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ORIGINAL PAPER

Pharmacological and genetic identification of serotonin receptor subtypes on *Drosophila* larval heart and aorta

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Abstract Serotonin, 5-hydroxytryptamine (5-HT), plays various roles in the fruit fly, *Drosophila melanogaster*. Previous studies have shown that 5-HT modulates the heart rate in third instar larvae. However, the receptor subtypes that mediate 5-HT action in larval cardiac tissue had yet to be determined. In this study, various 5-HT agonists and antagonists were employed to determine which 5-HT receptor subtypes are responsible for the positive chronotropic effect by 5-HT. The pharmacological results demonstrate that a 5-HT2B agonist significantly increases the heart rate; however, 5-HT1A, 5-HT1B, and 5-HT7 agonists do not have a significant effect on the heart rate. Furthermore, 5-HT2 antagonist, ketanserin, markedly reduces the positive chronotropic effect of 5-HT in a dose–response manner. Furthermore, we employed genetic approaches to confirm the pharmacological results. For this purpose, we used RNA interference line to knock down 5-HT2ADro and also used 5-HT2ADro and 5-HT2BDro insertional mutation lines. The results show that 5-HT2ADro or 5-HT2BDro receptor mutations reduce the response of the heart to 5-HT. Given these results, we conclude that these 5-HT2 receptor subtypes are involved in the action of 5-HT on the heart rate in the larval stage.

Keywords 5-HT · 5-HT2ADro and 5-HT2BDro receptors mutations · 5-HT receptors agonists and antagonists · Heart rate

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Introduction

Several recent studies have demonstrated that serotonin (5-hydroxytryptamine, 5-HT) plays many versatile roles in insects, for instance, in blowfly salivary gland secretion (Röser et al. 2012) and locust swarming behavior (Anstey et al. 2009). Furthermore, many studies have shown that 5-HT is involved in many physiological and behavioral aspects in the fruit fly, *Drosophila melanogaster*, such as, modulation of the heart rate (Dasari and Cooper 2006) and neural circuit (Dasari and Cooper 2004), as well as behaviors associated with sleep regulation (Yuan et al. 2006), circadian patterns (Nichols 2007), courtship behavior (Becnel et al. 2011), learning and memory (Johnson et al. 2011) and feeding behavior (Gasque et al. 2013). Various 5-HT receptor subtypes have been cloned and characterized in *Drosophila* (Tierney 2001): 5-HT1Adro, 5-HT1Bdro which are negatively coupled to adenylyl cyclase (AC) (Saudou et al. 1992); 5-HT2Adro (Colas et al. 1995), the intracellular signaling pathway has not yet been investigated for 5-HT2 receptor in *Drosophila*; 5-HT2B receptor was recently identified in *Drosophila* (Gasque et al. 2013); 5-HT7 is positively coupled to AC (Witz et al. 1990). It has been already shown that application of exogenous 5-HT to semi-intact larvae increases the heart rate in *Drosophila* larvae (Dasari and Cooper 2006); however, the subtype of 5-HT receptor that

mediates 5-HT action in larval heart has not yet been studied pharmacologically. Interestingly, 5-HT modulates the heart rate in other invertebrates, such as, hard clam *Mercenaria mercenaria*, garden snail *Helix aspersa*, *Aplysia* (Villalón and Centurión 2007), crayfish (Listerman et al. 2000), tobacco hornworm *Manduca sexta* (Platt and Reynolds 1986), and cockroach *Periplaneta americana* (Collins and Miller 1977).

Drosophila has an open cardiovascular system which consists of a simple dorsal vessel, anterior aorta and posterior heart. *Drosophila* larval heart is myogenic and hemolymph-borne modulators can have direct effect on cardiac tissue. The larval *Drosophila* heart has been used to investigate the electrophysiological properties of cardiomyocytes in normal and mutant larvae by recording cardiomyocyte action potentials (Lalevée et al. 2006; Desai-Shah et al. 2010) and it is proven to be a good model to study the role of ions in the generation of heart beat (Johnson et al. 1998; Desai-Shah et al. 2010). Recently, the detailed structure of *Drosophila* heart has been studied (Lehmacher et al. 2012), which opens up avenues to further investigate cardiac physiology of *Drosophila*. *Drosophila* heart has also been used to investigate the role of genes which are important for mammalian cardiac physiology (Johnson et al. 2001) and development since they share similar crucial transcription factors during development (Olson 2006). The morphology of heart in *Drosophila* and vertebrates is different, even though the molecular mechanisms that underlie heart development in *Drosophila* and vertebrates share a large degree of similarity (Bodmer and Venkatesh 1998). Moreover, *Drosophila* and vertebrate hearts are functionally indexed by similar physiological measurements, such as, cardiac output, rate and time in systole or diastole (Choma et al. 2011). Various studies have demonstrated that 5-HT receptors are important for cardiac physiology and development in mammals. The 5-HT1A receptor was shown, in 5-HT1A knockout mice, to be crucial to resist the stress-induced cardiac problems (Carnevali et al. 2012). Also, it has been revealed that loss of function of 5-HT2B receptor is lethal due to cardiac abnormalities during development (Nebigil et al. 2000). Moreover, increasing 5-HT2A receptor activity has shown to be related to cardiac hypertrophy in mice (Lairez et al. 2013).

In this study, we used pharmacological approaches to characterize the 5-HT receptor subtypes that mediate the positive chronotropic effect of 5-HT on the larval heart. Many signaling pathways might be involved in the modulation of the heart rate in *Drosophila*; thus, various 5-HT receptor subtypes could account for the action. We have shown that activation of 5-HT2 receptor is the primary subtype to account for the increase in heart rate. To corroborate the pharmacological results, we used genetic approaches. We have demonstrated that alterations in 5-HT2 receptor subtypes reduce the responsiveness of the heart to 5-HT. These results indicate that 5-HT2 receptors are implicated in the action of 5-HT; however, we cannot rule out the involvement of the other 5-HT receptor subtypes having some role.

Materials and methods

Fly rearing and culturing

In this study, canton-S (CS), w^{1118}, PBac-5-HT2A, Mi-5-HT2B, UAS-RNAi-5-HT2A, and 5-HT2A-Gal4 lines were used. The fly lines PBac-5-HT2ADro (stock #19367, 5-HT2ADro insertional mutant line), Mi-5-HT2BDro (stock #40810, 5-HT2BDro insertional mutant line), 5-HT2A-Gal4 (stock #49574), and UAS-RNAi-5-HT2A (stock #31882) were obtained from the Bloomington *Drosophila* Stock Center at Indiana University. The flies were reared at room temperature (22–23 °C), unless otherwise stated, in vials containing cornmeal-agar-dextrose-yeast medium.

Visual heart rate measurement

Third instar larvae were washed with distilled water to remove the food on the larvae. The larvae were dissected in the ventral side up position. The internal organs, intestine, and fat bodies were removed to observe the heart in modified HL3 saline (NaCl 70 mM, KCl 5 mM, $MgCl_2.6H_2O$ 20 mM, $NaHCO_3$ 10 mM, trehalose 5 mM, sucrose 115 mM, $CaCl_2.2H_2O$ 1 mM, and BES 50 mM and pH 7.0 (pharmacology study) or Trizma acid 25 mM and pH 7.1 (genetic study)). We modified the type and concentration of buffer in HL3 saline since the heart rate is very sensitive to pH change and the HL3 with BES 5 mM does not have a stable pH (deCastro et al. 2013).The detailed protocol for the larvae dissection and heart visualization is explained in Cooper et al. (2009). The heart rate was measured by directly counting heart beats on the television screen. The heart beats were counted over 1 min at various times to obtain beats per minute (BPM) at given time periods. Various agonists and antagonists were applied. The preparation was left for 1 min in saline after dissection, and then the rate was obtained for the following minute (this is considered as a first minute in the experiments). The saline was exchanged with saline containing agonist or antagonist. Preparations were left for 1 min with exposure to compounds and then the rates were obtained for the following minute. The preparations were also left for 10 min and then the rates were obtained again for 1 min. When an antagonist was employed, the preparation was incubated inside an antagonist for 10 min and then the

saline containing an antagonist was exchanged with saline containing 5-HT and antagonist altogether. Afterwards, a percentage change of heart rate was determined.

Chemicals

8-Hydroxy-DPAT hydrobromide (5-HT1A agonist), CP 93129 dihydrochloride (5-HT1B agonist), α-methyl-5-hydroxytryptamine maleate (5-HT2 agonist), TCB-2 (5-HT2 agonist), AS19 (5-HT7 agonist), WAY 100635 (5-HT1A antagonist), GR 55562 (5-HT1B antagonist), ketanserin tartrate (5-HT2 antagonist), SB 258719 (5-HT7 antagonist) were purchased from Tocris Bioscience (Bristol, UK). 1-(3-chlorophenyl) piperazine 2HCl (5-HT2C agonist) was purchased from Research biochemical international (RBI) (MA, USA). (±)-DOI hydrochloride was purchased from Sigma (St. Louis, MO, USA). Serotonin (5-HT) hydrochloride was purchased from Sigma (Steinheim, Germany). All chemicals were dissolved in fly saline, but AS19 was dissolved inside dimethyl sulfoxide (DMSO). 1 mM stock solution was made. Then, a fresh solution of specific concentrations was made from the stock solution every time before starting the experiment. The DMSO concentration in AS19 100 μM, which is the highest concentration used, was about 0.03 %.

