time course.

Likewise, each experimental line tested at 10 °C exhibited an average positive percent change with four out of the five lines displaying a change that was higher than the control line following the initial blue-light exposure. This result is rather interesting in that it has previously been shown that the application of exogenous DA and Ach after acute cold (10 °C) exposure induces negative percent changes in HR; however, 5-HT induces a positive percent change in a semi-intact preparation (Zhu et al. 2016b). It is important to note that the baseline rates in the intact preparation and the semi-intact preparations vary greatly and this may be due to the lack of synergistic effect on HR that may be present in the whole animal, as the physiological saline used in the semi-intact approach may lack additional cardioactive substances. However, we show here that the enhanced responses in the cold temperature matched closely with the room temperature observations, suggesting that the initial excitatory responses observed at room temperature were preserved at 10 °C.

In addition, it was noted that the elevated responses observed following blue-light activation were suppressed after 10, 20, and 30-min continued exposure. The relative stability of HR in the experimental lines following these time periods shows that the action of the modulators in driving an increase in HR was diminished; however, as noted, the HRs in each case were elevated above baseline throughout the experimental time course. It is, therefore, apparent that the excitatory responses exhibited in response to the circulation of the modulators in the hemolymph were sustained. Interestingly, the change in the rates over time in the intact animal and in the semi-intact preparations followed an amazingly similar trend. In each case, the initial response to the application of a controlled concentration of modulator was noted and the change in HR following a 10-min continued exposure was calculated for Ach, 5-HT, and DA (Titlow et al. 2013; Majeed et al. 2014; Malloy et al. 2016). In response to each modulator, the first minute following the exchange of a solution with an added modulator, the HRs displayed positive percent changes; however, after a 10-min exposure, the positive percent change was dampened but remained above baseline (Titlow et al. 2013; Majeed et al. 2014; Malloy et al. 2016).

We initially considered the possibility that the stabilization in HR over time observed here could be due to reduced probability of release of our targeted modulators from the nervous system. We considered that the continuous exposure to blue light might desensitize the rhodopsin channels, thereby reducing cation current and subsequently reducing vesicle fusion and release. Alternatively, we considered the potential that enhanced Ca2+ and/or Na+ influx over time may induce depletion of readily releasable vesicles or may cause neuronal refractory though Na+ channel inactivation or Ca2+-dependent K+ channel activation. While there may be some contribution due to synaptic depression, additional use of optogenetics in our behavioral analyses, whereby excitatory responses at the neuromuscular junction are observed well beyond the time course observed here, suggests that this is not likely the cause in diminished enhancement of HR over time. Although it has been shown that there is spike frequency adaption in neurons expressing different ChR2 variants, including Chr2-H134RII (Pulver et al. 2009), in response to constant blue light, the release of modulators persists, and the robust behavioral effects observed in our analysis suggests that the channels remain functional. We suspect the diminished increase in HR corresponds to reduced responsiveness of the heart to continued modulator interaction. The similarities observed in the semi-intact studies help to corroborate this notion. We reason that the action of these modulators increases HR enough to meet the energetic demands of the animal through the initial activation of receptors expressed in cardiac tissue. The energetic cost of continued enhancement in HR in response to prolonged modulator action is likely unnecessary and inactivation of intracellular cascades and/or receptor desensitization in cardiac tissue may result. Follow-up examinations manipulating receptor expression...
in the heart in the presence of continued blue-light activation may help to address this question and also may shed light on the receptor subtypes that may be important in regulating the changes in HR in response to physiological and environmental changes. This may help to strengthen the work that has been done in previous analyses identifying receptors that mediate the positive chronotropic responses and increased rates in Drosophila larvae.

Conclusion and future studies

We have identified, through the use of an optogenetic technique, neural populations that display modulatory effects on HR in an intact larva and have shed light on the probability that humoral factors are likely at play in modulating HR under a variety of conditions. The responses in HR correlate with what has been observed in semi-intact preparations in prior studies. We have shown that Ach, DA, and 5-HT may play important roles in regulating HR in response to environmental changes. While the neurons targeted in this study are known to release these modulators, we cannot rule out the synergistic effects that may arise from release of other cardioactive substances that were not directly targeted in this study. Given that the activation of these neural populations likely causes release of additional modulators/hormones, it would be important to address the circulating concentrations of other endocrine factors that may modulate HR. Future analysis of the hemolymph may be performed using high performance liquid chromatography (HPLC) or mass spectrometry following optogenetic stimulation of various neuronal ensembles to address this, and subsequent analysis using multiple techniques can be used to identify additional compounds that may be crucial in regulating Drosophila cardiac function. Further investigation into the receptors that mediate these neuroendocrine influences should be performed as well. Cardiac tissue-specific RNAi knockdown of various receptor subtypes and neuronal activation and analysis of cardiac response may help to assess the mechanisms underlying neuroendocrine regulation of HR in addition to what has already been reported. In addition, the use of optogenetics in long-term developmental assays is coming to the forefront. As a result, it is important to identify potential detrimental effects that may arise from chronic stimulation of subsets of excitable cells. Due to the fact that a number of targeted neuronal populations used in this study have been shown to release cardioactive modulators, it stands to reason that use in long-term studies targeting similar cells may affect heart function and development. Not only is it important to identify the effects of systemic neuromodulator and hormones on acute heart function, but also allows for the investigation of the potential detrimental effects of long-term optogenetic studies involving indirect influence on vital organs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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