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ORIGINAL PAPER

Pharmacological identification of cholinergic receptor subtypes on *Drosophila melanogaster* larval heart

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Abstract The *Drosophila melanogaster* heart is a popular model in which to study cardiac physiology and development. Progress has been made in understanding the role of endogenous compounds in regulating cardiac function in this model. It is well characterized that common neurotransmitters act on many peripheral and non-neuronal tissues as they flow through the hemolymph of insects. Many of these neuromodulators, including acetylcholine (ACh), have been shown to act directly on the *D. melanogaster* larval heart. ACh is a primary neurotransmitter in the central nervous system (CNS) of vertebrates and at the neuromuscular junctions on skeletal and cardiac tissue. In insects, ACh is the primary excitatory neurotransmitter of sensory neurons and is also prominent in the CNS. A full understanding regarding the regulation of the *Drosophila* cardiac physiology by the cholinergic system remains poorly understood. Here we use semi-intact *D. melanogaster* larvae to study the pharmacological profile of cholinergic receptor subtypes, nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs), in modulating heart rate (HR). Cholinergic receptor agonists, nicotine and muscarine both increase HR, while nAChR agonist clothianidin exhibits no significant effect when exposed to an open preparation at concentrations as low as 100 nM. In addition, both nAChR and mAChR antagonists increase HR as well but also display capabilities of blocking agonist actions. These results provide evidence that both of these receptor subtypes display functional significance in regulating the larval heart's pacemaker activity.

Keywords Acetylcholine · Nicotinic receptor · Muscarinic receptor · *Drosophila melanogaster* · Heart · Pharmacology

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Introduction

The *Drosophila melanogaster* larval heart is a popular cardiac disease model for mammalian heart pathologies. Various studies have shown a number of genes in *Drosophila* regulating cardiac function, including muscle contractile proteins and ion channels, are similar to those in mammals (Bier and Bodmer 2004; Cammarato et al. 2011; Ocorr et al. 2007; Wolf et al. 2006). In addition, because of the wealth of molecular tools available to alter expression of ion channels and membrane receptors, one can utilize this organism to better understand the physiological mechanisms which may underlie dysfunctions that are manifested in cardiac disease states. *Drosophila* use many of the same neurotransmitters and receptor subtypes as mammals and use similar mechanisms for transmitter release, recycling and general neuronal function (Hurst et al. 2013; Martin and Krantz 2014). One of these neurotransmitters, acetylcholine (ACh), is prominent in the nervous system and has been confirmed to exhibit modulatory effects on various tissues within *Drosophila*. In vertebrates, ACh is a chemical transmitter of the autonomic, somatic, and central nervous system (CNS). In insects, it is the predominant excitatory neurotransmitter of the sensory neurons and interneurons within the CNS (Martin and Krantz 2014). Acetylcholine receptors (AChRs) consist of two major

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subtypes: the metabotropic muscarinic acetylcholine receptors (mAChRs), and the ionotropic nicotinic acetylcholine receptors (nAChRs), both of which are activated by ACh and the agonists, muscarine and nicotine, respectively. The nicotinic receptor is part of the cys-loop family of ligand-gated ion channels that facilitates fast synaptic transmission (Wonnacott and Livingstone 2010). Muscarinic receptors are metabotropic and act indirectly with ion channels through second messenger G proteins to generate a cellular response (Collin et al. 2013). The *Drosophila* genome contains ten nAChR subunits and two mAChR types, A-type (encoded by gene CG4356) and B-type (encoded by gene CG7918), have been cloned in this organism (Collin et al. 2013). The expression of these subunits and pharmacological profiles had not been characterized in the larval heart.

Drosophila have an open circulatory system that consists of a simple dorsal vessel with a posterior heart and anterior aorta. The larval dorsal vessel is a myogenic tube that spans the rostral: caudal axis of the animal (Gu and Singh 1995). 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 myogenic (Dowse et al. 1995; Gu and Singh 1995; Johnson et al. 1998, 2001; Rizki 1978) meaning action potentials in this tissue are initiated in the absence of neural innervation within the cardiac muscle itself (Cooper et al. 2009; Desai-Shah et al. 2010). In the late 3rd instar there appear to be neurons innervating the rostral tissue of the aorta, but the function of this innervation has not been addressed (Johnstone and Cooper 2006). Because of these characteristics and additional similarities in physiology and ease of manipulating developmental expression of genes, the *Drosophila* larval heart can be used as a model for ionotropic and chronotropic actions as well as investigations into the ionic basis for pacemaker activity.

In mammals, the cholinergic system is implicated in a number of cardiac diseases. In fact, studies show that cardiac regulation by the parasympathetic nervous system is mediated primarily by ACh binding to the M_2 muscarinic ACh receptor (M_2-AChR) in many vertebrates (Gavioli et al. 2014). In insects, neuromodulators travel in the hemolymph and affect non-neuronal tissues in addition to acting as the primary mediator of communication between cells of the nervous system (Majeed et al. 2014). A number of neuromodulators that are prominent in larvae, including dopamine (Neve et al. 2004; Titlow et al. 2013), serotonin (Dasari and Cooper 2006; Majeed et al. 2013) and octopamine (Johnson et al. 1997), have also shown to exhibit modulatory effects on the heart. It has previously been shown that ACh at concentrations between 1 mM and 1 M, decreases heart rate (HR) in *Drosophila* at the larval, pupal, and adult stages with no significant changes at concentrations lower than 1 mM (Zornik et al. 1999); however, these studies were performed in the intact, whole animal with injections into the hemolymph. Many compounding actions may come into play with the stress of injections and the presence of other cardioactive substances other than those injected. Additionally, the pharmacological characterization of the cholinergic receptor subtypes involved in modulating HR has not been characterized in isolation of compounding variables with a well-defined physiological saline. The pupal metamorphic stage is also an active period of transition in hormones and development not only for the skeletal muscle and the nervous system but also the heart (Consoulas et al. 2005; Zeitouni et al. 2007).

This stage in *Drosophila* development is commonly used for investigating cardiac function since the pupa is stationary for injection and observation, but the dynamic process in this transitional stage make it somewhat problematic. In addition, the adult heart is modulated by neuronal inputs, which complicates addressing the function of the intrinsic cardiac pacemaker and ionic regulation in an intact heart (Dulcis and Levine 2003, 2005). The larval heart is easily exposed, myogenic, and its activity can be maintained for hours with a newly developed physiological saline (de Castro et al. 2014). Whereas previous research has utilized intact pupa or larvae with drug administration via injection, we directly expose an open preparation with pharmacological agents at known concentrations. This technique isolates the heart from the nervous system and prevents the action of additional modulation from various endogenously released substances.

Because regulation of the *Drosophila* cardiac physiology by modulators remains poorly understood, it is important to determine how endogenous modulators separately act on, and influence cardiac pacemakers in altering HR. The aim of this research is to gain insight into the role of the cholinergic system and specific receptor subtypes in modulating the *D. melanogaster* larval heart. The findings of this study enhance our understanding of the role of modulators and ion channels in affecting HR, adding to the ever-increasing knowledge regarding endogenous messengers on cardiac tissue. Homologous genes control early developmental events as well as functional components of the *Drosophila* and vertebrate hearts (Bier and Bodmer 2004); thus, the fly is a useful model in which to study cardiac function and the molecular mechanisms underlying heart disease in humans. Mutations affecting ion channels and second messenger systems are readily accessible in *Drosophila*, and it is important to understand the pharmacological profiles of specific receptors in order to utilize these mutants to study the mechanisms which regulate cardiac function.

Materials and methods

Fly rearing and stocks

Wild type *Canton S* (CS) flies were used for HR analyses via the semi-intact method. This strain has been isogenic in the lab for several years and was originally obtained from Bloomington Fly Stock. In order to obtain staged larvae, the flies were held for a few days at 25 °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).

Pharmacology

Acetylcholine (CAS #: 60-31-1), nicotine (CAS #: 65-31-6), clothianidin (CAS # 210880-92-5), muscarine (CAS #: 2936-25-6), atropine (CAS #: 51-55-8), and scopolamine (CAS #: 6533-68-2) were purchased from Sigma-Aldrich (St. Louis MO, USA) (Milwaukee WI, USA). Tubocurarine (curare) (Cat #:2820) and benzoquinonium dibromide (Cat #:0424), were purchased from Tocris Bioscience (Minneapolis, MN, USA). Fly saline, modified Hemolymph-like 3 (HL3) (Stewart et al. 1994) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25*N*,*N*-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) was used. The following modifications were made to the HL3 saline: pH was decreased from 7.2 to 7.1 and BES buffer was increased from 5.0 to 25.0 mmol/L to maintain a stable pH (de Castro et al. 2014).