Statistical analysis

All data are expressed as mean ± SEM. SigmaPlot (version 12.0) was employed for statistical analysis. ANOVA test was used for multiple comparisons among treatments. Bonferroni *t* test was used as a post hoc test to compare the percentage change of treatments heart rate with control heart rate. Student's *t* test (paired) was used in some experiments to compare the percentage change in heart rate before and after adding compounds. Also, Student's *t* test (unpaired) was used to compare the treatments with control in case when there were two groups. $P \leq 0.05$ is considered as statistically significant.

Results

Heart rate variation over time

Control experiments were carried out to show the effect of the incubation time on heart rate. When saline is exchanged with fresh saline, heart rate increased by a small percentage (Fig. 1a1, a2). These results suggest that the heart is sensitive to the mechanical disturbance. After 10 min incubation period, the heart rate will slightly decrease. The heart rate stabilizes and remains relatively constant for the duration of time used in these experiments. We used these results as a control for the rest of the experiments unless otherwise stated. We performed control experiments over the same length of times for the various experimental paradigms. 5-HT significantly increases the heart rate and the heart rate does not noticeably change over the subsequent 10 min (Fig. 1b1, b2).

Distribution of in situ basal heart rates

There is a wide variation in the initial heart rates of preparations. The distribution from 50 to >200BPM is quite remarkable. There are few occurrences of preparations with low initial heart rates (50–59). The majority of rates were between 60 and 170 (Fig. 2a). This variation in rate is noted even with using the same saline within one experimental sitting and these experiments were repeated over several different experimental days. Each day a fresh saline was used, but wide variation was still observed. Exposure to 5-HT (100 nM) statistically ($P \leq 0.001$) increases heart rate (Fig. 2b, c). This is consistent with a previous study (Dasari and Cooper 2006).

5-HT dose–response relationship

5-HT concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM) in saline were used to apply directly to the exposed heart. 1 nM and 10 nM 5-HT did not significantly increase the heart rate in comparison to the control exposure of saline; however, 100 nM, 1 μM, and 10 μM 5-HT all significantly increased the heart rate (Fig. 3a, b). After the exposure to 5-HT, the increased rate is relatively consistent for up to 10 min (Fig. 3a). Dose–response relation was obtained by plotting the data acquired from the percentage change in the rate before and during exposure to 5-HT. The control percentage change (exchanging saline with saline) value was subtracted from the percentage change of 5-HT action on the larval heart rate. These adjusted values are depicted by open circles (Fig. 3c).

A simple regression analysis was carried out to determine if there is a relationship between initial heart rate and percentage change of the heart rate. The results generally indicate that there is positive relationship between initial heart rate and percentage change with low initial heart rates having a higher percentage change (Fig. 3d).

Action of 5-HT agonists

Various 5-HT agonists (5-HT1A, 5-HT1B, 5-HT2, and 5-HT7) were investigated for their effect on altering heart rate. 5-HT2 agonist α-methyl-5-HT (100 μM) markedly increased the heart rate upon exposure. The preparations were left for 10 min to observe any further effects of agonists. No further significant changes were observed after the 10-min period. This indicates that the heart rate

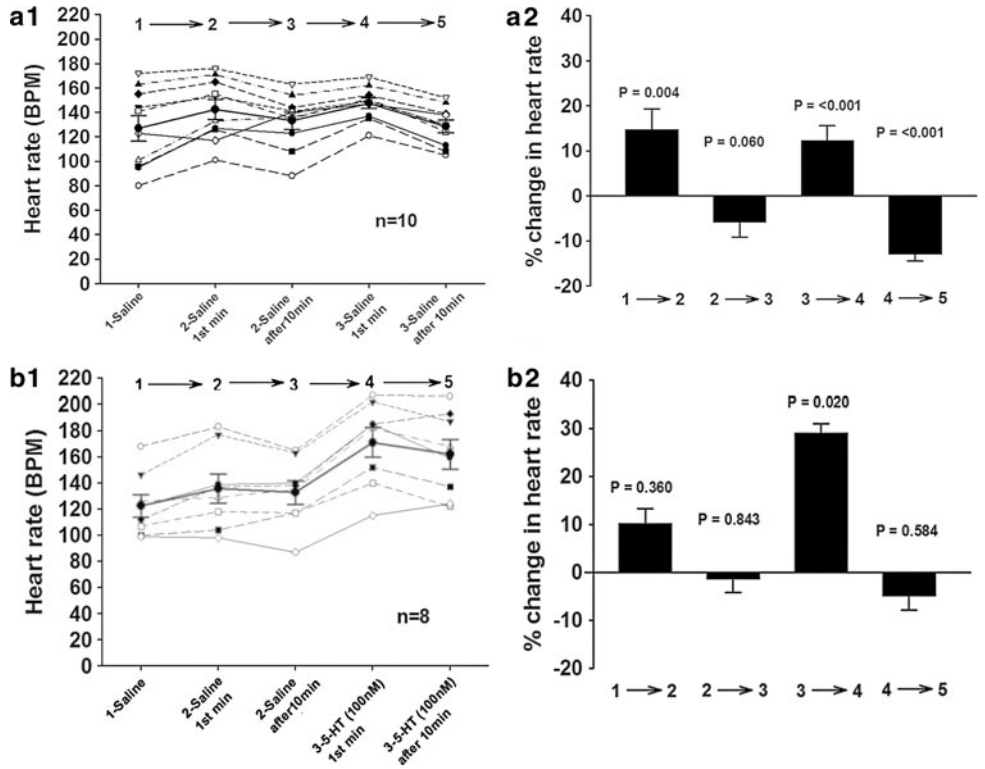

Fig. 1 Change in heart rate for control trails and exposure to 5-HT. **a1** The raw data of the heart rate of individual preparations ($n = 10$). **a2** The percentage change of heart rate before and after changing saline with saline as a control. **b1** Changes in heart rate during the entire experiment before and after application of 5-HT. **b2** 5-HT 100 nM significantly increases the heart rate (note the third change in bathing solution). The preparation was 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 preparation was left for 1 min and subsequently rate was obtained over the next minute. The preparation was left for 10 min and then the heart rate was counted for 1 min. Saline (2-Saline) was exchanged with saline (3-Saline), the preparation was left for 1 min before counting the rate in the next minute. The preparations were left for 10 min and then the heart rate was obtained for 1 min. Data are presented as mean ± SEM

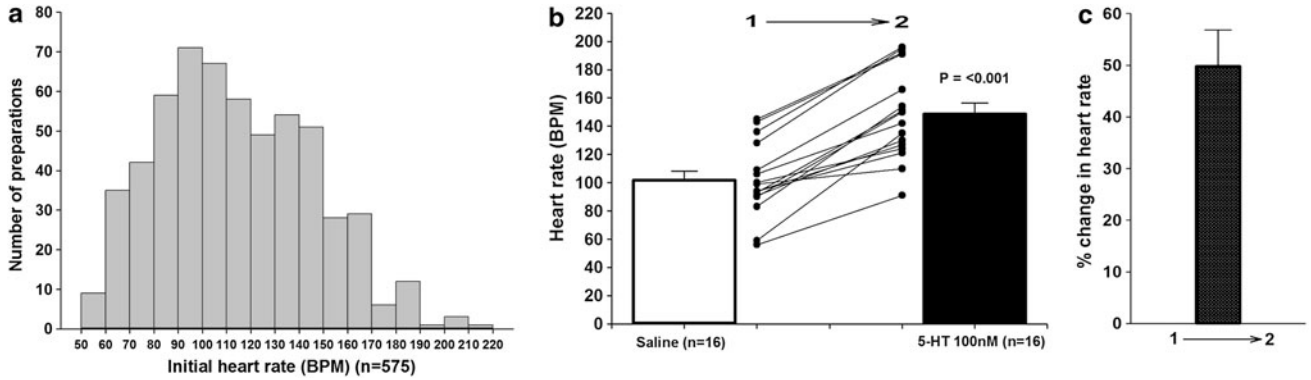

Fig. 2 **a** Distribution of the initial heart rates of preparations used in this study ($n = 575$). **b** There is a positive chronotropic effect of 100 nM 5-HT on heart rate (paired t test). **c** The percentage change of heart rate when saline was exchanged with 5-HT (100 nM). All data presented as mean ± SEM

does not decline after the initial effect within the first few minutes (Fig. 4a, b).

