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Direct influence of serotonin on the larval heart of *Drosophila melanogaster*

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Abstract The heart rate (HR) of larval *Drosophila* is established to be modulated by various neuromodulators. Serotonin (5-HT) showed dose-dependent responses in direct application within semi-intact preparations. At 1 nM, HR decreased by 20% while it increased at 10 nM (10%) and 100 nM (30%). The effects plateaued at 100 nM. The action of 5-HT on the heart was examined with an intact Central Nervous System (CNS) and an ablated CNS. The heart and aorta of dorsal vessel pulsate at different rates at rest and during exposure to 5-HT. Splitting the heart and aorta resulted in a dramatic reduction in pulse rate of both the segments and the addition of 5-HT did not produce regional differences. The split aorta and heart showed a high degree of sensitivity to sham changes of saline but no significant effect to 5-HT. Larvae-fed 5-HT (1 mM) did not show any significant change in HR. Since 3,4-methylenedioxymethamphetamine (MDMA) is known to act as a weak agonist on 5-HT receptors in vertebrates, we tested an exogenous application; however, no significant effect was observed to dosage ranging from 1 nM to 100 μM in larvae with and without an intact CNS. In summary, direct application of 5-HT to the larval heart had significant effects in a dose-dependent manner while MDMA had no effect.

Keywords Cardiovascular · CNS · Drugs of abuse · MDMA · Ecstasy

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Introduction

The various functional aspects of heart regulation from development to normal maintenance throughout the larval life of *Drosophila melanogaster* and its metamorphosis are being extensively addressed (Ashton et al. 2001; He and Adler 2001; Molina and Cripps 2001; Ponzielli et al. 2002; Sláma and Farkaš 2005; Wessells and Bodmer 2004). In addition, regulation of the heart via hormonal and direct neural innervation continues to be an active research area using this model organism (Dulcis and Levine 2005; Dulcis et al. 2005; Johnson et al. 2002; Miller 1997; Papaefthimiou and Theophilidis 2001). Relatively recently 5-HT receptors (5-HT1B/1CR and 5-HT2A/2BR subtypes) were shown to be present in the vertebrate aortic and mitral valve cells of the heart (Fitzgerald et al. 2000; Roy et al. 2000) and linked to some forms of cardiac valve disease with altered regulation (Jian et al. 2002). Since the larvae develop quickly and the age can be precisely timed, the development of the heart from the embryo stage and throughout the growth of the larvae, as well as the metamorphosis from larvae to adult, can be readily examined within a matter of 1–2 weeks. High throughput screening is possible to assess multitudes of pharmacological agents or mutational screens (Gu and Singh 1995).

The *Drosophila* heart, better known as the dorsal vessel, is a continuous tube extending from the last abdominal segment to the dorso-anterior region of the cerebral hemisphere. The heart is divided into anterior aorta and posterior aorta (Rizki 1978). The heart does not pulsate constantly as is noted when larvae are preparing to crawl (Rizki 1978). When a larva is engaged in feeding, the pulsation of the heart and the motion of the mouth hooks are seen to be functionally related. This is partly due to the fact that the terminal ligaments of the aorta are attached to the pharyngeal region of the alimentary canal.

In the larvae, the origin of the heartbeat is known to be myogenic (Dowse et al. 1995; Johnson et al. 1997);

however, the larval heart has not been shown to have innervation to date. Whereas in adults, Dulcis and Levine (2003) have shown that the heart is innervated. Thus, the current dogma is that the larval heart is myogenic without innervation. The heart rate (HR) in larvae can be altered by neurotransmitters and neuromodulators present in the hemolymph (Johnson et al. 1997, 2000; Nichols et al. 1999; Zornik et al. 1999). Injections in third instars and early pupa (P1 stage, transition between larva and pupa) of serotonin (5-HT), dopamine (DA), acetylcholine (Ach), octopamine (OA), and norepinephrine (NE) increase the HR (Johnson et al. 1997). Effects of *Drosophila* peptides on HR have also been examined (Johnson et al. 2000; Nichols et al. 1999; Zornik et al. 1999).

In pupa, injection of 5-HT (1 μM/l) caused the HR to increase by 46% from the base line (Johnson et al. 1997). A similar study by Zornik et al. (1999) also showed, in the wandering third instar larva, that 5-HT increases HR by 111% with a concentration of 10^{-5} M (10 μM/l) injected into the animal. In the Zornik et al. (1999) study, it was determined that the adult heart is more responsive to 5-HT than the wandering third instar larvae or the pupal heart.