Heart rate assay

Semi-intact preparations were used throughout. After collection, early third instar larvae were pinned ventral side up on a glass plate and dissected in a droplet of saline (Cooper et al. 2009). The *Drosophila* heart is very sensitive to pH (Gu and Singh 1995); therefore, the saline is adjusted to pH 7.1 and maintained with the high concentration of buffer as described in de Castro et al. (2014). The larval dissection is described in detail by Gu and Singh (1995) and in video by Cooper et al. (2009). An illustration of the preparation used can be found in Desai-Shah et al. (2010). In short, third instar larvae were opened by an incision in the ventral midline and the internal organs were washed aside by saline in order to expose the intact heart to various solutions. The preparation was then left untouched for 2 min after dissection to allow the heart to recover from the larval dissection. The heart was then visualized through a dissecting microscope and the rate was measured by directly counting the contractions in the posterior "heart" region of the dorsal vessel. In order for ease of counting the HR, one can readily observe the trachea movements as a consequence of the heart pulling on the ligament attachments. The baseline counts were collected with saline and then the saline solution was carefully removed and exchanged with saline solutions containing various agents. The solutions, consisting of agonists and antagonists of both nAChRs and mAChRs at varying concentrations, were introduced onto the open preparation. After exchanging the saline with an agent of interest, the preparation was allowed to sit for 1 min prior to counting the HR. Following a 1 min waiting period, the heart contractions were examined for 1 min, in order to calculate the HR in beats per minute (BPM). After the initial 1 min count, the solution was left on the preparation for 10 min and a 2nd count was performed in order to measure the effects of the agents after a longer period. Hearts that did not continuously beat throughout the paradigm or did not reach 50 beats in 1 min upon initial exposure to saline were not used in our analyses. As a control, fresh saline was used to replace the first saline solution. Once the HR was counted, the average BPMs and percent change in initial HRs as well as the percent change in the HRs after a 10 min period were calculated and graphed. All the experiments were performed at room temperature (21–23 °C) during the hours of 9–5 pm.

Statistical analysis

The data presented is expressed as mean ± SEM. The program, SigmaPlot (version 12.0) was used for graphing and statistical analysis. One-way ANOVA test was used for multiple comparisons among the concentration treatments by each individual drug. Student's *t* test was used in order to compare the HR treatments to the controls, with a confidence level of $P \leq 0.05$ as considered statistically significant. Tukey's test was used as a post hoc test to compare the percentage changes of HRs.

Results

Mechanical disturbance and time effect on HR

As previously reported, mechanical disturbance plays a role in altering HR in a semi-intact, open preparation (Majeed et al. 2013). In addition, the HR generally slows down over time. In order to obtain a baseline reading for the effects of mechanical disturbance and time, control experiments were conducted in which saline was washed out and exchanged for fresh saline of the same composition. The newly added saline was then left on the preparation for 10 min in order to analyze the role of time on HR. A simple saline exchange resulted in a small increase in HR initially and a decrease over a time period of 10 min (Fig. 1a). In addition, the raw

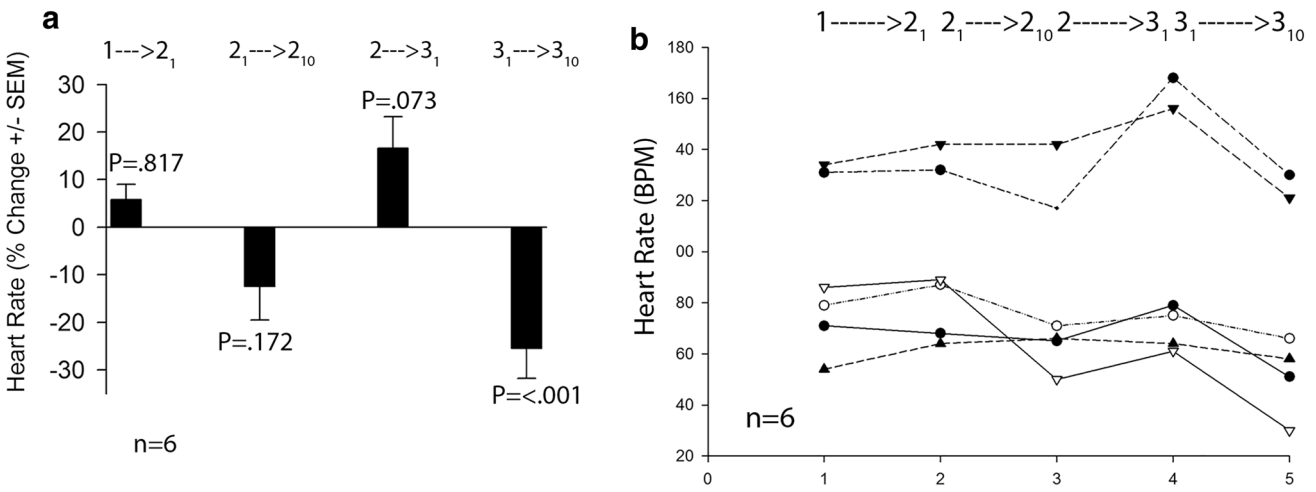

Fig. 1 Change in HR as a result of mechanical disturbance upon changing solutions. **a** The percent change in HR after exchanging saline solutions. The preparations were left inside saline for 1 min and then the rate was obtained for the following minute. Saline (1-Saline) was exchanged with saline (2-Saline). The preparations were left for 1 min and subsequently rate was obtained over the next minute. The preparations were left for 10 min (subscript 1 to 10) and then the HR was counted for 1 min. Saline (2-Saline) was exchanged with saline (3-Saline), the preparations were left for 1 min before counting the rate in the next minute. The preparations were left for 10 min and then the HR was obtained for 1 min. Data are presented as mean ± SEM. **b** The raw change in HRs in response to saline to saline solution exchanges. The changes in solutions are noted, with the subscripts illustrating time points during which solutions were left on the preparations (1 to 10 min period)

data for average BPM at five time points was recorded over a 10 min period for each individual preparation (Fig. 1b). The control experiment was used to account for changes in HR upon solution exchange when various compounds are added. Percent change in rates were compared to controls in order to obtain a true reading of the percentage change in HR due to the action of the added compounds. Results are provided as a percent change of basal rate since there were variations in baseline HRs among preparations, which were calculated based on initial saline counts for each separate trial. The initial change in HR increases 5.77 ± 3.22 % (Fig. 1a) after a saline to saline exchange and then drops 12.40 ± 7.03 % after 10 min bathed in saline. Exchanging saline for a second time, after the preparation is untouched for 10 min, induces a positive percent change of 16.60 ± 6.67 % before falling 25.40 ± 6.32 % following an additional 10 min period.

Acetylcholine dose–response relationship

After noting the change in HR induced by saline to saline exchange, the effect of ACh modulation on the heart was tested. Four different concentrations of ACh in saline were applied directly to the open preparation, and the percent change in HR after initial exchange and following a 10 min period was determined. 100 nM, 10 μM, 100 μM, and 1 mM concentrations of ACh in saline were used. Each concentration of ACh induced an initial increase in HR when compared with the saline to saline control (Fig. 2a, b, d). At the intermediate concentration tested, the average HR increased significantly when a saline solution was exchanged for one containing 10 μM ACh (Fig. 2b). Applying 100 nM concentration of ACh to the open heart induced an initial positive percent change of 26.3 ± 8.91 % from baseline, indicating an increase compared to control. The dose–response relationship reveals that increasing concentration of ACh did show a slight but insignificant increase in the mean percent change in HR (Fig. 2b). This indicates the ACh receptors may be saturated and desensitized after exposure to ACh concentrations as low as 100 nM. Data for each concentration of ACh was graphed and displays the variation in alteration in HRs over the 10 min time course. The averages in the responses are shown in Fig. 2a. The data indicates that there were variations among baseline rates among preparations; however, at each concentration, ACh displayed a positive effect on the HR. In addition, the preparations exposed to ACh did not show dramatic reductions in HR after a 10 min period, suggesting that the addition of ACh to saline helped stabilize the hearts for a more extended period. This is in contrast to controls, which showed more dramatic reductions in HR over the full experimental time period (Fig. 2a, d).

nAChR and mAChR agonists dose–response relationship

Following examination of the effect of ACh on the heart, the role of the three primary cholinergic agonists in modulating