The average of heart rates with different treatments is shown for the entire experimental paradigms (Fig. 5a). Various 5-HT2 agonists were employed to observe if they could modulate the heart rate. The 5-HT2 agonist α-methyl-5-HT (100 μM) markedly increased the heart rate within the initial exposure. The preparations were also left for 10 min. No further significant changes were observed over the 10 min period. This indicates that the heart rate remains stable to the effect of 5-HT or is not altered by a prolonged exposure. Screening of these agonists indicated

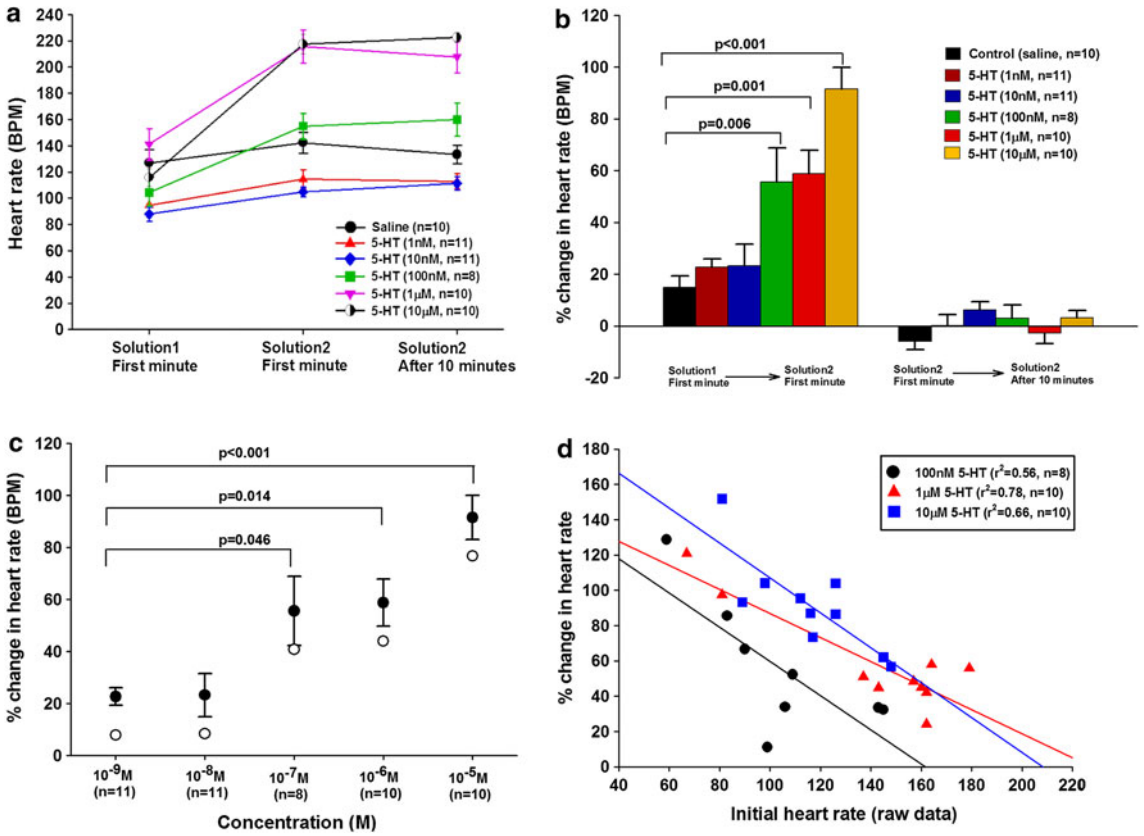

Fig. 3 Effect of various concentrations of 5-HT on larval heart rate. **a** The trend of heart rate at various 5-HT solution concentrations. **b** At first minute (first block of columns) of 5-HT application, heart rate markedly increased for 100 nM, 1 μM, and 10 μM concentrations in comparison to control. After 10 min (second block of columns), the heart rate stayed at the same level as no further % change was noted. **c** Dose–response relation of 5-HT action on larval heart rate. *Open circles* represent the subtraction of control saline exchanges from various concentrations of 5-HT action (Bonferroni's *t* test was used for comparison). **d** Simple linear regression analysis between initial heart rate and percentage change of heart rate. There is generally a positive relationship between initial heart rate and percentage change in heart rate. Data presented as mean ± SEM

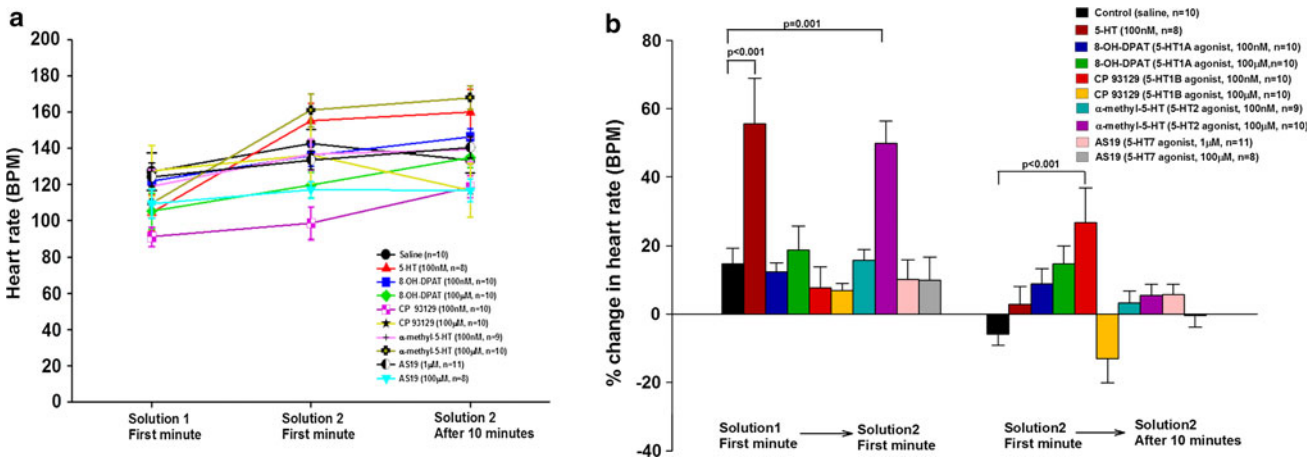

Fig. 4 Effect of various 5-HT agonists on heart rate. **a** The raw data of heart rate throughout the experiment. **b** 5-HT (100 nM) and α-methyl-5-HT (100 μM) significantly increased the heart rate (Bonferroni's *t* test was used to compare the rest of the treatments to control). Data presented as mean ± SEM

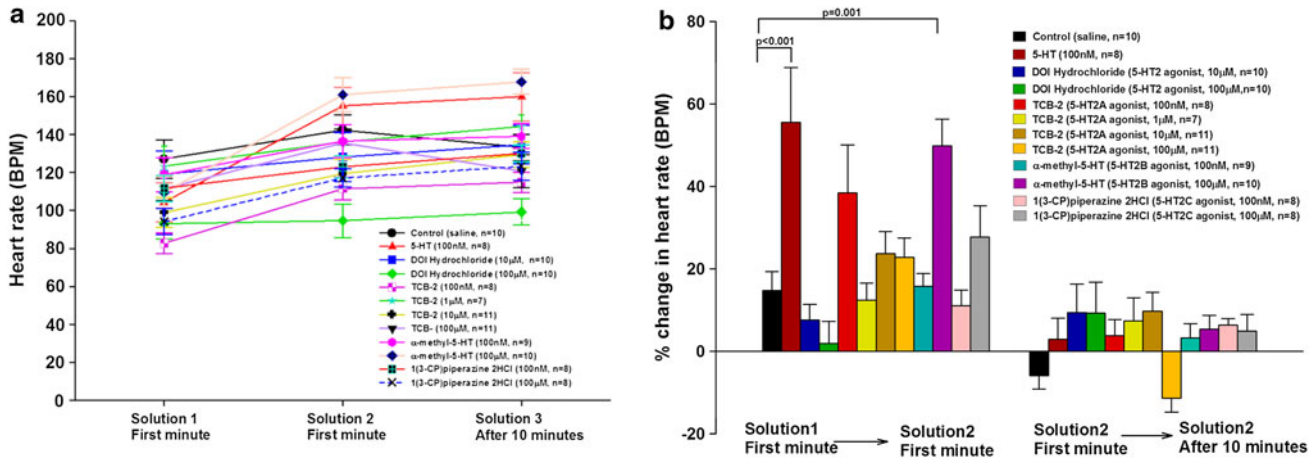

Fig. 5 The effect of various 5-HT2 receptor agonists on larval heart rate. **a** The initial long-term effect of the agonists on heart rate. **b** 5-HT (100 nM) markedly increases the heart rate. Also, α-methyl-5-HT significantly increases the heart rate. TCB-2 and 1(3-CP) piperazine show a slight trend of increasing the rate, but are not statistically significant at $P < 0.05$. The rates were obtained inside saline (solution 1). The saline was exchanged with saline containing an agonist (solution 2). The percentage change in heart rate was measured from solution 1 to solution 2 (first block of columns) and from solution 2 (first minute) to solution 2 after 10 min (second block of columns). Bonferroni's *t* test was used to compare the rest of the treatments with control. Data presented as mean ± SEM