Injection through the larval body wall or into the pupal case of biogenic amines can cause the activation or release of many other compounds, related to stress or injury, into the hemolymph from endogenous sources that cannot be controlled during the experiment. Thus, we opened the larva in a bath of physiological saline that washes away the hemolymph to test the direct effects of compounds on the heart within a defined saline. Since we are not aware of any attempt to clearly determine if the larval heart is or is not innervated, we assayed the effects of compounds with and without the CNS intact. Also, since exogenous application of a neuromodulator could cause the local release of neuroendocrine compounds, we wanted to eliminate the possibility. In addition, we have shown earlier that OA, DA, and 5-HT have direct actions on the larval CNS neural activity (Dasari and Cooper 2004). It is also known that in adult flies, the rate of stress with age is correlated with heart failure. In addition, HR decreases with age (Wessells et al. 2004). So, to avoid the stress induced variables on HR that could occur with injections, we examined if feeding larvae 5-HT would have an effect on HR in intact preparations.

Many drugs of abuse and mind-altering therapeutic medicines have an impact on the serotonin–ergic system, with some altering the presynaptic reuptake mechanisms or acting directly as an agonist/antagonist against the 5-HT receptors. One compound, MDMA (3,4-methylenedioxymethamphetamine, ecstasy), and its direct action on 5-HT receptors is particularly interesting. Invertebrates have proven to be useful on this front in studying drugs of abuse since they possess physiological systems with few compounding variables and relatively fast development times (Hirsh 2001; Rothenfluh and Heberlein 2002; Sparks et al. 2004; Wolf and Heberlein 2003). MDMA is a ring-substituted amphetamine and is a widely abused drug among young people worldwide. MDMA is a potent releaser and also reuptake inhibitor of 5-HT, DA and NE (Green et al. 2003). In humans the acute adverse effects that are seen after MDMA ingestion are the following: elevated blood pressure, increased heart rate, nausea, chills, sweating tremor, bruxism probably through actions on the autonomic nervous system (de la Torre and Farre 2004; Green et al. 2003; McCann et al. 1996; Peroutka et al. 1988). Previously it was demonstrated that MDMA has direct action on neuronal 5-HT receptors in an arthropod; thus, parallel to 5-HT's effect, we also examined the action of MDMA on the *Drosophila* heart with an intact and ablated CNS.

The purpose of this study is to observe the acute effects of 5-HT and MDMA on heart rate in intact and semi-intact third instar larval *Drosophila* preparations. In addition, we wanted to know if an intact CNS altered the responsiveness of the heart to these compounds possibly through direct innervation of the heart or aorta.

Preliminary results of this study were presented in an abstract form (Dasari et al. 2004).

Methods

Stock and staging of larvae

The common 'wild-type' laboratory strain of *D. melanogaster*, Canton S, was used in these studies. The methods used to stage fly larvae have been described previously (Campos-Ortega and Hartenstein 1985; Li and Cooper 2001). All animals were maintained in vials partially filled with a cornmeal–agar–dextrose–yeast medium. Larvae of the early third instar phase were used in these experiments. The general dissection technique and HL3 saline content has been previously reported (Cooper and Neckameyer 1999; Stewart et al. 1994). The HL3 saline was derived from direct measures with ion sensitive electrodes of larval hemolymph and maintains normal function of larval neuromuscular junctions and the CNS (Ball et al. 2003; Dasari and Cooper 2004). In brief, the HL3 saline was prepared in the lab from component reagents (Sigma) and contained the following: 1.0 mM $CaCl_2{\cdot}2H_2O$, 20 mM $MgCl_2$, 70 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM BES (*N*,*N*-bis [2-Hydoxy-ethyl]-2-aminoethane-sulfonic acid). The HL3 was freshly controlled for pH and temperature prior to experimentation, as the pH will drift during storage.

Heart rate measures

With a microscopic method, the movements of the trachea or heart were used for direct counts of HR. A microscope (adjustable zoom 0.67–4.5; World Precision Instrument, FL, USA) fitted with a 10× eye objective was used for visual observations. With visual inspection,

one can readily observe the heart beating or the trachea movements as a consequence of the heart pulling on the ligament attachments (Fig. 1a). The movements of the trachea are commonly used to monitor *Drosophila* larval heart rate because of the clear contrast of the structures (Dasari and Cooper 2005; Johnson et al. 1997; Miller 1985; White et al. 1992).