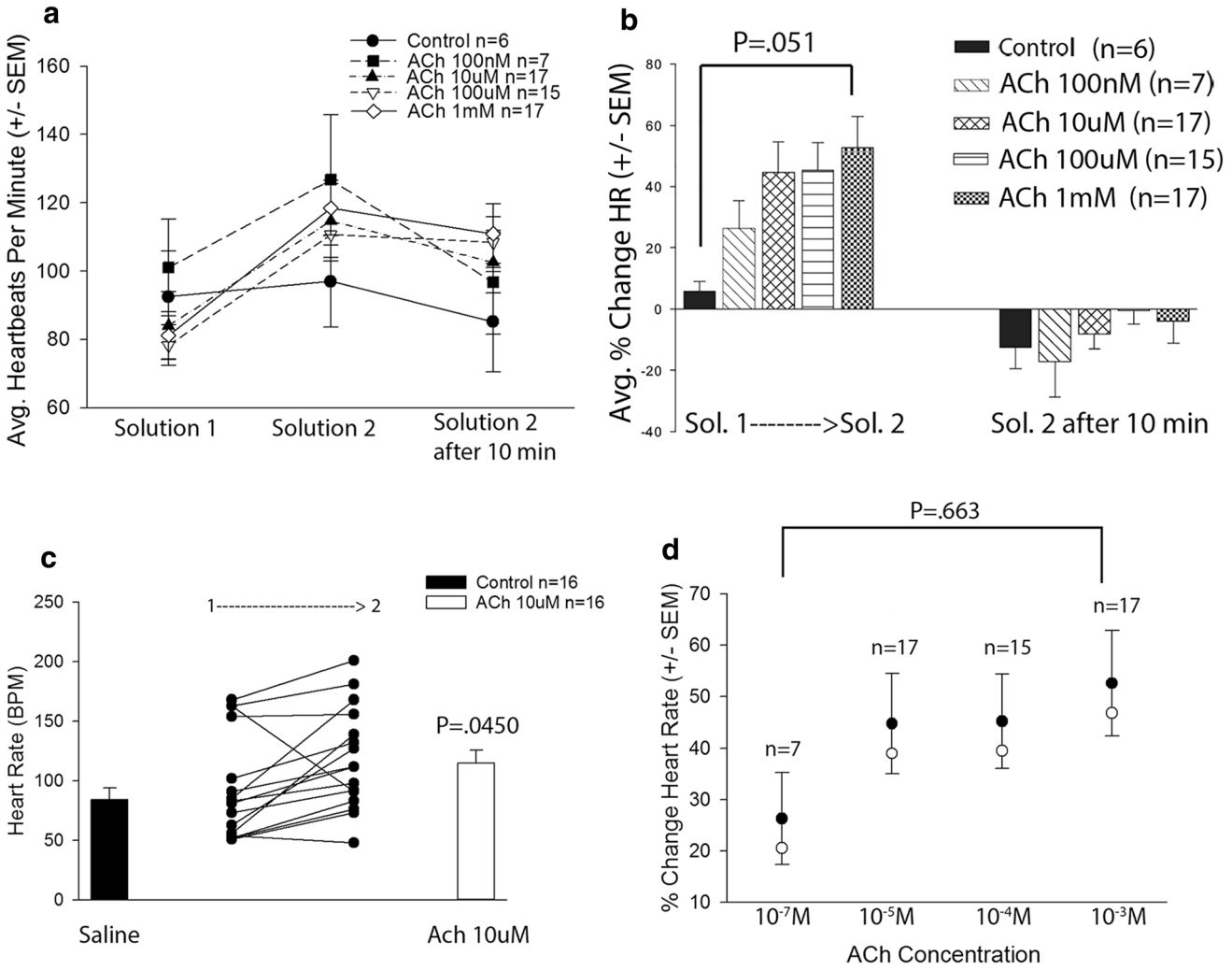

Fig. 2 Change in HR in response to various concentrations of ACh. **a** The average change in HR in response to saline (solution 1) to saline + ACh (solution 2) exchanges. At each concentration, ACh induced a more substantial change in beats per minute (BPM) when compared to controls as evidenced by the increased slope. In addition, preparations bathed in ACh solutions for 10 min displayed less dramatic reductions in HR after the time period. **b** The percent changes in HR after exchange from solution 1 to solution 2. **c** Change in average HR in exchange from saline to ACh 10 µM with raw changes for each preparation. The addition of ACh induced a significant change in average HR. (Student's *t* test was used for comparison). **d** Dose–response relation of ACh action on larval HR. *Open circles* represent the subtraction of control saline exchanges from various concentrations of ACh. One-way ANOVA was used for comparison

HR was examined. Nicotine and clothianidin concentrations of 100 nM, 10 µM, 100 µM, and 1 mM were exposed to open preparations. Muscarine concentrations of 100 nM, 10 µM, and 1 mM were used in order to reveal a dose–response relationship. For each concentration tested, new larvae were used. The initial percent change after solution exchange as well as percent change after a 10 min period was calculated and is shown in Fig. 3a. Average HR counts for hearts exposed to 10 µM of each agonist solution were also calculated and agonists that induced significant changes in HR are presented (Fig. 3b, c). In addition, the dose–response curve for each agonist was analyzed and displayed (Fig. 3d). Exposure to nicotine at a concentration of 100 nM increased HR, displaying a percent change of 25.54 ± 9.82 % from baseline (Fig. 3a). Exposure to nicotine increased average HR significantly at a concentration of 10 µM upon initial exchange (Fig. 3b), displaying a percent change of 74.43 ± 19.44 %. At higher concentrations, the percent change was not as dramatic. In addition, after bathing the preparations in nicotine, the HRs did not slow down as dramatically as preparations exposed to saline without added nicotine. The average decrease in HR after 10 min for each of the preparations exposed to nicotine was approximately −7.93 ± 6.04 % BPM for all concentrations whereas the preparations bathed in saline alone showed a decrease of approximately −12.48 ± 7.03 % BPM (Fig. 3a). Nicotine