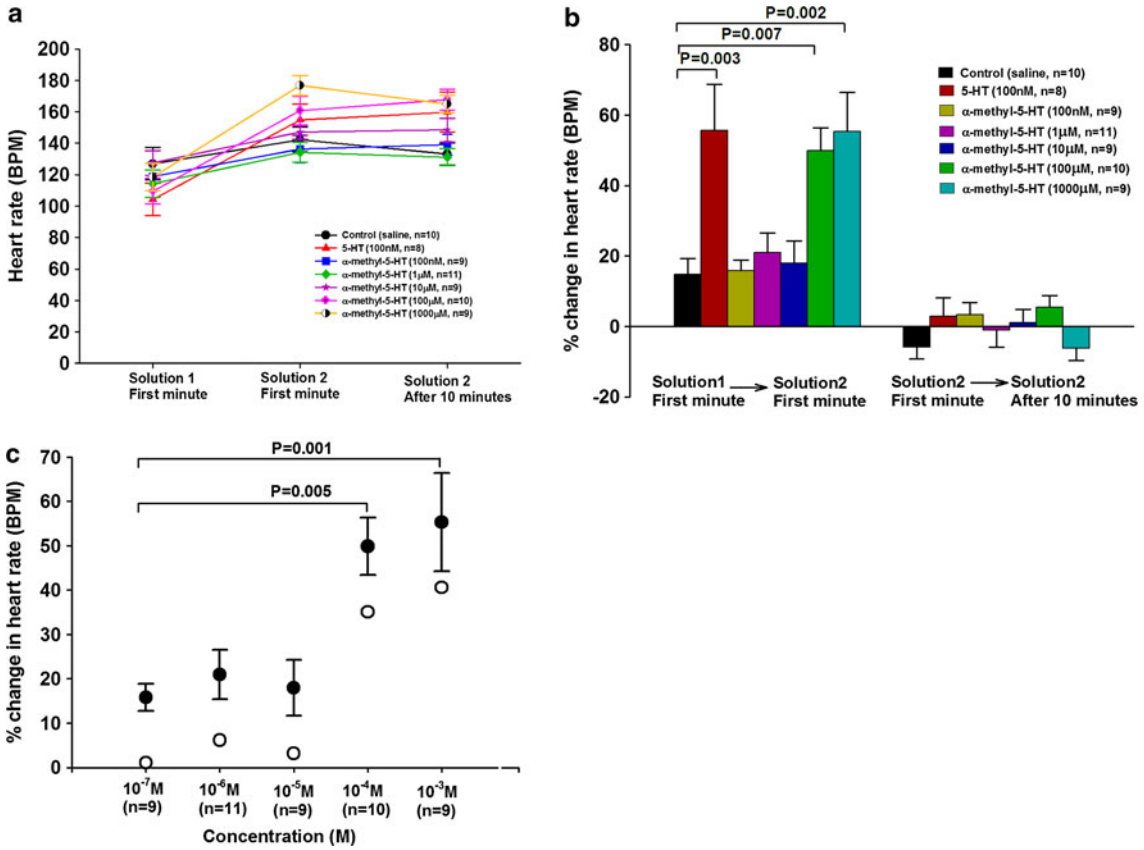

Fig. 6 **a** Heart rate during exposure of α-methyl-5-HT maleate. **b** The effect of various concentrations of α-methyl-5-HT maleate (5-HT2B receptor agonist) on heart rate. α-methyl-5-HT maleate (100 μM) significantly accelerates the heart rate (Bonferroni's *t* test was used to compare the effect of various concentrations of α-methyl-5-HT maleate to control). **c** Dose–response relation of α-methyl-5-HT maleate (5-HT2 agonist). The control (saline) value was subtracted from the values of α-methyl-5-HT maleate action on heart rate (*open circles*) (Bonferroni's *t* test was used to compare the effect of various concentrations of α-methyl-5-HT maleate to 100 nM α-methyl-5-HT maleate). Data presented as mean ± SEM

that TCB-2 (100 nM) and 1(3-CP) piperazine (100 μM) produced an increase in rate (Fig. 5b).

Action of α-methyl-5-HT maleate

The trend in the rates is shown for the various concentrations of α-methyl-5-HT maleate (Fig. 6a). The concentrations of 100 nM, 1 μM, 10 μM, 100 μM and 1000 μM were used. The preparations were left inside saline for 1 min and then heart beats counted for the next minute (solution 1). Afterwards the saline was exchanged with saline containing one of the α-methyl-5-HT concentrations indicated. The preparation was left for an additional minute during the exposure and the rate counted the following minute (solution 2). The

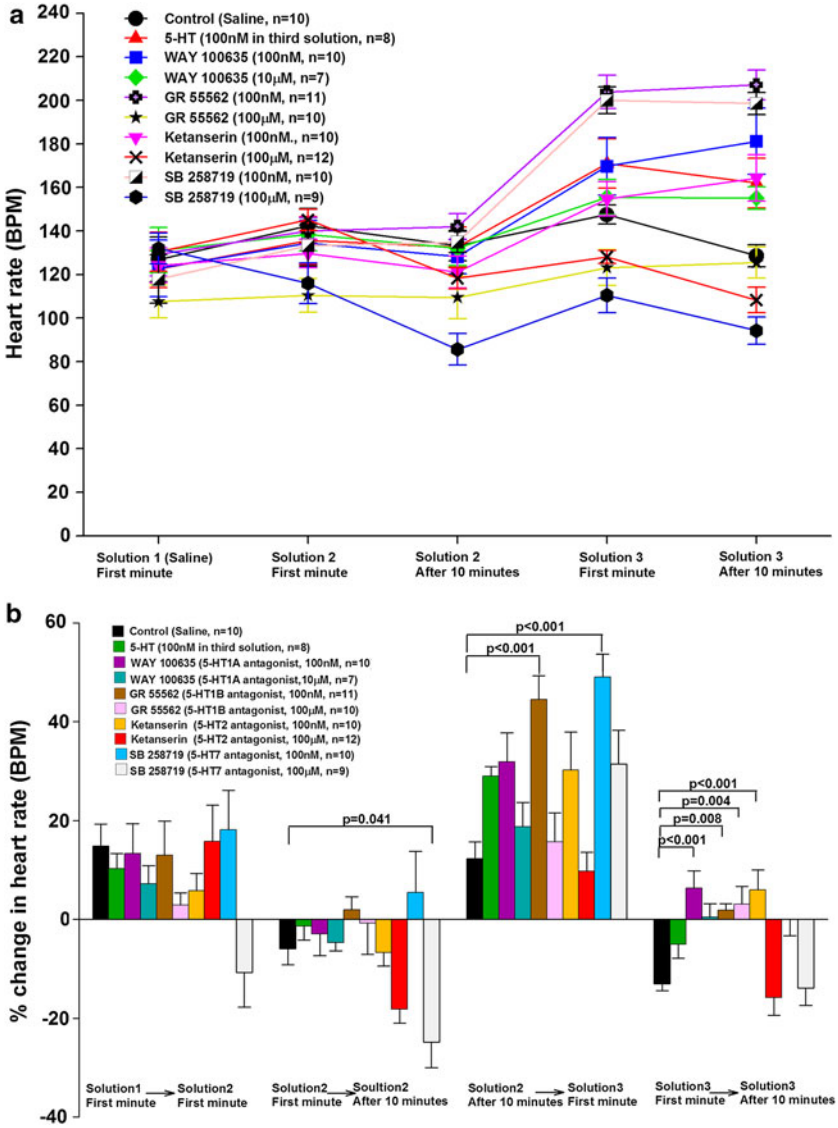

Fig. 7 Effect of various 5-HT receptor antagonists on the action of 5-HT on heart rate. **a** The trend of heart rates during the period of the experimental paradigm. **b** Preparations were left inside saline for 1 min and the rate was counted over the next minute (BPM) (solution 1). The saline was exchanged with saline containing one of the various antagonists. The preparations were left for 1 min, and the heart rate was counted over the following minute (solution 2). The percentage change (first group of histogram) was calculated from saline to saline containing antagonist. The preparations were left inside antagonist for 10 min and the rate counted. The percentage change was calculated from saline containing antagonist (first minute) to saline containing antagonist (after 10 min). Afterwards, the saline containing the antagonist was exchanged with saline containing a combination of 5-HT (100 nM) and the same antagonists. These preparations were left for 1 min and the rate was counted (solution 3). The percentage change was calculated from saline containing antagonist to saline containing 5-HT and antagonist. A percentage change was calculated from saline containing 5-HT and antagonist (first minute) to saline containing 5-HT and antagonist (after 10 min). Ketanserin (100 μM) blocks the positive chronotropic action of 5-HT in larval heart in comparison to control larvae. 5-HT7 antagonist (SB 258719) also reduced the heart rate in the absence of the 5-HT; however, 5-HT7 antagonist (SB 258719) did not block the 5-HT action (Bonferroni's *t* test was used to compare the treatments with control). Data presented as mean ± SEM

percentage change (first block of columns) was calculated from saline to saline containing agonist. The preparation was left exposed to agonist for 10 min and the heart rate counted in the following minute. The percentage change was calculated from saline containing agonist (first minute) to saline containing agonist (after 10 min). α-methyl-5-HT maleate 100 and 1000 μM significantly increased the heart rate. The preparations were left inside agonists for 10 min. After that the heart rate did not decrease (Fig. 6b). The α-methyl-5-HT maleate at 100 or 1000 μM significantly increased the heart rate. However, 100 nM α-methyl-5-HT maleate did not have a significant effect on the heart rate. The 1 and 10 μM α-methyl-5-HT maleate exposure slightly increased the heart rate. The changes measured for exchanging saline with saline as a control are subtracted from the responses and the results are shown as open circles (Fig. 6c).