Semi-intact preparation

The HR was also used to examine the direct effects of MDMA and 5-HT, at different concentrations, in exposed hearts of larvae with an intact and ablated CNS. These semi-intact animals were of early third instar that had been dissected ventrally and pinned on four corners (Fig. 1b). Guts and all the visceral organs (including the brain for deinnervation) were removed in such a way that the heart is intact and still attached to the rostral and caudal ends of the larvae. This dissection technique has been used to directly assess pharmacological agents on the heart of *Drosophila* larvae (Gu and Singh 1995). The dissection time was 3–6 min. The preparation was allowed to relax while bathed in HL3 saline for 3–5 min after dissection. The heartbeats were counted for a minute at every 5 min for 20 min, initially in saline and then with 5-HT containing saline. Time '0' is taken as the first minute of counting in saline. 5-HT is added to the preparation at the 7th minute and a fresh dose is added again at the 12th minute. The average heartbeat at the 1st and 5th minute for saline and at the 15th and 20th minute for 5-HT or MDMA was used for analysis.

The HL3 saline was carefully controlled to be at pH 7.2 since HR slows down at a high pH and speeds up at a low pH (Badre et al. 2005). Gu and Singh (1995) used pH 7.0 for the pharmacological analysis of the heart and also showed maintained viability. During these trials the saline remained aerated by agitation of solution through repetitively injecting saline, with a 21-gauge needle, into a beaker.

To quantify HR, either direct observations were used or the images were recorded onto VHS tapes and analyzed by a photodiode. In the cases in which the photodiode was used, the detector (model 276–142, Radio Shack, USA) was placed at the back of a black plastic 35 mm film canister and the open end was held over the region on the monitor screen in which the heart and the caudal end of the larvae was magnified. The output of the photodiode was amplified by use of an impedance amplifier. The impedance detectors (UFI, model 2991) allowed HR to be monitored as a measure of dynamic change in the light path across the photodiode during each heart contraction. These signals were recorded online to a PowerMac 9500 via a MacLab/4s interface (AD Instruments, Australia). Events were measured and calibrated with the MacLab Chart software version 3.5.6 (AD Instruments) with an acquisition rate set at 4 kHz. The HR was determined by direct measures with a window discriminator, which measured a running average of instantaneous events. The values were then

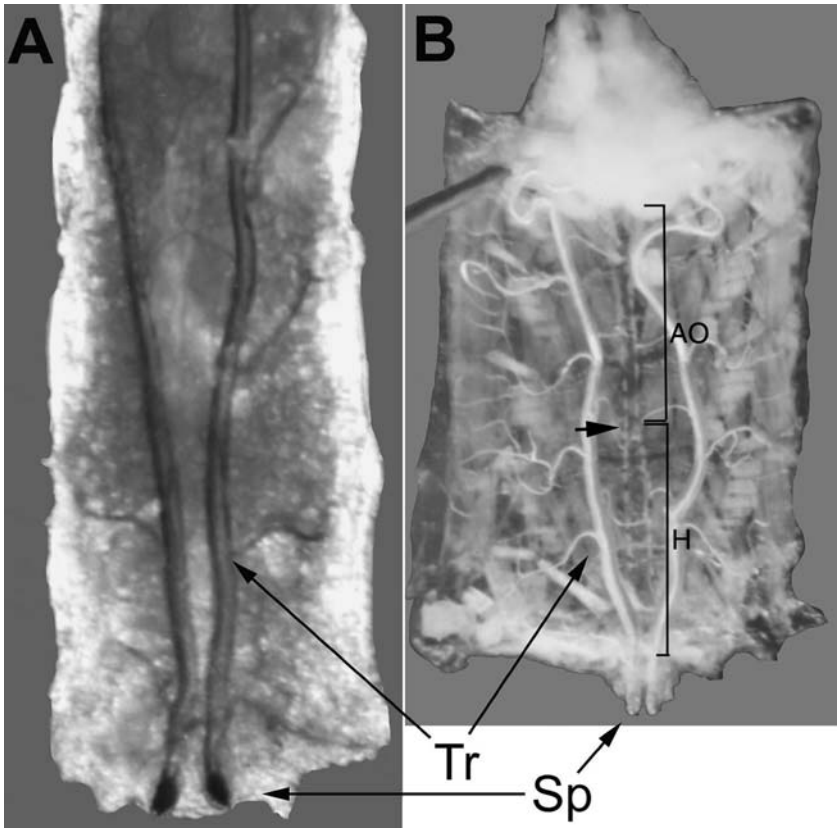

Fig. 1 **a** Dorsal view of an intact third instar larva. The movement of trachea due to pulling of the attachments from heart is used to observe the heart rate. **b** Ventral dissection of a third instar to view the heart directly. Pinning of the animal on its back after dissection is used to directly apply the compounds on the heart with or without the CNS intact. *Small arrow* indicates where the heart and aorta are separated for transected dorsal vessel studies (see below). *Tr* Trachea, *Sp* spiracles, *H* heart, *AO* aorta