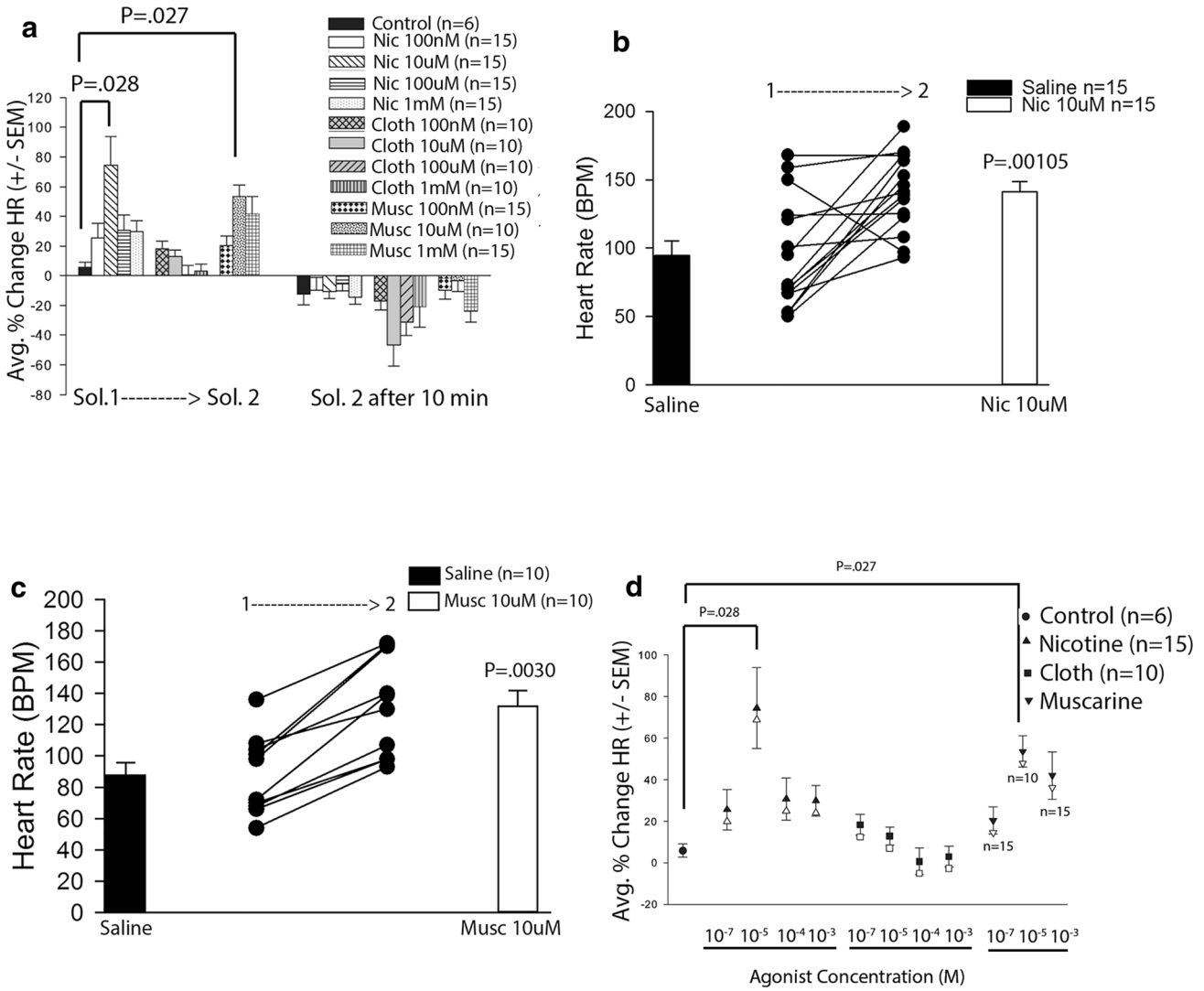

Fig. 3 Change in HR in response to various concentrations of AChR agonists. **a** The percent changes in HR after exchange from solution 1 to solution 2. Solution 2 contained various concentrations of nicotine (Nic), clothianidin (Cloth) or muscarine (Musc), as indicated. The percent change in HR after 10 min is noted in the second group of columns. The addition of both agonists induced a positive percent change in HR. **b** Change in average HR in exchange from saline to Nic 10 µM with raw changes for each preparation. The addition of Nic induced a significant change in average HR. **c** Change in average HR in exchange from saline to Musc 10 µM with raw changes for each preparation. The addition of muscarine induced a significant increase in average HR. (Student's *t* test was used for comparison). **d** Dose–response relation of Nic, Cloth, and Musc action on larval HR. *Open shapes* represent the subtraction of control saline exchanges from various concentrations of agonists. One-way ANOVA was used for comparison

induces a more dramatic change in increasing HR when exchanged compared to a simple saline to saline exchange and maintains a higher HR over the observed time period (Fig. 3a). When the open preparation was exposed to an additional nAChR agonist, clothianidin, it was found that no significant change in HR resulted. There was an insignificant positive percent change of 18.20 ± 5.09 % when the preparation was exposed to 100 nM clothianidin (Fig. 3a). At increased concentrations, the HR did not show a positive change and even dropped in the presence of high concentration of clothianidin, signifying this agonist did not influence HR. This was in stark contrast to nicotine, which induced a significant positive percent change at a concentration of 10 µM, suggesting nicotine may act via a separate mechanism to promote changes in HR.

In addition to exposing preparations to various concentrations of nicotine and clothianidin, muscarine solutions were tested in order to observe the effects of this mAChR agonist on HR. Much like nicotine, exchanging saline with a 100 nM muscarine solution induced a positive percent change in HR. In addition, a 10 µM muscarine solution induced a significant increase in average HR (Fig. 3c), rising 53.53 ± 7.43 % from baseline (Fig. 3a). Exposure to the highest concentration of muscarine did not yield as dramatic an increase in HR, again suggesting these receptors

may be desensitized at a lower concentration. Initial change in HRs was higher when compared to controls at each concentration, following the same trend observed with ACh and nicotinic solutions. The hearts of preparations exposed to saline containing low concentrations of muscarine displayed a smaller percent decrease on average after a 10 min waiting period compared to controls (Fig. 3a). Overall, two agonists, nicotine and muscarine, were capable of inducing positive initial change in HR when exchanged from saline and both maintained hearts at higher rates after a 10 min period, indicating that adding these drugs to a saline solution enhanced the ability of the heart to maintain a more rapid beat over a prolonged period of time. Clothianidin, however, did not affect HR, which may suggest that nicotine could influence HR through alternative mechanisms due to characteristics unique to the drug.

nAChR and mAChR antagonist dose–response relationship

Various cholinergic receptor antagonists were examined to test their ability to block the action of the agonists. Antagonists for both receptor subtypes were used in this examination. A total of four antagonists were examined. Each antagonist in various concentrations was used to test effect on the HR. In addition, following analysis of the effect of each antagonist on HR, the solutions were exchanged a second time, and the third solution exchanged contained a 10 μM concentration of either nicotine or muscarine in order to examine the ability of each antagonist to block the positive response induced by each agonist. The initial percent change in HR after each solution was exchanged was calculated and the change in HR after a 10 min bathing was calculated as well (Fig. 4a, b). In addition, the averages of HRs at exchange point was calculated as well for each intermediate concentration (Fig. 4c, d). As can be seen in Fig. 4a, nAChR antagonists, benzoquinonium dibromide (BD) and curare both displayed agonist-like characteristics, as they increased HR after initial exchange, inducing a positive chronotropic response. At a concentration of 100 nM, BD induced a positive percent change in HR of 27.56 ± 8.56 %, indicating this compound is capable of acting as a potent agonist in this model. Changes in HR were not dramatic with increasing concentration. In addition, curare also increased HR after initial exchange from saline. When compared to saline to saline exchanges alone, curare induced a higher positive percent change in HR at low concentrations, but induced a negative percent change at a high dose (10 μM) (Fig. 4a). Both nAChR antagonists also were capable of maintaining higher HRs over a 10 min period compared with controls. At 100 nM, hearts exposed to curare displayed an increase in HR after 10 min exposure and hearts exposed to BD displayed a small decrease of 4.19 ± 5.36 %. This compares to a decrease of 12.48 ± 7.03 % in hearts bathed in saline alone for a 10 min period (Fig. 4a).

In addition, mAChR antagonists atropine and scopolamine were examined for their effect in altering HR. Similar to nAChR antagonists tested, both mAChR antagonists induced a positive chronotropic response in HR upon initial exchange from saline. Specifically, at each concentration, both atropine and scopolamine increased HR from baseline. At 10 μM, atropine increased HR 36.51 ± 15.23 % from baseline, a 31 % difference in percent change when compared to a saline to saline exchange alone (Fig. 4b). Scopolamine displayed agonist-like characteristics at a higher concentration, increasing HR 35.47 ± 13.51 % from baseline at 1 μM (Fig. 4b). Both displayed a greater ability to maintain the HR over the course of 10 min, which is longer than compared to saline alone (Fig. 4b).

After examining the effect these antagonists alone had on HR, their ability to block the action of nAChR and mAChR agonists was tested. The same preparations were used, and a third solution exchange was performed after allowing the antagonist-containing solutions to sit on the preparations for a 10 min period. The third solution contained a 10 μM concentration of the agonist along with varying concentrations of the antagonists. Only one preparation is used for each antagonist-agonist combination trial. As can be seen in Fig. 4a, curare displays a slight ability to block nicotine action initially, as the percent change in HR is lower after initial exchange with this solution compared to a saline to saline exchange; however, after a 10 min period, the HRs do not decrease as substantially as they do when bathed in a solution containing saline alone. This is similar to what was found when nicotine was added to saline without the addition of curare, suggesting this drug does not block the action of nicotine over the observed time period. In addition, BD does not inhibit the ability of nicotine to induce a positive response at low doses, but does appear to attenuate the action of nicotine at higher concentrations (Fig. 4a). Similarly, the mAChR antagonist scopolamine does not block the ability of muscarine to induce a positive percent change in HR. Muscarine induces a dramatic change in HR in solutions containing scopolamine, increasing HR as high as 87.17 % from baseline (Fig. 4b). In contrast, our analysis shows that the mAChR competitive antagonist, atropine attenuates the substantial increase in HR exhibited by a muscarine solution, suggesting this antagonists is capable of blocking muscarine action. In the presence of 10 μM muscarine, a 10 μM atropine solution induces a 5.02 ± 3.99 % reduction in HR after initial exchange (Fig. 4b). However, the positive response in HR observed when atropine is in solution without muscarine is surprising. The averages for each intermediate concentration of antagonist tested was calculated and compared with saline averages. In addition, averages after exchange with a third solution containing antagonists plus each agonist were calculated and compared (Fig. 4c, d).