Action of 5-HT antagonists

Several 5-HT antagonists (5-HT1A, 5-HT1B, 5-HT2, and 5-HT7) were employed to determine if they blocked the action of 5-HT. The 5-HT7 antagonist (100 μM, SB 258719) significantly decreased heart rate even in the absence of 5-HT (gray bar in first group of histograms, Fig. 7a, b); whereas the 5-HT2 antagonist (100 μM, ketanserin) completely blocked the action of 5-HT on heart rate, but had no effect on its own in the absence of 5-HT within the first minute. However, 5-HT7 antagonist did not block the action of 5-HT on heart rate (third group of histograms, light blue and gray bars, Fig. 7). 5-HT1A antagonist (100 μM, WAY 100635) and 5-HT1B agonist (100 μM, GR 55562) reduced the action of 5-HT on larval heart rate (Fig. 7b).

Action of ketanserin

Ketanserin 100 nM, 1 μM, 10 μM, and 100 μM were used to address the antagonist action to 5-HT. The trend of heart rates over the duration of the experimental paradigm was examined (Fig. 8a). Ketanserin completely blocked the action of 5-HT (100 nM) in a dose-dependent manner (Fig. 8b). When the preparations were incubated in ketanserin (100 μM) alone for 10 min, the heart rate significantly decreased ($P = 0.029$) (Fig. 8b). However, exposure to 5-HT with low levels of ketanserin resulted in an increase in heart rate, but the higher concentrations (1, 10, and 100 μM) blocked the 5-HT induced increase. Ketanserin (5-HT2) antagonist decreased the heart rate in a dose–response manner and as noted 100 μM completely blocked the action of 5-HT (Fig. 8c).

Effect of 5-HT and 5-HT2 agonist and antagonist on aorta rate

The effect of 5-HT, 5-HT2 agonist (α-methyl-5-HT) and 5-HT antagonist (ketanserin) was examined on heart and aorta rates concurrently. The results demonstrate that aorta responds to 5-HT, 5-HT2 agonist as well as 5-HT2 antagonist. 5-HT significantly increases the aorta rate (Fig. 9a1, a2). 5-HT2 agonist (α-methyl 5-HT) markedly increases the aorta rate (Fig. 9b1, b2). Moreover, 5-HT2 antagonist (ketanserin) blocks the action of positive chronotropic effect of 5-HT on heart and aorta rates (Fig. 9c1, c2).

5-HT action in 5-HT2 receptor mutant larvae

A dose–response relationship for 5-HT in w^{1118} larvae was performed. In the w^{1118} line, 5-HT increases the heart rate in a dose–response manner (Fig. 10a1, a2, a3). 5-HT2A receptor insertional mutant larvae were used to observe how mutation in 5-HT2A affects on 5-HT action on heart rate. 5-HT (100 nM) does not significantly increase the heart rate (Fig. 10b1, b2, b3); however, 5-HT (1 and 10 μM) increased the heart rate. The 5-HT (10 μM) action was lower than the 5-HT (1 μM) action.

The RNA interference (RNAi) approach was used to knock down 5-HT2ADro receptor expression. The results demonstrate that 5-HT2A knockdown reduces the action of 5-HT; however, it is not statistically significant (Fig. 11a1, a2). The flies were raised at 30 °C to increase the efficiency of RNAi-mediated knockdown since Gal4 activity is temperature dependent (Duffy 2002). A 5-HT2BDro mutant line was used to probe the role of 5-HT2BDro in the action of 5-HT. The results show that the 5-HT2BDro mutant flies were less responsive to 5-HT in comparison to w^{1118} flies (Fig. 11b1, b2).

Discussion

The *Drosophila* heart is used as a model to obtain insights into the underlying molecular mechanisms of heart diseases in other organisms (Piazza and Wessells 2011). One of the advantages of *Drosophila* is its genetic amenability. Genetic screens in *Drosophila* can be easily carried out to find genes that might be culprits and implicated in cardiac dysfunctions (Bier and Bodmer 2004; Wolf et al. 2006). The *Drosophila* larval heart is modulated by neurotransmitters and neuromodulators (5-HT, dopamine, octopamine) (Johnson et al. 1997; Dasari and Cooper 2006; Titlow et al. 2013). Serotonergic neurons in larval CNS synthesize 5-HT and they release 5-HT into the hemolymph when the larvae are under the stress. This results in

