5-HT stimulation of heart rate in Drosophila does not act through cAMP as revealed by pharmacogenetics

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Majeed ZR, Nichols CD, Cooper RL. 5-HT stimulation of heart rate in Drosophila does not act through cAMP as revealed by pharmacogenetics. J Appl Physiol 115: 1656–1665, 2013. First published October 3, 2013; doi:10.1152/japplphysiol.00849.2013.—The fruit fly, Drosophila melanogaster, is a good experimental organism to study the underlying mechanism of heart rate (HR) regulation. It is already known that many neuromodulators (serotonin, dopamine, octopamine, acetylcholine) change the HR in Drosophila melanogaster larvae. In this study, we investigated the role of cAMP-PKA signaling pathway in HR regulation and 5-HT positive chronotropic action. In order to obtain insight into the 5-HT mechanism of action in larvae cardiomyocytes, genetic and pharmacological approaches were used. We used transgenic flies that expressed the hM4Di receptor [designer receptors exclusively activated by designer drugs (DREADDs)] as one tool. Our previous results showed that activation of hM4Di receptors (modified muscarinic acetylcholine receptors) decreases or arrests the heart from beating. In this study, it was hypothesized that the positive chronotropic effect of serotonin [5-hydroxytryptamine (5-HT)] are mediated by serotonin receptors coupled to the adenylyl cyclase pathway and downstream cAMP and PKA activity. Activation of hM4Di by clozapine-N-oxide (CNO) was predicted to block the effects of serotonin by inhibiting adenylyl cyclase activity through Goi pathway activation. Interestingly, we found here that manipulation of adenylyl cyclase activity and cAMP levels had no significant effect on HR. The ability of hM4Di receptor activation to slow or stop the heart is therefore likely mediated by activation of GIRK channels to produce hyperpolarization of cardiomyocytes, and not through inhibition of adenylyl cyclase.

5-HT; M4D receptor; CNO; adenylyl cyclase; PKA

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neered receptor has high affinity for a chemical that is consid-
ered physiologically inert, clozapine-N-oxide (CNO) that has 
full agonist efficacy at DREADD receptors (1, 4, 29). With this 
approach one can rule out off-target effects of the natural 
ligand to specifically and remotely control effector pathway 
activity in defined target tissues that the DREADD receptor is 
expressed in.

In the pupal stage of *Drosophila* forskolin does not produce 
a change in the HR (21), indicating that cAMP levels are not 
important in the pupa; however, in the pupal stage significant 
alterations in endocrine function accompanied by significant 
morphological changes are occurring, and mechanisms of car-
diac function may be different than in the larva. We previously 
demonstrated that 5-HT increases HR in larvae; therefore, we 
examine here the potential mechanisms of action of 5-HT in 
the larval stage heart. Our working model was that 5-HT 
activates GoS, which activates adenylyl cyclase that in turn 
increases the level of cAMP. Increased cAMP leads to an 
increase in active PKA signaling and positive chronotropic 
modulation of the heart. In this scenario, blockade of adenylyl 
cyclase activity with pharmacological methods, or activation of 
Gsi through activation of hM4Di receptors expressed in the 
heart, would be predicted to decrease cAMP levels and PKA 
activation and block the positive chronotropic effects of 5-HT 
on the heart. Our data, however, indicate that cAMP levels do 
not contribute to either modulation of HR or the positive 
chronotropic effects of 5-HT in the *Drosophila* heart. It has 
been reported that forskolin does not noticeably change the HR 
in P1 pupal stage of *Drosophila* (21). This confirms that 
*Drosophila* HR in larvae also might not be markedly changed 
by activation of AC-PKA pathway; although, AC-PKA path-
way might increase the contractility of heart.

**MATERIALS AND METHODS**

*Transgenic fly strains.* In this study, two fly strains were used, 
UAS-hM4Di and 24B-GFP (a strain heterozygous for the 24B-GAL4 
driver element that expresses in all larval muscle, and is also homozy-
gous for the UAS-GFP element; from Dr. Charles Nichols, Louisiana 
State University Health Sciences Center). The UAS-hM4Di strain was 
crossed with 24B-GFP strain in order to express hM4Di (Gsi) 
coupled receptor in muscle fibers, including cardiomyocytes. In the F1 
generation, half the flies contained the 24B-GAL4 expression ele-
ment, detected by GFP expression, and the other half did not. The 
GFP-positive, hM4Di-expressing larvae were used as the experimen-
tal animals, and the non-GFP-positive, non-hM4Di-expressing (but 
UAS-hM4Di containing) ‘littermates’ were used as controls (back-
ground). The flies were reared at room temperature (22°C) in 
vials containing cornmeal-agar-dextrose-yeast medium.