converted to beats per minute (BPM). Similar procedures in the use of an impedance amplifier were used as described in earlier studies for obtaining heart rates and ventilatory rates in crayfish (Dasari and Cooper 2005; Li et al. 2000; Listerman et al. 2000; Schapker et al. 2002).

Aorta and heart separation

Wandering third instars are opened as described above. HR was measured for 2 min and then the heart and aorta were separated by severing them at the junction with a fine pair of scissors. HR was measured for another 2 min before applying saline containing 5-HT.

Intact preparation

Early third instar larvae were glued ventrally on a glass slip using super glue in such a way that mouth hooks are free to move. Care was taken not to glue the spiracles so that only the ventral aspect within the mid-length was adhered to the glass. Yeast mixed in either 5-HT or water was placed over the head of the animal. The yeast stimulated the animal to eat, thus consuming the yeast and the compounds. The larvae were glued down and HR measured for 2 min with water and yeast followed by another 15 min while feeding on 5-HT containing solution. Heart beats were counted for 30 s intervals starting initially at the time periods between 1–2 and 6–8 min.

Statistics

All the groups were first tested with one-way ANOVA and followed with Bonferroni's post hoc analysis. Some of the groups were also analyzed using Student's *t* test.

Results

Semi-intact preparation

The direct exposure of the heart tube allows individual compounds to be directly assayed on the heart without compounding variables introduced from endogenous hormones and substance contained in the hemolymph. After pinning the dissected larvae open, the saline bathing solution was changed to one containing 5-HT or MDMA. Six different concentrations of 5-HT and three different concentrations of MDMA were examined. The 1 nM 5-HT exposure resulted in a significant decrease in HR (Fig. 2a, ANOVA $P < 0.05$, $n = 10$) as compared to the saline sham exposures. Saline exchange by itself results in a slight increase in HR in some preparations but it does not have a significant effect in all animals. However, we felt that experimentally it was important to provide sham controls, as this is a valid issue when examining the effects of 5-HT and MDMA in isolated heart segments (see below). The increased concentration of 5-HT at 10 nM produced a substantially increased HR (Fig. 2a, ANOVA $P < 0.05$, $n = 10$). However, at 100 nM, the increase in HR from sham control reached the maximum effect (ANOVA $P < 0.05$, $n = 10$) when compared to 1, 10, and 100 μM. There was no further significant increase at these higher concentrations compared to 100 nM but all were significantly greater in increased HR from the saline control (ANOVA $P < 0.05$, $n = 10$). Thus, the 5-HT receptors were likely saturated at 100 nM with only modest increases in the mean HR for the higher concentrations. It should be noted that each preparation was treated individually and not exposed to a series of experiments. At concentrations of 10 μM 5-HT and above, the heart is in tetany and cannot relax well between beats. This is likely the prime reason for the saturating effect in further increases of HR with exposure to higher concentrations.

Exposure to MDMA at various concentrations did not have a significant effect on the HR (Fig. 2a, b). The animals were followed for at least 20 min without any notable consequences related to HR.

Since there is no known innervation from the brain to the dorsal vessel, we examined larva with the CNS left intact (Fig. 2a, b) and in ones where it was carefully removed (Fig. 2c, d). To test whether a potentially deinnervated heart showed any differences in HR compared to larvae with an intact CNS, the dissected larvae with and without the CNS were exposed to 5-HT and the change in HR noted. There was significant decrease in HR to 5-HT at 1 nM (Student's *t* test $P < 0.05$, $n = 10$) but no significant decrease with 5-HT at 10 nM and MDMA at 1 μM ($n = 10$) as compared to saline exposure. As with the intact CNS preparations 100 nM 5-HT showed a significant increase in HR (ANOVA $P < 0.05$, $n = 10$, Fig. 2c). Comparing the differences between intact and ablated CNS, the effects of MDMA caused a decreased HR at 1 μM in larvae without a CNS (Student's *t* test $P < 0.05$, $n = 10$). In addition, a reversal in the effects of 10 nM 5-HT in ablated CNS preparations was observed. Without the CNS, the 10 nM 5-HT had a significant effect in reducing the HR (Student's *t* test $P < 0.001$, $n = 10$; compare Fig. 2a, c for 10 nM). The mean, median, 95% confidence levels and the range of the distributions in the data sets are shown in the whisker box plots for the intact CNS (Fig. 2b) and for the preparations with an ablated CNS (Fig. 2d).