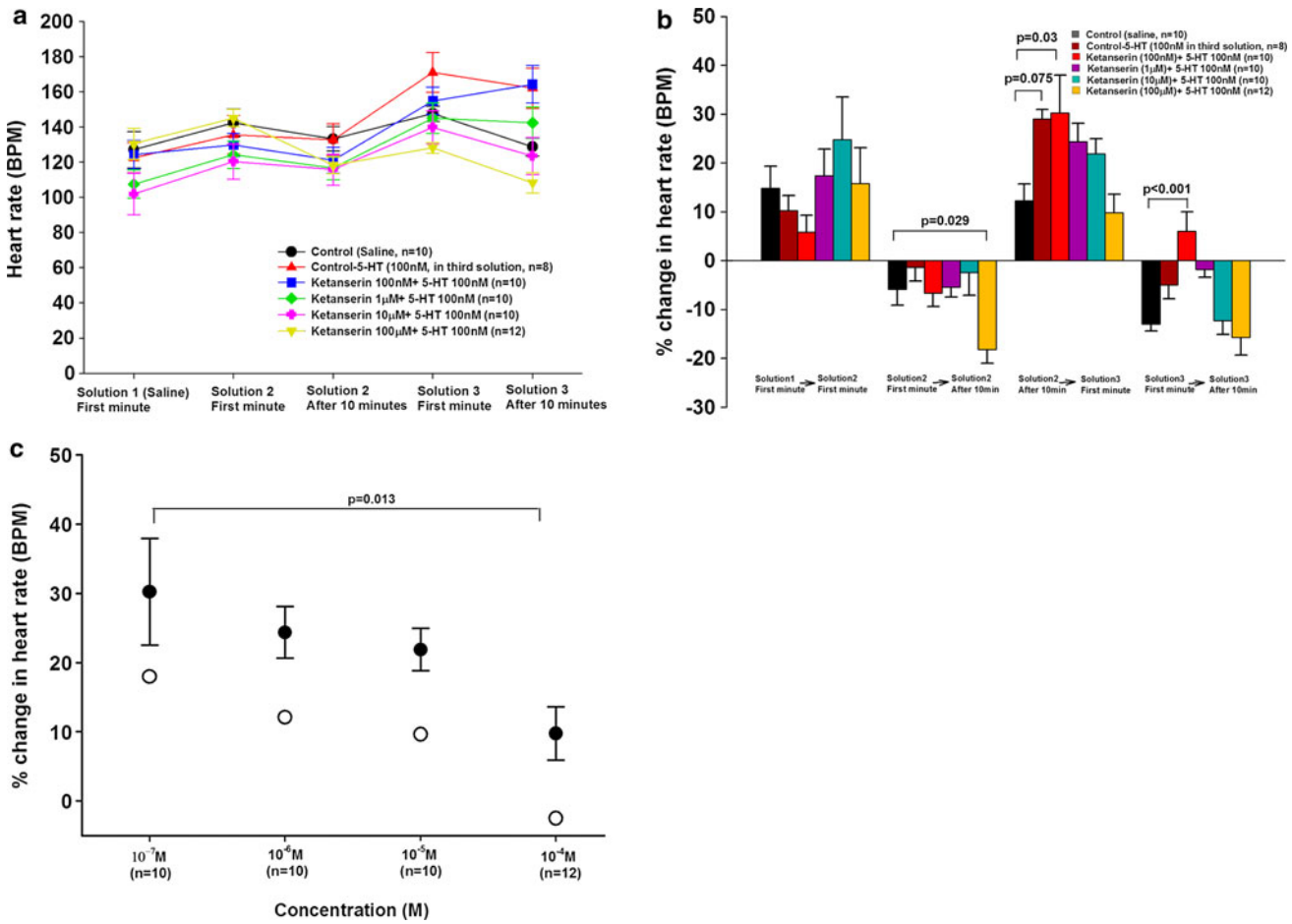

Fig. 8 Effect of various concentrations of ketanserin on the action of 5-HT on heart rate. **a** Heart rate over the period of experimentation. **b** The preparations were left inside saline for 1 min and the rate was counted over the next minute (BPM) (solution 1). Saline was exchanged with saline containing one of the ketanserin concentrations. The preparations were left for 1 min, and the heart rate was counted over the next minute (solution 2). The percentage change was calculated from saline to saline containing ketanserin. The preparations were left inside ketanserin for 10 min and the rate was counted for the next minute. The percentage change was calculated from saline containing ketanserin (first minute) to saline containing ketanserin (after 10 min). Afterwards, saline containing ketanserin was exchanged with saline containing a combination of 5-HT (100 nM) and ketanserin. The preparations were left for 1 min, and the heart rate was counted over the following minute (solution 3). The percentage change was calculated from saline containing ketanserin to saline containing 5-HT and ketanserin. The preparations were left inside 5-HT and ketanserin for 10 min. The heart rate was counted for 1 min after 10 min. The percentage change was calculated from saline containing 5-HT and antagonist (first minute) to saline containing 5-HT and antagonist (after 10 min). Ketanserin (100 μM) noticeably blocks the action of 5-HT on heart rate. However, ketanserin (100 μM) decreases the heart rate in the absence of 5-HT. (Bonferroni's *t* test was used to compare the treatments with control). **c** Dose–response relationship of ketanserin (5-HT2 antagonist). The control (saline) value was subtracted from the values of ketanserin action on heart rate (*open circles*) (Bonferroni's *t* test was used to compare the effect of various concentrations of ketanserin to 100 nM ketanserin). Data presented as mean ± SEM

the heart rate acceleration. In this study, the 5-HT receptor that mediates the positive chronotropic action of 5-HT was investigated using a pharmacological approach. Various 5-HT agonists and antagonists were used to investigate the 5-HT receptor subtypes in *Drosophila* larval heart. This study broadens our understanding regarding the action of 5-HT in the heart. 5-HT also modulates heart rate in mammalians; however, the effect of 5-HT in mammalian heart is biphasic, which causes tachycardia and bradycardia, due to the presence of different 5-HT receptors in cardiovascular system (Villalón and Centurión 2007).

5-HT dose–response relationship

5-HT is a monoamine neurotransmitter as well as neuromodulator (Coleman and Neckameyer 2005) and is synthesized from tryptophan. There are two enzymes that catalyze the biosynthesis of 5-HT, DTRH, a rate-limiting enzyme of 5-HT synthesis in serotonergic neurons, and DTPHu which mediates peripheral synthesis (Neckameyer et al. 2007). As we established, the *Drosophila* larval heart is very sensitive to 5-HT with even a 100 nM significantly accelerating heart rate. We also investigated varied

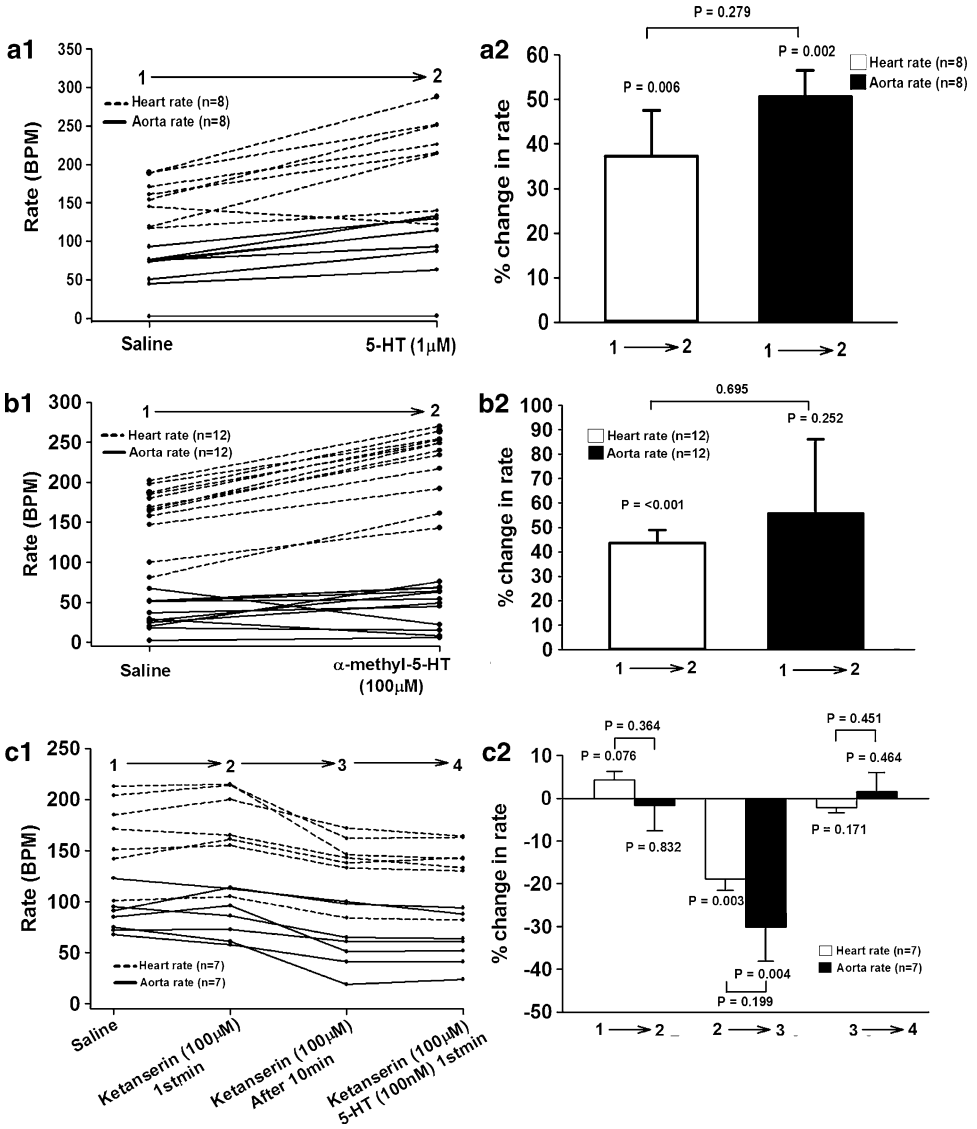

Fig. 9 Action of 5-HT on heart and aorta independently. **a1** Heart and aortic rates before and during 5-HT application. The preparations were left for 1 min and then the heart and aorta rates were counted simultaneously for 1 min. **a2** The effect of 5-HT (1 μM) on heart and aorta. 5-HT (1 μM) significantly increases the aorta rate as well as heart rate. The aorta rate is lower than the heart rate; although, there was no significant difference between the heart and aorta rates percentage changes (Paired *t* test was used to compare the heart rate before and after 5-HT application). (Student's *t* test (non-paired) was used to compare heart and aorta percentage change). **b1** Heart and aortic rates before and during 5-HT2 agonist (α-methyl-5-HT) application. **b2** 5-HT2 agonist (α-methyl-5-HT) (100 μM) application markedly increases both heart and aorta rates. **c1** The trend of heart rate before and during 5-HT2 antagonist (ketanserin) application. **c2** The effect of 5-HT2 antagonist (ketanserin) on heart and aorta rates. 5-HT2 antagonist (ketanserin) (100 μM) significantly blocks the action of 5-HT on the heart and aorta. Hearts were separated from aortas prior to the chemicals being applied. The preparations were left for 1 min and then the heart and aorta rates were counted for 1 min simultaneously in the same preparation. All the chemicals have effect on aorta rate as well as on heart rate. Data presented as mean ± SEM

concentrations to determine if a similar biphasic response might be present as that in mammals. It was observed that high 5-HT concentration (10 μM) remarkably elevates the heart rate and the heart rate remains high even after 10 min of exposure. 5-HT increases the heart rate in a dose–response manner and even 10 μM might not have resulted in a plateau in the increase in heart rate. Interestingly, 5-HT also modulates the heart rate in other invertebrates (Collins and Miller 1977; Platt and Reynolds 1986). Moreover, mammalian heart is modulated by 5-HT. This modulation can even occur in a fetal heart from a disturbance in physiological 5-HT levels in the mother. Such fetal alterations will alter the physiology of the heart in offspring (Fligny et al. 2008).