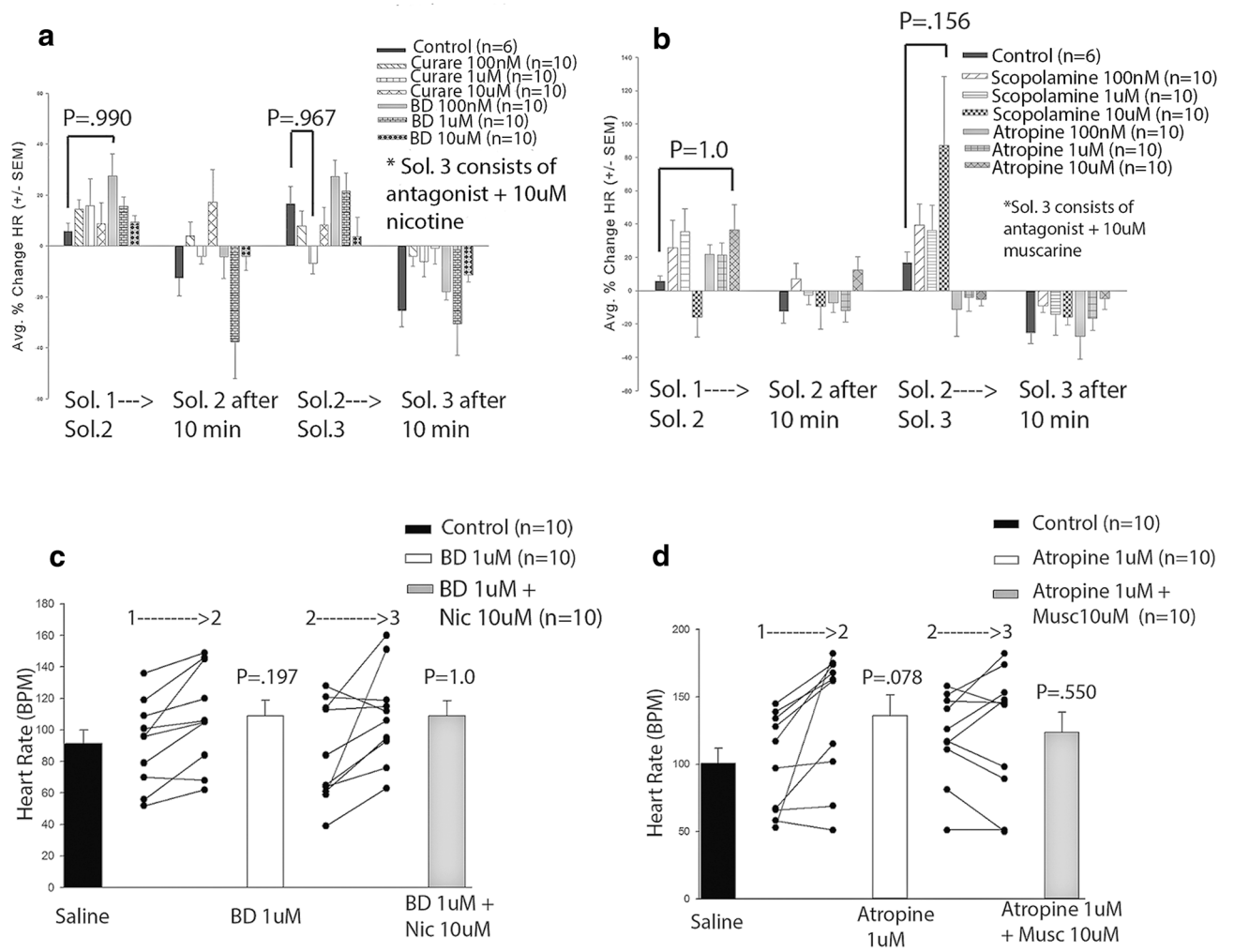

Fig. 4 Effects of AChR antagonists on HR. **a** The average percent change in HR when saline is exchanged for nAChR antagonists curare and BD. Solution 2 consists of saline + antagonist. The observed change after a 10 min period is noted. In addition, the ability of each antagonist to block the action of nicotine was tested. Solution 3 consists of saline + antagonist + 10 μM nicotine. **b** The average percent change in HR when saline is exchanged for mAChR antagonists scopolamine and atropine. Solution 3 consists of saline + antagonist + 10 μM muscarine. Atropine blocks the positive action of muscarine at each concentration, but, like nAChR antagonists, displays agonist-like characteristics of its own. Scopolamine does not block muscarine action. **c** Change in average HR in exchange from saline to BD 1 μM with raw changes for each preparation. The change in average HR is recorded then solution 2 is exchanged with solution 3 containing 1 μM BD + 10 μM nicotine (Student's *t* test was used for comparison). **d** Change in average HR in exchange from saline to atropine 1 μM with raw changes for each preparation. The addition of atropine induced an increase in average HR that was not statistically significant. In addition, the change in average HR is noted then solution 2 is exchanged with solution 3 containing 1 μM atropine + 10 μM muscarine (Student's *t* test was used for comparison)

Discussion

This analysis adds to the increasing understanding of *Drosophila* cardiac physiology, and aids in promoting the larval model as a useful tool in analyzing modulatory systems and diseases affecting the heart. The availability of a wealth of molecular tools make this model attractive for genetic studies. In addition, *Drosophila* serve a valuable model in understanding physiology at the cellular level, particularly as it relates to regulation of cardiac function (Piazza and Wessells 2011). One can utilize this genetically tractable organism in order to screen for mutations in ion channels and receptors that may be crucial in regulating the heart.

Mechanical disturbance activates stretch-activated ion channels

Control saline exchanges induced a positive percent change in initial HR. The small percent change examined is potentially indicative of a response resulting from activation of stretch-activated ion channels. It is well known that these ion channels are present in cardiomyocytes of vertebrates

and are sensitive to mechanical stimuli (Baumgartner et al. 2012). In addition, Piezo proteins are documented in *Drosophila* and are also sensitive to mechanotransduction (Coste et al. 2012). These ion channels are activated by mechanical disturbance and activation results in the intracellular accumulation of positively charged ions, such as Ca^{2+} and Na^{+} (Baumgartner et al. 2012). This leads to activation of downstream signaling cascades within the cell. The mechanical disturbance caused by exchanging solutions most likely activates these channels and induces cellular response.

Acetylcholine increases HR

Cholinergic receptors are known to play an integral role in cardio regulation throughout the animal kingdom (McCann 1970). A number of diseases of the heart are associated with dysfunctions of cholinergic receptors in mammals, and it is known that ACh receptors are ubiquitous in the CNS of *Drosophila*, but their expression in cardiac tissue had yet to be fully determined (Gundelfinger and Schloss 1989; Nurminen et al. 1991; Schuster et al. 1993; Wadsworth et al. 1988). Whether or not ACh acts through peripheral neurons to modulate *Drosophila* HR in adults is currently unknown. Activation of peripheral neurons could lead to the release of ACh into the hemolymph where it would interact with cholinergic receptors in cardiac pacemaker cells even for larvae. Previous studies performed in intact larvae suggest that ACh and nicotine both decrease larval HR, but show contrasting modulation in the adult fly. In addition, no evidence had yet been provided suggesting the presence of muscarinic receptors in this tissue. Conflicting results in previous work suggest that receptors in larval cardiac tissue are not solely nicotinic (Zornik et al. 1999). In fact, our analysis may rule out the possibility of functional nicotinic receptor presence in the plasma membrane of cardiomyocytes altogether. The peculiar actions of nicotine may mask any findings resulting from studies of an intact animal, as it is known that this agonist is lipophilic and can have additional actions within the cell. This previous research does, however, provide evidence that cholinergic receptors are present in this tissue and their activation contributes to modulation of HR (Zornik et al. 1999). A more thorough investigation into the mRNA expression of the receptor subtypes present at the larval stage will help to delineate the role of the cholinergic system in modulating HR in this model.

Since we were able to maintain hearts in a physiological saline for long periods of time, we were now able to address the effects of modulators known to be in hemolymph on cardiac function directly. It was found that ACh increased HR at concentrations as low as 100 nM. There was a substantial increase in HR upon exposure to 100 nM ACh suggesting the presence of cholinergic receptors in larval heart tissue. Higher concentrations show little additional positive effect on HR, suggesting ACh desensitizes receptors at low concentrations, thus resulting in decreased sensitivity to additional ACh activation at concentrations above 10 µM. In this analysis, semi-intact preparations were used allowing for the exposure of the larval heart directly to select compounds without the influence of compounding variables. We determined that ACh is capable of inducing an increase in HR suggesting this modulator is activating receptors present in cardiomyocytes, resulting in depolarization of the membrane and a positive chronotropic action on the heart in this model.