Heart and aorta separated

During the initial experiments, when exposing the heart through a ventral dissection, it became apparent that the dorsal vessel had a different rhythm along its length. These regional differences in pulse rates were not observed in the intact preparations. In addition, exposure of the entire dorsal vessel in the ventral dissected preparations to 5-HT suggested regional differences in the

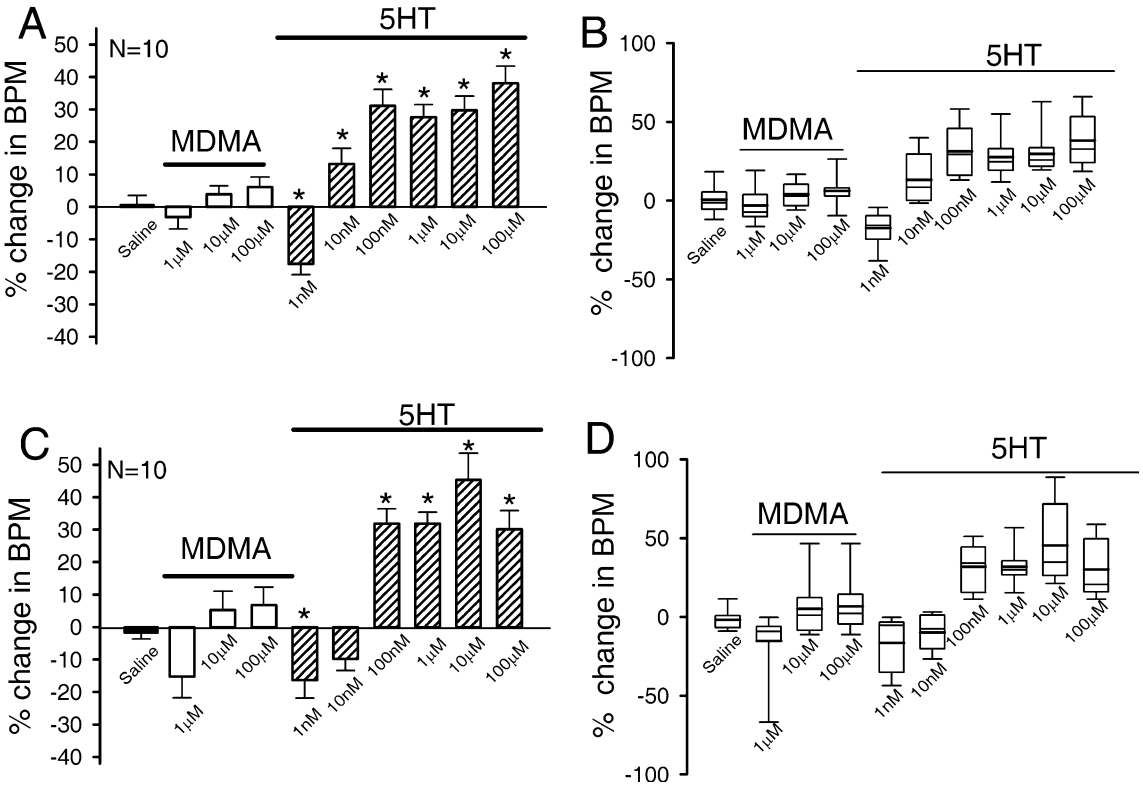

Fig. 2 Heart rate of third instar semi-intact preparations: **a** percent change in heart rate from saline per minute in innervated preparations (brains attached). **b** 95% confidence intervals for the same data set as in (**a**). **c** Percent change in the heart rate from saline in deinnervated preparations (CNS ablated). **d** 95% confidence intervals for the same data set as in (**c**). *BPM* beats per minute. A *star* denotes ANOVA, $P < 0.0001$, followed by Bonferroni test, $P < 0.05$

actions of 5-HT. To address this issue, the heart and aorta were transected at their junction. The rate of beating of aorta and heart was observed in saline and during exposure to 5-HT (100 nM). We chose 100 nM concentration as this level produced a significant increase in HR from saline without inducing a tetanic state of the heart in the whole dorsal vessel. MDMA was not used in these preparations, as it did not show any effect on direct exposure to the heart.