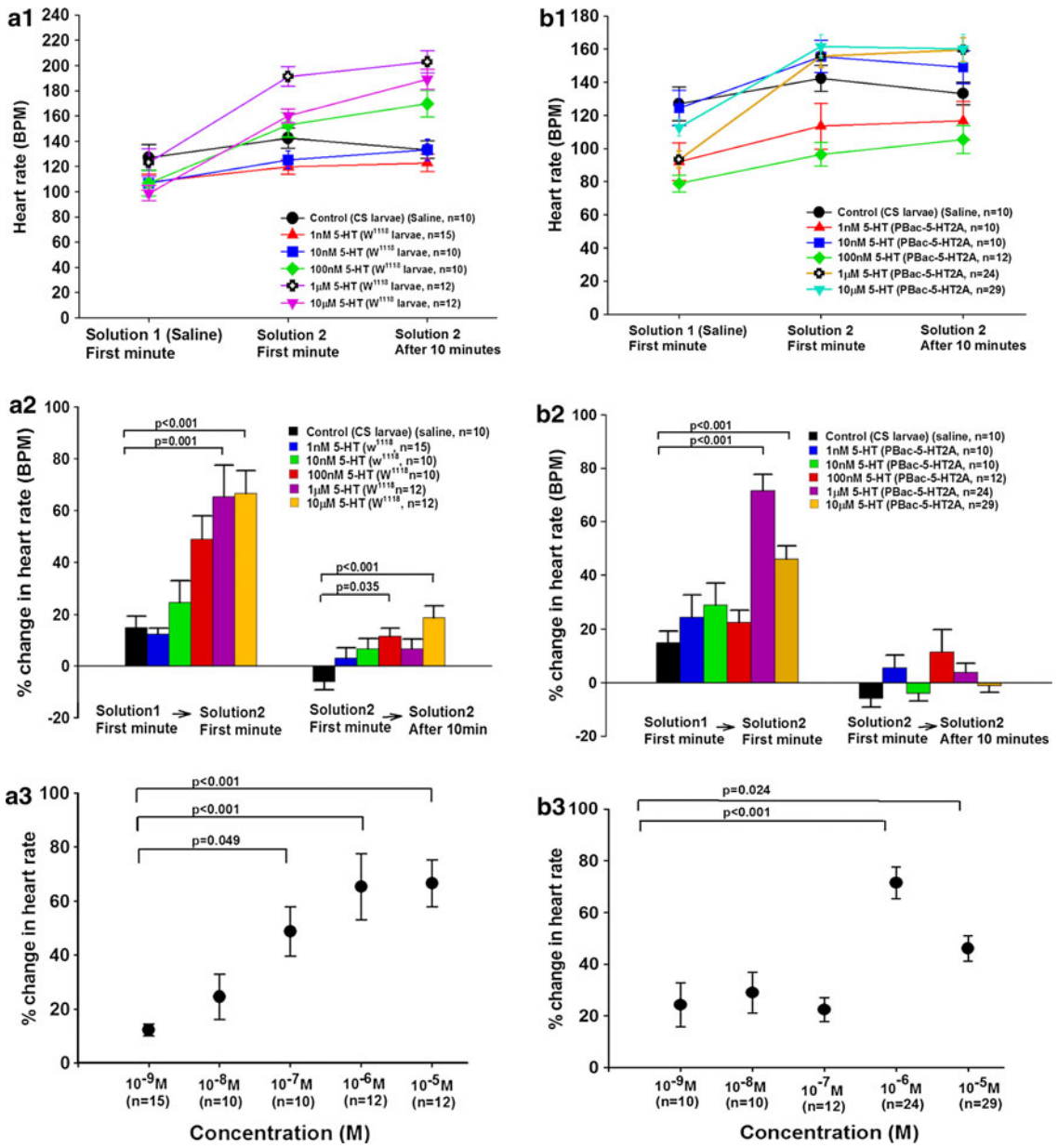

Fig. 10 5-HT action on heart in 5-HT2 receptor mutant larvae. **a1** The trend of heart rate for the same period of time as the experimental paradigm. **a2** The effect of various 5-HT concentrations on heart rate in w^{1118} larvae. The heart rates of w^{1118} larvae are compared to canton-S (CS) larvae (control). The preparations were left inside saline for 1 min and afterwards the heart rate was counted for 1 min. The preparations were left for 10 min, and then the heart rate was counted for 1 min. **a3** Dose–response-relationship of 5-HT in w^{1118} larvae. **b1** Heart rate for the duration of the experimental protocol. **b2** The effect of various 5-HT concentrations on heart rate in 5-HT2ADro insertional mutant larvae. 5-HT (100 nM) does not have a marked effect on heart rate. 5-HT (1 and 10 μM) significantly increased the heart rate. However, the action of 5-HT (100 μM) is less than the action of 5-HT (10 μM). **b3** Dose–response relationship of 5-HT in 5-HT2ADro receptor insertional mutant larvae

5-HT2 agonists' actions on heart rate

In examining the 5-HT agonists, we noted α-methyl 5-HT maleate significantly increased the heart rate at 100 and 1000 μM (Figs. 4, 5, 6). TCB-2 and 1(3-CP) piperazine 2HCl had effect on heart rate; however, the action of TCB-2 and 1(3-CP) piperazine 2HCl was not statistically significant (Fig. 5). The affinity of α-methyl 5-HT maleate might be weaker than 5-HT to the 5-HT2 receptor since 5-HT noticeably increases the heart rate at 100 nM and α-methyl 5-HT maleate markedly increases the heart rate at 100 μM. A study has shown that α-methyl-5-HT has some potency for 5-HT2ADro ($pK_i = 6.8$), but 5-HT is more potent for 5-HT2ADro ($pK_i = 7.8$) (Colas et al. 1995). Using

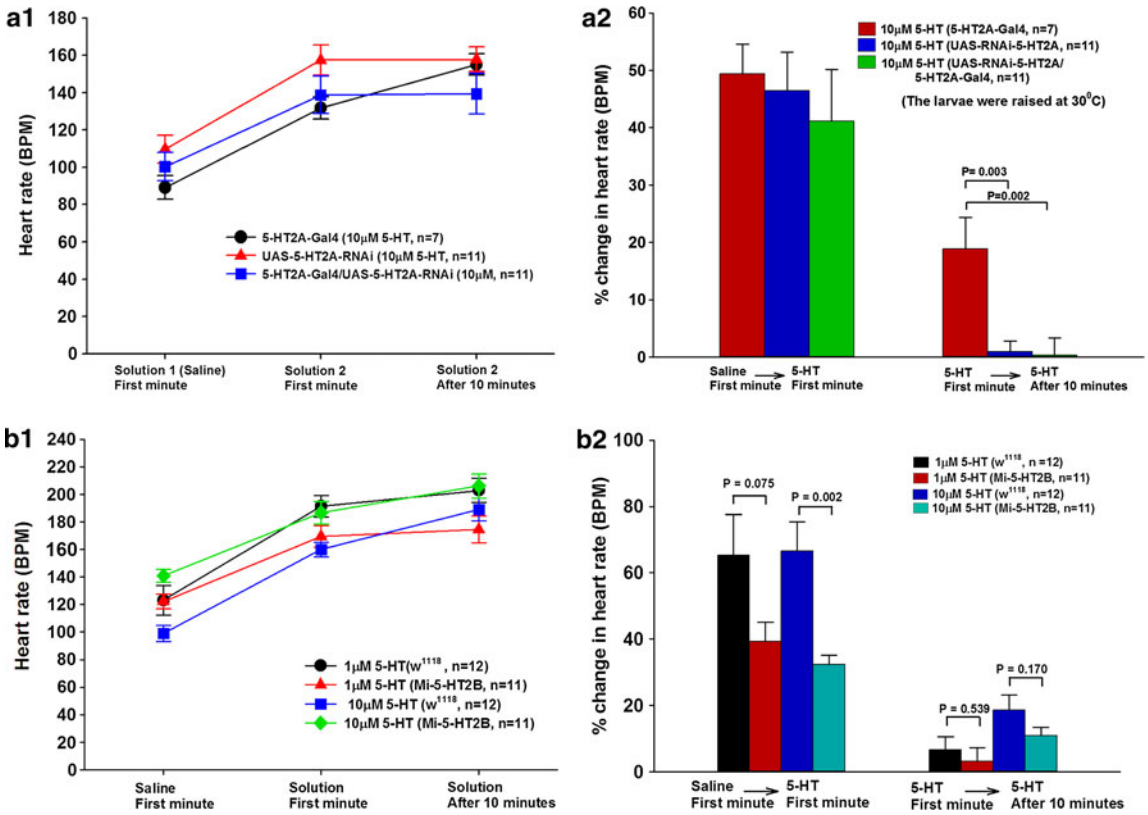

Fig. 11 **a1** The trend in heart rate for the duration of the experimental time frame. **a2** 5-HT action in 5-HT2A knockdown larvae. The flies were raised at 30 °C to increase the activity of Gal4 transcriptional activator. 5-HT (10 μM) markedly increased the heart rate in all fly lines. The 5-HT2A receptor knockdown tends to reduce the effect of 5-HT action on heart rate, but the reduction is not statistically significant. When the preparation was left inside the 5-HT solution for 10 min, the heart rate noticeably increased in 5-HT2A-Gal4 driver line; however, this increment was not observed in UAS-RNAi-5-HT2A and UAS-RNAi-5-HT2A/5-HT2A-Gal4 lines (Bonferroni test was used for this comparison). **b1** The trend of heart rate during the experimental conditions. **b2** 5-HT action in 5-HT2BDro receptor mutant larvae. There is a marked difference between w^{1118} (control) and 5-HT2B receptor mutant larvae regarding response to 5-HT. The response of 5-HT2B receptor mutant larvae hearts to 5-HT is weaker compared to w^{1118} larvae hearts (unpaired *t* test was used for this comparison)