Muscarine and nicotine increase HR

Since ACh induces an increase in HR when exposed directly to the larval heart it is likely that cholinergic receptors are expressed in this tissue. Previous studies have shown that ACh decreases HR and the nAChR agonist, nicotine increases HR (Zornik et al. 1999); however, pupa were injected with the substance and compounds were not selectively examined directly on the heart in a well buffered saline. There has been no evidence supporting the presence of muscarinic receptors in *Drosophila* larval cardiac tissue to date. In order to elucidate the cholinergic receptor subtypes which may play a role in altering HR, we first added various concentrations of nicotine, clothianidin or muscarine to the open preparations and then examined if selective antagonists blocked agonist actions. The findings indicate that functional mAChRs are likely present in cardiomyocytes at the larval stage. These receptors function to induce a significant enhancement in pacemaker activity, resulting in an increase in HR. Although we cannot definitively rule out the expression of nAChRs in larval cardiac tissue, the finding that clothianidin does not affect HR and the inability of nAChR antagonists from blocking nicotinic action suggests the absence of functional nAChR in the plasma membrane of pacemaker cells. More thorough expression analysis is needed to confirm this finding.

The results demonstrate nicotine influences HR significantly when exposed to the heart directly. While our findings show there may be an absence of nAChRs in the plasma membrane, the influence of nicotine may very well act in an alternative manner to induce an increase in HR. It is known that nicotine not only activates plasma membrane receptors but is well known to have direct effects on intracellular function since the compound is lipophilic and crosses cell membranes rapidly, particularly in more alkaline environments (>6.5 pH) (Hukkanen and Benowitz 2005). Considering the saline solution used to bathe the open preparations is measured at a pH of 7.1, it is likely that nicotine exists in a more unionized state in this

solution, and thus may cross the cell membrane quickly. Nicotine may also stay within a membrane depending on its ionized state. This is an important characteristic that likely enhances the action of nicotine within the cell. Once in the cell the role of nicotine in modulating HR remains poorly understood. However, recent imaging analysis of membrane proteins, including nAChRs, performed by Moonschi et al. (2015) shows evidence of nAChR receptor presence in Endoplasmic Reticulum (ER) derived microsomes. Not only does this group confirm the presence of nAChR subunits in microsomes, but they also, through the use of Ca^{2+} flux imaging, show that these receptors are functional. In addition, previous findings indicating rapid desensitization of membrane nAChRs, such as that of Colombo et al. (2013), could also support nAChR activity in the ER in other cell types, as these receptors could be desensitized prior to incorporation into the plasma membrane. Therefore, we speculate the presence of functional nAChRs in the ER that may act to dump Ca^{2+} in the presence of nicotine, inducing an increase in HR. Although difficult in larval cardiac pacemaker cells due to the trouble in fluorescent imaging of a contractile organ, one may test this hypothesis in additional cell types through a Ca^{2+} flux imaging experiment where nAChR release from the ER is blocked via Brefeldin A. One could then look for changes in calcium binding with a calcium sensitive fluorophore (fluo-4) upon exposure to ACh or nicotine. The additional actions of nicotine, including the activation of other membrane receptors, such as the transient receptor potential A1 channel (Talavera et al. 2009), as well as the blocking of additional surface receptors including 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) (Schreiner et al. 2014) could play a role of altering HR in vivo as well. These findings open the door to further investigation of the mechanistic actions of nicotine in modulating HR.

While the presence of functional nAChRs in the ER remain a possibility, our analysis suggests that the identity of cholinergic receptors on larval pacemaker plasma membranes are primarily muscarinic. In testing the role of muscarine, an agonist that activates metabotropic mAChRs, in regulating HR, it was found that muscarine increased HR at both low and high concentrations, suggesting the presence of mAChRs in larval cardiac tissue. As stated, two subtypes of mAChRs are expressed in *Drosophila*, A-type and B-type. The activity of these two receptor subtypes are crucial in regulating the excitability of the cell. In mammals, five muscarinic receptor subtypes have been identified (M1–M5) and classified pharmacologically (Felder 1995). These subtypes have been grouped into two groups based on their mobilization of intracellular Ca^{2+} (M1, M3, and M5) or their ability to inhibit adenylate cyclase (M2 and M4) (Felder 1995). M2 receptor is known to be present on human hearts and acts to slow down HR by inhibition of adenylate cyclase and decrease of intracellular cAMP. The functional characterization of the two muscarinic receptor subtypes in *Drosophila* has been more problematic; however a comprehensive analysis of the function of A-type and B-type mAChRs in this model was performed by Collin et al. (2013). The group measured relative A-type and B-type mAChR expression at various stages of the life cycle by extracting mRNA from the head, thorax, and whole-body of individual animals. Their expression analysis shows that each subtype is expressed at each developmental stage throughout the body; however, the pharmacological profiles of these receptor subtypes appear to be distinct (Collin et al. 2013). The A-type receptor can be activated by both low concentrations of ACh and muscarine, whereas the B-type receptor is not responsive to muscarine binding (Collin et al. 2013). In addition, sequencing analysis shows the binding pocket for ACh in the A-type receptor is highly similar to the binding domain in mammalian M1–M5 receptors, but less so in the B-type receptor, suggesting the different pharmacological profile is most likely due to structural differences between the two receptor subtypes (Collin et al. 2013). In our analysis, the heart was responsive to low concentrations of both ACh and muscarine, suggesting the presence of A-type mAChRs in larval cardiac tissue. It is noted that the addition of muscarine significantly increases average HR when compared to controls, indicating a stimulatory effect and potential activation of a 2nd messenger cascade that mediates intracellular Ca^{2+} levels. As stated, M2 mAChR receptor subtype is present in mammalian cardiac tissue and was shown to attenuate adenylate cyclase activity, thereby reducing the intracellular levels of cAMP through G_i (Felder, 1995). Our analysis suggest that the mAChRs present in cardiac tissue at the larval stage act through a stimulatory cascade that is not regulated by adenylate cyclase, as it has been shown that HR stimulation by 5-HT does not act through cAMP (Majeed et al. 2013). In a recent study by Ren et al. (2015), the group showed that A-type mAChRs couple to the $G_q/_{11}$ signaling pathway, whereas B-type mAChR couple to the $G_i/_0$ pathway. Their findings that A-type receptors do not act through the inhibitory $G_i/_0$ pathway supports our evidence that this receptor subtype is present in larval heart tissue, as the stimulatory effects on HR suggest. However, the tissue from which mAChR mRNA was extracted was not described in their analysis, so the 2nd messenger signaling pathway through which these receptors act in larval heart tissue must be examined.

Understanding how cardiomyocytes pace the *Drosophila* heart has been in question. A study by Desai-Shah et al. 2010 provided a comprehensive analysis of the role of three important calcium pumps in modulating HR, the Na^+/Ca^{2+} exchanger (NCX), the plasma membrane Ca^{2+}-ATPase (PMCA), and the sarcoplasmic/

endoplasmic reticulum Ca^{2+}-ATPase (SERCA). It was found that compromising these exchangers individually or together had a dramatic effect on the HR of a semi-intact preparation. The analysis lead to the conclusion that $[Ca^{2+}]_i$ and $[Na^+]_i$ are tightly regulated in *Drosophila* larval hearts. A proposed model indicates that when *Drosophila* hearts are in diastole, depolarization and a slow release of Ca^{2+} from the sarcoplasmic reticulum (SR) by ryanodine receptors (RyR) leads to a rise in $[Ca^{2+}]_i$. The SERCA pumps Ca^{2+} back into the SR and the NCX removes $[Ca^{2+}]_i$ in exchange for Na^+ ions across the plasma membrane of the cell. The influx of Na^+ ions leads to a depolarization of the plasma membrane, thus opening low voltage-gated T-type Ca^{2+} channels (VCa) (Huser et al. 2012) and potentially voltage-gated Na^+ channels. The influx of Ca^{2+} acts on the RyR to cause the ER (endoplasmic reticulum) to dump Ca^{2+} which results in a calcium induced inhibition of the RyR. Until the $[Ca^{2+}]_i$ is reduced by the SERCA and NCX, the RyR stays inhibited but will start leaking Ca^{2+} as $[Ca^{2+}]_i$ returns to a low level to then repeat the cycle (Subramani and Subbanna 2006). In addition, it is understood that the pacing cells act as contracting myocytes and that they can also generate action potentials, suggesting the presence of voltage gated Na^+ channels (Gu and Singh 1995). Given the fact that ER nAChRs have been shown to permit Ca^{2+} influx (Moonschi et al. 2015) and A-type mAChRs act through a stimulatory signaling cascade, it can be determined that activation of these receptors could lead to an initial increase in Ca^{2+} concentration in the cell, as the Ca^{2+} conductance increases. This increased Ca^{2+} conductance in turn activates the NCX, which pumps Ca^{2+} out of the cell, and Na^+ into the cell, leading to membrane depolarization in cardiac pacemaker cells and an increase in HR.