The number of beats within the aorta and heart were measured prior to transection and then again after a saline wash or a saline wash containing 5-HT. Comparisons were made by determining a percentage difference for the separated aorta and heart to the intact state of the aorta and heart. The effect of direct exposure of 5-HT (100 nM) produced a substantial increase in HR (Fig. 3a, ANOVA $P < 0.05$, $n = 10$) in the heart when the dorsal vessel is left intact. However, after the transection the preparation became very sensitive to changes in the bathing media. When sham controls for mechanical disturbances on the heart rate were compared to saline changes containing 5-HT, there is no significant effect of 5-HT. Saline exchange alone increased the rate of the aorta significantly (Fig. 3b, ANOVA $P < 0.05$, $n = 14$). A small effect was also produced on the true heart with saline exchange (Fig. 3b, ANOVA $P < 0.05$, $n = 14$). The exposure to 5-HT also had a substantial effect on the rate of both the heart and aorta but not significantly over the sham control effects ($n = 7$). Therefore, we concluded that 5-HT did not show any differential effect on the rate of the two segments when the dorsal vessel segments were isolated from each other.

Intact preparation

In examining the possibility of 5-HT gaining access to the hemolymph and altering HR, we fed early third instars 5-HT (1 mM) mixed with yeast. Control experiments of water and yeast were also conducted for the same period of time. The controls maintained a steady HR for 8–10 min, however, the larvae eating a 5-HT tainted diet after 6–8 min dropped in HR for some larvae but not for others. The change was not significant (Student's *t* test) for 5-HT fed larvae compared to controls. The same larvae feeding on 5-HT were followed further for 12–15 min, and in some preparations HR was not noticeable even though the larvae were alive as indicated by mouth hook movements. The cardiac arrest was not due to tetany of the heart. In other larvae, no change in HR occurred before or after 15 min of eating the 5-HT containing food. Thus, there was a large variation observed in the 5-HT fed larvae as compared to controls.

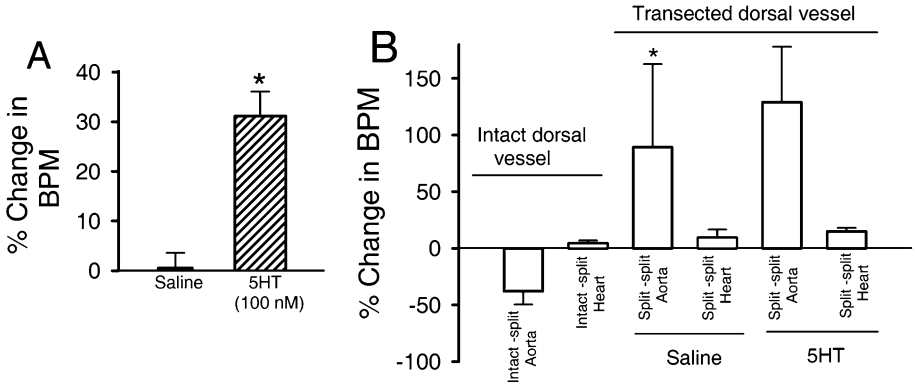

Fig. 3 The effects of 5-HT on intact and on the isolated heart and aorta segments. The number of beats were measured before and after severing the dorsal vessel at the aorta and heart junction. **a** Percent change in the heart rate from saline to exposure to exogenous 5-HT (100 nM) in an intact dorsal vessel. **b** The beat rate was measured in aorta and heart separately before and after dividing the vessel. A percent change is calculated for intact to split aorta and heart in saline ($n = 14$). The percent change in HR before and after saline wash in the split aorta and heart preparations revealed a significant increase in rate ($n = 14$; ANOVA, $P < 0.0002$, Bonferroni test, $P < 0.05$). Exposure to 5-HT (100 nM) produced a larger mean effect but there was also considerable variation among the preparations; thus no effect to 5-HT exposure was observed as compared to the sham saline effects ($n = 7$)