*Heart rate measurement.* Third instar larvae were dissected in the 
ventral side up position to expose the dorsal vessel (heart) in HL3 
(saline solution) (NaCl 70 mM, KCl 5 mM, MgCl 2.6H2O 20 mM, 
NaHCO3 10 mM, Trehalose 5 mM, sucrose 115 mM, BES 5 mM, and 
CaCl2.2H2O 1 mM with the pH adjusted to 7.1). Because heart 
performance is very sensitive to pH change, the pH was tightly 
regulated and adjusted as needed. Recently, the effects of various 
buffers have been probed to optimize the saline for monitoring heart 
rate (14). Drugs were applied at various concentrations as indicated in 
the Results. The preparation was left for 1 min in saline after 
dissection, and then heart beats were counted for the following 
minute. Exceptions are indicated on graphs. The difference in the HR 
before and after application of drugs was used to measure the effects 
of the various compounds.

**Chemicals.** All the chemicals, serotonin hydrochloride [5-hydroxy-
tryptamine (5-HT)], forskolin, SQ 22,536, N6,2’-O-Dibutyryl adeny-
osine 3’,5’ cyclic monophosphate sodium salt (dbcAMP), 2’,5’-dide-
oxoxyadenosine were purchased from Sigma-Aldrich, St. Louis, MO. 
Clozapine-N-oxide was kindly synthesized by Dr. David Nichols 
(Purdue University). 5-HT stock solution (1 mM) was prepared with 
HL3 saline and immediately before use, a 1 μM serotonin solution 
was made from the stock solution. Forskolin was dissolved in 1% 
dimethyl sulfoxide (DMSO) to obtain a 1 mM stock solution. The 
final concentration of DMSO was less than 0.1% (roughly about 
0.03%) in 30 μM forskolin and 0.5% in 100 μM forskolin. 300 μM 
and 1 mM of dbcAMP were also prepared with HL3 saline. 200 μM 
of SQ 22,536 was made with saline from a 1 mM stock solution. A 
1 mM dideoxyadenosine stock solution was made in DMSO, then 50 
μM (0.04% final DMSO concentration) and 500 μM (0.2% final 
DMSO concentration) with dilutions in saline.

*Statistical analysis.* All data are expressed as mean ± SEM. The 
paired t-test (before and after) was used to compare the difference of 
HR change after exchanging solution with saline containing chemi-
cals. T-test was used to compare between background (UAS-hM4Di) 
and hM4Di expressing (UAS-hM4DiX24B-Gal4) larval HRs (Sigma-
Plot version 11.0). P ≤ 0.05 is considered as statistically significant. 
The number of asterisks are considered as P ≤ 0.05 (*), P ≤ 0.02 
(**), and P ≤ 0.001 (**).
markedly increases the HR, even in the presence of CNO (Fig. 3, A and C). These results confirm the positive chronotropic effects of 5-HT and that CNO does not block the plasma membrane receptor binding site for 5-HT. Only when hM4Di DREADD receptor expression was induced did CNO have an effect on the positive chronotropic effect of 5-HT (Fig. 3, B and C). We observed that CNO by itself slightly decreased HR in background larvae (Fig. 3, A and C). This may be due to leaky expression of the UAS-GAL4 element present in the background genotype rather than a direct effect of CNO on the heart. When the CNO + 5-HT containing saline was exchanged with saline (4→5), the HR significantly increased in both background and hM4Di-expressed larvae. This might be due to the 5-HT residual effect on the HR. The increase in HR on this final wash was 2-fold greater in the hM4Di expressing larvae, suggesting a rebound effect occurring from the removal of the repressing CNO mediated hM4Di signaling.

After 5-HT is applied to increase HR, the addition of CNO reverses the positive chronotropic effects of 5-HT. 5-HT was applied prior to exposure of CNO in order to see if CNO can also reverse the effects of 5-HT in addition to block, as demonstrated above. 5-HT markedly increases the HR (Fig. 4). The addition of CNO reversed the effects of 5-HT (Fig. 4). When CNO + 5-HT saline was exchanged with saline alone (4→5), the HR increased again, as observed in the experiments shown in Fig. 3, indicating a possible rebound effect from removal of the repressing hM4Di signaling.

CNO blocks the effects of forskolin. In order to know more about the effect of hM4Di expression on cardiac muscle fiber second messengers, forskolin, an AC activator was examined. In the presence of CNO forskolin increased the HR in background larvae (Fig. 5A). The presence of CNO blocked the positive chronotropic effect of forskolin in hM4Di-expressing larvae. Furthermore, not only was the HR completely arrested by CNO application, but forskolin was unable to overcome this blockade (Fig. 5, B and C).

Forskolin (30 μM) alone was observed to increase the HR (Fig. 6). The subsequent addition of CNO had no inhibiting effect in the background larva but dramatically halted the HR in the hM4Di-expressing larvae (Fig. 6). Since forskolin was not able to overcome the effects of hM4Di activation this may indicate that cAMP levels alone are not the primary mediator of HR. The effect of forskolin did not show a significant difference between two genotypes (Fig. 6 C).