nAChR and mAChR antagonists increase HR

In addition to testing the role the two cholinergic receptor agonists in regulating HR, classical competitive antagonists were tested in order to deduce their ability to block the action of nicotine and muscarine. It would be assumed that since it is evident that both agonists significantly increase HR, the addition of competitive antagonists in the presence of the agonists would block this response. Surprisingly, we found that each antagonist actually increases HR initially and only atropine displays the ability to block the action of the mAChR agonist (muscarine) at each concentration tested. Although this may seem rather peculiar, it is well established that the pharmacological profile of nicotine and nAChRs is quite complex. In numerous studies involving mice, including those by Buccafusco et al. (2009) and Paradiso and Steinbach (2003), the description of nicotine as a simple nAChR agonist appears to be quite simplistic (Buccafusco et al. 2009). These studies, along with many others, have found that the actions of nicotine often mimic the actions of classic nAChR antagonists, including *d*-tubocurarine and α-bungarotoxin (Ropert and Krnjevic 1982). We found similar results testing BD and curare. As stated previously, this phenomenon may be explained by the ability of nicotine to activate and desensitize receptors quite rapidly (Buccafusco et al. 2009) and compensatory upregulation of expression of nAChR subunits could result (Buccafusco et al. 2009). However, it is noted in this analysis, nAChR antagonists were bathed on the preparation prior to the addition of nicotine. Thus, the ability of nicotine to induce a positive response in the presence of these competitive antagonists may not be due to its ability to quickly desensitize receptors. Had the preparation been bathed in nicotine first, one could assume that a change in conformation of the receptors would alter the ability of competitive antagonists to block further agonist action. Instead, it can be assumed that the difference in nAChR pharmacology in this model may likely be explained by structural differences in the associated receptor proteins. Additionally, the actions of nicotine on ER nAChRs could also play a role in rapid desensitization.

In addition, the ability of mAChR antagonists to block the action of muscarine were tested. Based on the results observed of muscarine altering HR and comparison with previous studies, it is likely that A-type receptors are present in larval cardiac tissue. Pharmacological data provided by Collin et al. (2013) shows both scopolamine and atropine are capable blocking the action of muscarine in *Drosophila*. While we found that atropine did indeed reduce HR in the presence of muscarine, scopolamine surprisingly did not show an ability to block this agonist. Moreover, both antagonists displayed agonist-like characteristics of their own. Although the pharmacology provided here suggests the presence of A-type mAChRs in larval cardiac tissue, the question regarding 2nd messenger cascade activation by these receptor subtypes in this tissue remains. Further pharmacological inhibition of particular 2nd messengers may be required in future studies to elucidate the role of mAChRs in modulating HR.

Conclusion

Analysis of the effects of cholinergic compounds on HR have not been previously administered in a manner that isolates the actions of the desired compound. In contrast with current understanding, our pharmacological analysis indicates cholinergic compounds modulate HR in larval *Drosophila*. Understanding the effects of neuromodulators

on regulation of HR and cardiac development can aid in understanding how exposure to increased concentrations of cholinergic drugs, such as nicotine in early development may alter the normal development of this vital organ. Alterations in these modulatory systems have shown to dramatically affect HR, showing the potential detriment posed to human fetuses in embryonic development (Horta et al. 1997). In addition, this study aids in providing a pharmacological profile for this organism and helps lay a foundation for future analysis in characterizing cholinergic receptor subtypes in cardiac tissue. Future studies surrounding potential nAChR function in the ER membrane in vivo can be performed to enhance knowledge regarding nicotinic action not only in cardiac pacemaker cells, but in additional excitable cells as well. The genetic amenability of *D. melanogaster* allow for thorough examination of functional expression of particular subunits of cholinergic receptors and the role of second messenger signaling cascades in regulation of cardiac physiology and development.

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Compliance with ethical standards

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

References

Baumgartner U, Greffrath W, Treede RD (2012) Contact heat and cold, mechanical, electrical and chemical stimuli to elicit small fiber-evoked potentials: merits and limitations for basic science and clinical use. Neurophysiol Clin Clin Neurophysiol 42(5):267–280. doi:10.1016/j.neucli.2012.06.002

Bier E, Bodmer R (2004) *Drosophila*, an emerging model for cardiac disease. Gene 342(1):1–11. doi:10.1016/j.gene.2004.07.018

Buccafusco JJ, Beach JW, Terry AV Jr (2009) Desensitization of nicotinic acetylcholine receptors as a strategy for drug development. J Pharmacol Exp Ther 328(2):364–370. doi:10.1124/jpet.108.145292

Cammarato A, Ahrens CH, Alayari NN, Qeli E, Rucker J, Reedy MC, Zmasek CM, Gucek M, Cole RN, Van Eyk JE, Bodmer R, O'Rourke B, Bernstein SI, Foster DB (2011) A mighty small heart: the cardiac proteome of adult *Drosophila melanogaster*. PLoS One 6(4):11. doi:10.1371/journal.pone.0018497

Campos-Ortega JA (1974) Autoradiographic localization of 3H-gamma-aminobutyric acid uptake in the lamina ganglionaris of *Musca* and *Drosophila*. Zeitschrift fur Zellforschung und Mikroskopische Anatomie 147(3):415–431

Collin C, Hauser F, de Valdivia EG, Li S, Reisenberger J, Carlsen EMM, Khan Z, Hansen NO, Puhm F, Sondergaard L, Niemiec J, Heninger M, Ren GR, Grimmelikhuijzen CJP (2013) Two types of muscarinic acetylcholine receptors in *Drosophila* and other arthropods. Cell Mol Life Sci 70(21):4197. doi:10.1007/s00018-013-1464-4

Colombo SF, Mazzo F, Pistillo F, Gotti C (2013) Biogenesis, trafficking and up-regulation of nicotinic ACh receptors. Biochem Pharmacol 86(8):1063–1073. doi:10.1016/j.bcp.2013.06.023

Consoulas C, Levine RB, Restifo LL (2005) The steroid hormone-regulated gene Broad-Complex is required for dendritic growth of motorneurons during metamorphosis of *Drosophila*. J Comp Neurol 485:321–337

Cooper AS, Rymond KE, Ward MA, Bocook EL, Cooper RL (2009) Monitoring heart function in larval *Drosophila melanogaster* for physiological studies. J Vis Exp. doi:10.3791/1596

Coste B, Xiao BL, Santos JS, Syeda R, Grandl J, Spencer KS, Kim SE, Schmidt M, Mathur J, Dubin AE, Montal M, Patapoutian A (2012) Piezo proteins are pore-forming subunits of mechanically activated channels. Nature 483(7388):176–181. doi:10.1038/nature10812

Dasari S, Cooper RL (2006) Direct influence of serotonin on the larval heart of *Drosophila melanogaster*. J Comp Physiol B-Biochem Syst Environ Physiol 176(4):349–357. doi:10.1007/s00360-005-0058-3

de Castro C, Titlow J, Majeed ZR, Cooper RL (2014) Analysis of various physiological salines for heart rate, CNS function, and synaptic transmission at neuromuscular junctions in *Drosophila melanogaster* larvae. J Comp Physiol A- Neuroethol Sens Neural Behav Physiol 200(1):83–92. doi:10.1007/s00359-013-0864-0

Desai-Shah M, Papoy AR, Ward M, Cooper RL (2010) Roles of the Sarcoplasmic/Endoplasmic reticulum Ca2-ATPase, plasma membrane Ca2-ATPase and Na/Ca2 exchanger in regulation of heart rate in larval *Drosophila*. Open Physiol J 3:16–36

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(3):153–168. doi:10.3109/01677069509083461

Dulcis D, Levine RB (2003) Innervation of the heart of the adult fruit fly, *Drosophila melanogaster*. J Comp Neurol 465(4):560–578. doi:10.1002/cne.10869

Dulcis D, Levine RB (2005) Glutamatergic innervation of the heart initiates retrograde contractions in adult *Drosophila melanogaster*. J Neurosci 25(2):271–280. doi:10.1523/jneurosci.2906-04.2005

Felder CC (1995) Muscarinic acetylcholine receptors—signal-transduction through multiple effectors. Faseb J 9(8):619–625

Gavioli M, Lara A, Almeida PWM, Lima AM, Damasceno DD, Rocha-Resende C, Ladeira M, Resende RR, Martinelli PM, Melo MB, Brum PC, Fontes MAP, Santos RAS, Prado MAM, Guatimosim S (2014) Cholinergic signaling exerts protective effects in models of sympathetic hyperactivity-induced cardiac dysfunction. PLoS One 9(7):9. doi:10.1371/journal.pone.0100179

Gu GG, Singh S (1995) Pharmacological analysis of heartbeat in *Drosophila*. J Neurobiol 28(3):269–280. doi:10.1002/neu.480280302