heterologous expression system, it has been shown that K_i of 5-HT in a COS-1 cell line for 5-HT2Dro is 200 ± 16 nM, notwithstanding the K_i for α-methyl 5-HT is 420 ± 23 nM (Schaerlinger et al. 2007). So, the affinity of 5-HT to 5-HT2Dro is higher than α-methyl 5-HT in these conditions. Recently, a study has revealed in *M. sexta* that 5-HT can activate 5-HT receptor at 100 nM; however, 5-HT2 agonist (DOI) weakly activates 5-HT2 receptor at 10 μM and produces a drastic activation of 5-HT2 receptor at 100 μM (Dacks et al. 2013). These data show that the invertebrate 5-HT receptor responses to vertebrate 5-HT agonist and antagonists are different. It has been demonstrated that (R)-DOI is effective in *Drosophila* (Johnson et al. 2009); even though, surprisingly, (±)-DOI did not increase larval heart rate (Fig. 4). We observed that TCB-2 also increases the heart rate at low concentration, but it does not increase the heart rate at higher concentration. TCB-2 might desensitize 5-HT receptors at high concentration or it might not have a high affinity for the *Drosophila* 5-HT receptors.

An insertional mutation of *Drosophila* DTRHn significantly decreases the heart rate in white prepupal stage (WPP) in *Drosophila* also indicating that the 5-HT receptors play a role in modulating heart rate (Neckameyer et al. 2007). Also, the *Drosophila* pupa shows an increase in heart rate when α-methyl 5-HT was injected into P1 pupal stage (Johnson et al. 2002). The α-methyl 5-HT maleate agonist appears to function like 5-HT across species so that this agonist may likely be viable to use in comparative studies of 5-HT receptor subtypes. Apparently, α-methyl 5-HT injection into crustacean (crayfish) raises the hemolymph glucose level just as an injection of 5-HT (Lee et al. 2000) and application of α-methyl 5-HT enhances synaptic transmission at crayfish neuromuscular junction (Tabor and Cooper 2002). The main 5-HT receptor subtype at the neuromuscular junction is also likely a 5-HT2 form. Even in the crayfish preparations, α-methyl 5-HT maleate does not fully reproduce the effect of 5-HT at the same concentration likely due to the presence of other 5-HT receptor

subtypes or differences in binding affinity to the 5-HT2 receptor subtype. In mammals, the 5-HT2B signaling pathway is indispensable for heart development and normal physiology of adult heart (Nebigil and Maroteaux 2001). So, it is likely that 5-HT2B has an evolutionarily conserved function in physiological regulation of heart function.

5-HT antagonists' actions on heart rate

Screening the various 5-HT receptor antagonists revealed that ketanserin (a 5-HT2 antagonist) and GR 55562 (5-HT1B antagonist) substantially block the 5-HT action on the larval heart rate at 100 μM (Fig. 7). However, after incubation of the preparation inside a combination of antagonist and 5-HT, the heart rate in GR 55562(100 μM)-incubated preparations significantly increased; whereas, the heart rate of ketanserin (100 μM)-incubated preparations strikingly decreased. The result of ketanserin blocking the 5-HT action on larval heart and that the heart rate decreases when incubated in ketanserin for 10 min suggests that ketanserin might work as an inverse agonist on heart 5-HT receptors. This is feasible if the 5-HT2 receptor has a constitutive activity (Nitanda et al. 2005) since ketanserin further reduces the heart rate after 10 min incubation. This is also consistent with the data that show ketanserin prominently inhibited cardioacceleratory action of 5-HT when it was injected into P1 pupal stage with 5-HT in *D. melanogaster* (Johnson et al. 2002). Also, it has been shown that ketanserin blocks the action of 5-HT on synaptic transmission in crayfish (Tabor and Cooper 2002) which is evidence that there is receptor homology in pharmacological function between invertebrates and vertebrates.

5-HT and 5-HT2 agonist and antagonist actions on aorta rate

The aorta was separated from the heart and afterwards the various compounds were applied to the preparations. The aorta and heart rates were counted simultaneously in the same preparations which demonstrated the intrinsic aorta rate is lower than the heart rate. 5-HT, 5-HT2 agonists (α-methyl-5-HT) noticeably increase the heart and aorta rates. Moreover, the 5-HT2 antagonist (ketanserin) significantly blocked the positive chronotropic action of 5-HT on aorta rate as well as heart rate. These results indicate that 5-HT receptors are activated in the aorta. The density of the 5-HT receptors in the aorta might be less than the receptors on the heart. Also, the ionic channel composition of pacemaker cells of the aorta might be different than for the heart.

5-HT2 receptors mutation and 5-HT action

It has been shown that 5-HT2ADro receptor mutant line, which is used in this study, has reduced 5-HT2A receptor expression. However, this insertional mutation is not a null mutation; it is a hypomorphic mutation since there is still low percentage rate of 5-HT2A mRNA expression (Nichols 2007). Here, we used the same 5-HT2ADro insertional mutant line to observe the action of 5-HT. 5-HT (100 nM) cannot increase the heart rate dramatically, even though the 5-HT (1 and 10 μM) significantly increases the heart rate. We observed that the 5-HT (10 μM) action was lower than 5-HT (1 μM). These results suggest that 5-HT2A receptor mutation reduces the action of 5-HT and high concentration of 5-HT (10 μM) might desensitize 5-HT receptors in 5-HT2A mutant larvae. We speculate that both 5-HT2ADro and 5-HT2BDro might be expressed in larvae cardiac tissue. It has been shown that the EC50 of 5-HT for 5-HT2ADro is lower (99 nM) compared to EC50 of 5-HT for 5-HT2BDro (293 nM) (Gasque et al. 2013). We employed 5-HT2BDro receptor mutant line to further our understanding of the 5-HT receptors that might be involved in positive chronotropic action of 5-HT. The 5-HT can increase the heart rate in 5-HT2BDro mutant larvae, although the response of 5-HT2BDro mutant larvae to 5-HT was lower in comparison to the response of w^{1118} larvae to 5-HT. These results suggest that 5-HT2BDro might be another 5-HT receptor which could modulate heart rate.

5-HT receptors are known to be G-protein coupled receptors (GPCR) in *Drosophila* (Yuan et al. 2006). In our study, the results show that 5-HT2 agonist increases the heart rate and 5-HT2 antagonist decreases the heart rate and can block 5-HT action on the larval heart. Also, the results indicate that the 5-HT2ADro or 5-HT2BDro mutation decreases the responsiveness of the heart to 5-HT. The intracellular signaling pathway of 5-HT2Dro has not been completely identified. By using various pharmacological agents that target the activity of PLC-PKC pathway and intracellular Ca^{2+} store release, one would be able to obtain a better understanding of 5-HT2 signaling pathway in larval heart. 5-HT2 receptor is coupled to Gαq protein in mammals. When 5-HT or 5-HT agonist binds to 5-HT2 receptor, Gαq will be activated and released from heterotrimeric G-protein complex. Gαq can activate phospholipase Cβ (PLC). This can result in the lipid membrane phosphatidylinositol 4,5-bisphosphate (PIP2) being cleaved by active PLC into two second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG). The rise in DAG can activate protein kinase C (PKC); whereas, IP3 binding to IP3 receptors on endoplasmic reticulum (ER) will cause Ca^{2+} release into cytosol. Elevated Ca^{2+} level in cytosol can lead to the activation of calcium/calmodulin-dependent kinase, subsequently this can activate calmodulin and lead to blocking of K^+ channels (Nichols and Sanders-Bush 2001). Active PKC is able to modulate L-type calcium channels in cardiac cells (Kamp and Hell

2000). We have already shown that inhibition of AC does not have effect on the 5-HT action on the heart rate (Majeed et al. 2013). Therefore, we suggest that activation of PLC-IP3-PKC pathway might be responsible for the 5-HT action through activation of 5-HT2 receptor in *Drosophila* larval heart. It has been shown that KEN-93, which is a CaM kinase II inhibitor, inhibits the 5-HT action on the heart in P1 pupal stage of *Drosophila*. CaM kinase II has the ability to modulate ion channels, such as, Ca^{2+} and K^{+} channels (Johnson et al. 2002).

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