Discussion

In this study, we showed that the larval *Drosophila* heart can be examined in a semi-intact state in which the hemolymph can be exchanged with saline in order to reduce compounding variables of endogenous neuromodulators or induce release due to systemic injections of pharmacologic compounds. The minimal HL3 saline maintains the HR; thus pharmacological assays can be run with an intact or ablated CNS for high output screening as to direct actions on cardiac function. The larval heart in a semi-intact state (heart and aorta connected) decreased its rate at 1 nM 5-HT but increased its rate with exposure higher than 10 nM with a plateau in the enhancement at 100 nM. Ablating the CNS resulted in a decline in HR for 5-HT exposure at 10 nM; however, it increased at higher concentrations. Separating the dorsal heart tube into the true heart and aorta while exposing the segments to 5-HT or saline both resulted in an increase in HR with no discernable effects to 5-HT alone over the sham effects of changing the saline bath. It is of interest to note in future pharmacological investigations that the heart is very sensitive to exchange of the bathing media. This was also noted in earlier studies in examining the effects of pH on HR (Badre et al. 2005). Lastly, 5-HT introduced in the diet at a high concentration of 1 mM did not alter HR significantly but did result in an increased variability in HR, thus suggesting that feeding larvae this particular biogenic amine to address effects on HR might not be feasible.

Direct exposure of the heart tube to 5-HT resulted in biphasic changes in the HR with a low concentration decreasing the HR and a high concentration increasing it. This beacons the possibility of different 5-HT receptor subtypes with varying degrees of binding affinity and/or activated second messenger cascades on the heart tissue (Johnson et al. 2002). It is known that the *Drosophila* genome contains four different 5-HT receptor-coding sequences (Colas et al. 1995; Monastirioti 1999; Saudou et al. 1992; Witz et al. 1990). However, posttranslational modifications or regulated RNA editing of the 5-HT receptors in *Drosophila* might occur as known in vertebrates for some 5-HT receptors subtypes (Paupard et al. 2000; Slominski et al. 2003; Visiers et al. 2001). Differences in RNA editing is also a possibility over the development of the larvae as shown for different types of receptors in mammals (Lee et al. 2001); thus one should consider pharmacological and molecular analysis at various life stages for a complete assessment in subtypes of 5-HT receptors. In this study, we only examined early third instars, which predominantly express the 5-HT_{2Dro} receptor subtype (Colas et al. 1995), however, previous studies did not specifically address expression in heart tissue developmentally. A complete pharmacological profile of agonists and antagonists to 5-HT receptors would be advantageous in helping to address the receptor subtypes in cardiac modulation within *Drosophila*. Although one should also be careful in assuming the vertebrate, pharmacological profiles correctly characterize the invertebrate systems (Sparks et al. 2003). Such differences might explain why the heart is not sensitive to MDMA but the larval CNS is very sensitive to exogenous application (Dasari et al. 2004).

A number of studies have addressed the effects of biogenic amines and peptides on cardiac function in larval and pupal stages (Johnson et al. 1997; Zornik et al. 1999). The pupal stage offers the advantage in that it does not require to be restrained. Even the larval heart can be viewed in freely wandering larvae but it is considerably harder to maintain a good assessment of heart function continuously (Dasari and Cooper 2005). Thus, gluing or taping larvae have been preferred methods to restrain the animal for viewing of the tracheal movements or the heart directly. The introduction

of pharmacological agents has also been preferred by injection through the cuticle into the hemolymph. Although such methods have certain advantages, one is not confident on the effects of the stress in relation to the animal dumping various endogenous biogenic amines and peptides into the hemolymph. One also needs to consider that heat stress is known to alter biogenic amine levels in *Drosophila* (Hirashima et al. 2000) In addition, circadian patterns of 5-HT are known to occur and could alter the responsiveness to exogenous applications (Fowler et al. 1972). This was one reason that we chose to dissect the larvae and expose the heart directly to 5-HT in a defined physiological saline at a given temperature and period of day (12:00–18:00). This semi-intact preparation also offers the advantage of introducing various compounds over time and wash out previously introduced substances (Gu and Singh 1995). An additional advantage of the dissected preparation that has not previously been addressed is the fact that the CNS can be left intact or removed. By leaving it intact and using a small volume of bathing media, one can assess if compounds from the CNS can be induced for release for examining its effect on cardiac performance. In addition, if any neuronal connection does exist on the larval heart it can be removed or left intact for assaying various exogenous agents. Earlier studies in which TTX was bathed on the exposed heart did not produce any significant alteration in HR (Gu and Singh 1995) and thus it was assumed that the larval hearts are myogenic but this does not prove that the hearts are not modulated by neuronal innervation as for mammals. Since we did note that differences do occur in sensitivity to 5-HT in preparations with an ablated CNS, this suggests that there may indeed be a neural input that is modulated by 5-HT possibly at presynaptic nerve terminals on the heart as for skeletal muscles in crustaceans (Southard et al. 2000). The activity of the CNS circuitry in larvae is known to be influenced substantially by 5-HT as well as by octopamine and dopamine (Dasari and Cooper 2004). This issue of innervation of the heart tube remains to be addressed anatomically in larval *Drosophila*.