Gundelfinger ED, Schloss P (1989) Nicotinic acetylcholine receptors of the *Drosophila* central nervous system. J Protein Chem 8(3):335–337. doi:10.1007/bf01674267

Horta BL, Victora CG, Menezes AM, Halpern R, Barros FC (1997) Low birthweight, preterm births and intrauterine growth retardation in relation to maternal smoking. Paediatr Perinat Epidemiol 11(2):140–151. doi:10.1046/j.1365-3016.1997.d01-17.x

Hukkanen J, Benowitz NL (2005) Metabolism and disposition kinetics of nicotine. Pharmacol Rev 57(1):79–115. doi:10.1124/pr.57.1.3

Hurst R, Rollema H, Bertrand D (2013) Nicotinic acetylcholine receptors: from basic science to therapeutics. Pharmacol Ther 137(1):22–54. doi:10.1016/j.pharmthera.2012.08.012

Huser A, Rohwedder A, Apostolopoulou AA, Widmann A, Pfitzenmaier JE, Maiolo EM, Selcho M, Pauls D, von Essen A, Gupta T, Sprecher SG, Birman S, Riemensperger T, Stocker RF, Thum AS

(2012) The serotonergic central nervous system of the drosophila larva: anatomy and behavioral function. PLoS One 7(10):23. doi:10.1371/journal.pone.0047518

Johnson E, Ringo J, Dowse H (1997) Modulation of *Drosophila* heartbeat by neurotransmitters. J Comp Physiol B-Biochem Syst Environm Physiol 167(2):89–97. doi:10.1007/s003600050051

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):1–24

Johnson E, Ringo J, Dowse H (2001) Dynamin, encoded by shibire, is central to cardiac function. J Exp Zool 289(2):81–89. doi:10.1002/1097-010x(20010201)289:2<81:aid-jez1>3.0.co;2-t

Johnstone AFM, Cooper RL (2006) Direct innervation of the *Drosophila melanogaster* larval aorta. Brain Res 1083:159–163. doi:10.1016/j.brainres.2006.02.007

Majeed ZR, Nichols CD, Cooper RL (2013) 5-HT stimulation of heart rate in *Drosophila* does not act through cAMP as revealed by pharmacogenetics. J Appl Physiol 115(11):1656–1665. doi:10.1152/japplphysiol.00849.2013

Majeed ZR, Stacy A, Cooper RL (2014) Pharmacological and genetic identification of serotonin receptor subtypes on *Drosophila* larval heart and aorta. J Comp Physiol B-Biochem Syst Environm Physiol 184(2):205–219. doi:10.1007/s00360-013-0795-7

Martin CA, Krantz DE (2014) *Drosophila melanogaster* as a genetic model system to study neurotransmitter transporters. Neurochem Int 73:71–88. doi:10.1016/j.neuint.2014.03.015

McCann FV (1970) Physiology of insect hearts. In: Smith, Ray F, Thomas E Mittler (eds) Annual review of entomology Vol 15 Vii + 502p Illus annual reviews, Inc, Palo Alto, pp 173–200

Molina MR, Cripps RM (2001) Ostia, the inflow tracts of the *Drosophila* heart, develop from a genetically distinct subset of cardial cells. Mech Dev 109(1):51–59. doi:10.1016/s0925-4773(01)00509-3

Moonschi FH, Effinger AK, Zhang XL, Martin WE, Fox AM, Heidary DK, DeRouchey JE, Richards CI (2015) Cell-derived vesicles for single-molecule imaging of membrane proteins. Angewandte Chemie-Int Edn 54(2):481–484. doi:10.1002/anie.201408707

Neve KA, Seamans JK, Trantham-Davidson H (2004) Dopamine receptor signaling. J Recept Signal Transduction 24(3):165–205. doi:10.1081/lrst-200029981

Nurminen ML, Paakkari I, Seppala T (1991) Serotonergic involvement in the cardiovascular stimulation by thyrotropin-releasing hormone (Trh) in anesthetized rats. Neurosci Lett 127(2):147–149. doi:10.1016/0304-3940(91)90781-n

Ocorr K, Reeves NL, Wessells RJ, Fink M, Chen HSV, Akasaka T, Yasuda S, Metzger JM, Giles W, Posakony JW, Bodmer R (2007) KCNQ potassium channel mutations cause cardiac arrhythmias in *Drosophila* that mimic the effects of aging. Proc Natl Acad Sci USA 104(10):3943–3948. doi:10.1073/pnas.0609278104

Paradiso KG, Steinbach JH (2003) Nicotine is highly effective at producing desensitization of rat alpha 4 beta 2 neuronal nicotinic receptors. J Physiol Lond 553(3):857–871. doi:10.1113/jphysiol.2003.053447

Piazza N, Wessells RJ (2011) Drosophila Models of Cardiac Disease. In: Chang KT, Min KT (eds) Progress in molecular biology and translational science, vol 100. Elsevier Academic Press Inc, San Diego, pp 155–210. doi:10.1016/b978-0-12-384878-9.00005-4

Ren GR, Folke J, Hauser F, Li S, Grimmelikhuijzen CJ (2015) The A- and B-type muscarinic acetylcholine receptors from *Drosophila melanogaster* couple to different second messenger pathways. Biochem Biophys Res Commun 462(4):358–364. doi: 10.1016/j.bbrc.2015.04.141

Rizki TM (1978) The circulatory system and associated cells and tissues. In: Ashburner M, Wright TRF (eds) The genetics and biology of *Drosophila*. vol 2b. Academic Press, USA

Ropert N, Krnjevic K (1982) Pharmacological characteristics of facilitation of hippocampal population spikes by cholinomimetics. Neuroscience 7(8):1963–1977. doi:10.1016/0306-4522(82)90011-2

Schreiner BSP, Lehmann R, Thiel U, Ziemba PM, Beltran LR, Sherkheli MA, Jeanbourquin P, Hugi A, Werner M, Gisselmann G, Hatt H (2014) Direct action and modulating effect of (+)- and (−)-nicotine on ion channels expressed in trigeminal sensory neurons. Eur J Pharmacol 728:48–58. doi:10.1016/j.ejphar.2014.01.060

Schuster R, Phannavong B, Schroder C, Gundelfinger ED (1993) Immunohistochemical localization of a ligand-binding and a structural subunit of nicotinic acetylcholine-receptors in the central nervous system of *Drosophila melanogaster*. J Comp Neurol 335(2):149–162. doi:10.1002/cne.903350202

Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF (1994) Improved stability of *Drosophila* larval neuromuscular preparations in hemolymph-like physiological solutions. J Comp Physiol A- Sens Neural Behav Physiol 175(2):179–191. doi:10.1007/bf00215114

Subramani S, Subbanna PK (2006) Calcium-transporters in myocardial cells. Indian J Physiol Pharmcol 50:99–113

Talavera K, Gees M, Karashima Y, Meseguer VM, Vanoirbeek JAJ, Damann N, Everaerts W, Benoit M, Janssens A, Vennekens R, Viana F, Nemery B, Nilius B, Voets T (2009) Nicotine activates the chemosensory cation channel TRPA1. Nat Neurosci 12(10):1293–1299. doi:10.1038/nn.2379

Titlow JS, Rufer J, King K, Cooper RL (2013) Pharmacological analysis of dopamine modulation in the *Drosophila melanogaster* larval heart. Physiol Rep 1(2):e00020. doi:10.1002/phy2.20

Wadsworth SC, Rosenthal LS, Kammermeyer KL, Potter MB, Nelson DJ (1988) Expression of a *Drosophila* melanogaster acetylcholine-receptor-related gene in the central nervous system. Mol Cell Biol 8(2):778–785

Wolf MJ, Amrein H, Izatt JA, Choma MA, Reedy MC, Rockman HA (2006) *Drosophila* as a model for the identification of genes causing adult human heart disease. Proc Natl Acad Sci USA 103(5):1394–1399. doi:10.1073/pnas.0507359103

Wonnacott S, Livingstone PD (2010) Nicotinic receptors and the modulation of transmitter release in the prefrontal cortex. Eur Neuropsychopharmacol 20:S193

Zeitouni B, Senatore S, Severac D, Aknin C, Semeriva M, Perrin L (2007) Signalling pathways involved in adult heart formation revealed by gene expression profiling in drosophila. PLoS Genet 3(10):1907–1921. doi:10.1371/journal.pgen.0030174

Zornik E, Paisley K, Nichols R (1999) Neural transmitters and a peptide modulate *Drosophila* heart rate. Peptides 20(1):45–51. doi:10.1016/s0196-9781(98)00151-x