Earlier reports have shown that injection of 5-HT into third instars and P1 pupal stage of *D. melanogaster* Oregon R and Canton S strains, respectively, causes an increase in HR (Table 1). In this study, we have shown that direct exposure of heart to 5-HT in third instars of Canton S strain also causes it to increase at high concentrations but at low concentrations of 1 nM it decreases. In the previous concentration ranges examined, 5-HT produced a larger increase in HR than what we report for semi-intact preparations.

The synergistic action of endogenous compounds on the injected 5-HT is one possibility for enhancing the effects of 5-HT. As shown in crustaceans and leech, 5-HT has different effects in the presence of octopamine as compared to 5-HT exposure alone (Djokaj et al. 2001; Mesce 2002). Given that there are many active substances contained in the larval hemolymph, the biogenic/peptide cocktails remains an interesting avenue for further research in isolated preparations. On the other hand, the dissection of the larvae and exposure to HL3 physiological saline might not provide the correct ionic environment for cardiac function. The HL3 saline was originally developed and assayed for skeletal neuromuscular transmission, however the ions, were determined from larval hemolymph samples (Stewart et al. 1994).

The ability to feed larval and adult *Drosophila* compounds to alter endogenous neuromodulators has been used with success (Neckameyer et al. 2001) but this approach is not likely to be feasible for readily degraded compounds by an acidic environment or that are photo liable such as 5-HT (Lisi et al. 2003; Strawn et al. 2001). Saliva of adult *Drosophila* contains enzymes known to digest chitin as well as amylose and cellulose (Gregg et al. 1990), thus possibly larvae also contain such abilities. Hence, we are not convinced that feeding the larvae food tainted with 5-HT (1 mM) resulted in any significant change in hemolymph levels since injection into larvae or direct application on to the heart at lower concentrations resulted in a change of HR.

Much to our dismay, the transected heart tube into the true heart and aorta became very sensitive to any alteration in changing of the bathing media that made it difficult to assay differential effects of the aorta and the true heart to exposure of 5-HT. It is established that the heart tube contains pacemaker activity but the extent and control of a major pacemaker in the heart and aorta segments has not been addressed in relation to biogenic modulation. Transecting the heart tube into the true heart and the aorta resulted in the two segments beating at their own intrinsic rates. The aorta significantly slowed down and the true heart increased in rate. Thus, uncoupling the caudal-anterior communication within the heart tube had some effect on possibly unmasking some feedback or inhibition of rate in the intact true heart where the lack of the master pattern activity of the heart on the aorta resulted in a slower aorta pacemaker

Table 1 Effects of 5-HT on Heart Rate (HR) as percent difference in HR from base line as reported in various studies for comparison

	1 mM	100 μM	10 μM	1 μM	0.1 μM	0.001 μM	Reference
Injection third instar	↑120 ± 6	↑123 ± 5	↑111 ± 4	NS	–	–	Zornik et al. (1999)
Injection P1 stage	↑75	↑55	↑28	↑46	↑15	–	Johnson et al. (1997)
Direct application third instar	–	↑38 ± 5	↑29 ± 4	↑27 ± 3	↑31 ± 5	↓17 ± 3	This study

The effect of 5-HT on HR. The percent difference in heart rate from the base line after injection of 5-HT as reported in earlier work is shown. Zornik et al. (1999) injected different concentrations of 5-HT in third instar larva. Johnson et al. (1997) injected 5-HT into P1 stage, the transition stage between third instar and pupa

activity, which is analogous to mammalian hearts among the pacemaker regions. Possibly after the heart tube is separated there is a reduction in filling the aorta or less peripheral resistance for the heart between pulses which could then effect myogenic stretch related activity. It is also conceivable that the micro-environments in the cut vesicles related to perfusion of the saline to luminal surface of the vessel is altered which might explain the heightened sensitivity to saline exchange and exposures to 5-HT.

Developing a means to introduce agents without causing mechanical disturbance of the bathing media will help in examining localized effects of pharmacological agents on the true heart and the aorta separately. We are currently examining explant cell culture of *Drosophila* heart tissue to determine its feasibility. Electrophysiological recordings would give an insight into the ionic properties of the pacemakers and the mechanistic effects of the various known modulators on the *Drosophila* heart.

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