A higher concentration of forskolin (100 μM) was used to determine whether or not the 30 μM concentration was maximal or near maximal and thus producing a saturating effect. We also incubated with the higher concentration of forskolin for 10 min to observe if there was a time-dependent effect present on heart rate for forskolin exposure compared with an acute exposure. We observed that forskolin at 100 μM increased the HR at the beginning; however, it was not greater.

Fig. 1. A: The effect of changing saline with saline on larval heart rate in UAS-M4D X 24B-GFP flies, n = 9. B: Difference in heart rate before and after changing solution. C and D: The effect of changing saline containing DMSO with saline containing DMSO on larval heart rate in UAS-M4D X 24B-GFP flies; n = 10. P ≤ 0.05 (*), P ≤ 0.02 (**).

Fig. 2. Concentration response curve for clozapine-N-oxide (CNO) at hM4Di receptors on the larval heart rate. The absolute change in rate [beats per minute (BPM)] is shown P ≤ 0.001 (***).
than that of 30 μM, and no further increase the HR occurred during the 10 min incubation time (Fig. 7, A and B), indicating that our original 30 μM concentration was likely saturating for forskolin.

To further probe the role of cAMP, we used the AC inhibitor SQ 22,536 (SQ). Application of SQ had no effect in the first minute (Fig. 8). Because no inhibitory effect of SQ on the HR was observed within the first few minutes, we prolonged the interval to 9 min. At the ninth minute of incubation the HR decreased significantly ($P < 0.02$), but it did not stop (Fig. 8). This decrement in HR might be due to the lengthy incubation period, rather than the drug. However, it was shown if the semi-intact heart preparation (Canton-S flies) that were incubated inside the saline (pH 7.00) for 10 min the HR do not show a marked change (data not shown). Taken together, these results show that SQ has a slightly inhibitory effect on the HR. When the SQ solution was exchanged with SQ + 5-HT solution, HR significantly increased (Fig. 8). These results indicate that SQ cannot override the positive chronotropic effect of 5-HT. Either the SQ is not effective in blocking Drosophila AC as it is for mammalian AC, or perhaps manipulating cAMP levels alone is not sufficient to mediate an increase in HR, in agreement with our results using forskolin.

We next tried another AC inhibitor, dideoxyadenosine. Application of dideoxyadenosine (50 and 500 μM) did not produce a noticeable change in HR in hM4Di expressing larvae.

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**Fig. 3.** Effect of activation of hM4Di receptors on the action of 5-HT in larval heart. A: Background UAS-M4D, $n = 10$. B: UAS-M4D X 24B-GFP, $n = 10$. C: Comparison between background (dark grey) and M4D expressing group (light grey). The differences between genotypes are represented by NS (not significant) or asterisk(s) above the lines. The differences, which are due to the changing of one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars. $P \leq 0.05$ (*), $P \leq 0.02$ (**), $P \leq 0.001$ (***)

**Fig. 4.** A: Effect of 5-HT application plus hM4Di activation on larval heart rate (UAS-M4D X 24B-GFP); $n = 9$. B: The difference in heart rate before and after change of solution. $P \leq 0.02$ (**), $P \leq 0.001$ (***)

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**Fig. 3.** A: Background UAS-M4D, $n = 10$. B: UAS-M4D X 24B-GFP, $n = 10$. C: Comparison between background (dark grey) and M4D expressing group (light grey). The differences between genotypes are represented by NS (not significant) or asterisk(s) above the lines. The differences, which are due to the changing of one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars. $P \leq 0.05$ (*), $P \leq 0.02$ (**), $P \leq 0.001$ (***)
Fig. 5. Effect of hM4Di activation plus forskolin application on larval heart rate. A: Background strain UAS-M4D and heart rate with solution changing; n = 6. B: Change of heart rate in UAS-M4D X 24B-GFP; n = 5, with the same solutions as in A. C: Comparison between background (dark grey) and M4D expressing group on the effect of CNO and forskolin (light grey). The differences between genotypes are represented by NS (not significant) or asterisk(s) above the lines. The differences, which are due to the changing one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars. P ≤ 0.05 (*), P ≤ 0.02 (**), P ≤ 0.001 (***)

(Fig. 9 and 10). By the second minute HR showed a very slight decrease in hM4Di expressing larvae. However, when dideoxyadenosine (50 or 500 μM) solution was exchanged with dideoxyadenosine + 5-HT, a marked increase in the HR occurred (Fig. 9 and 10).

In a different approach, we attempted to manipulate cAMP levels by using the cAMP analog dbcAMP, which both inhibits phosphodiesterase and mimics the effects of cAMP on downstream effectors like PKA. dbcAMP is resistant to cleaving by phosphodiesterase (19). Application of dbcAMP (300 μM) does not change the HR in background larvae (Fig. 11, A and C). Because no effect was observed with the initial exposure of dbcAMP on the HR, the HR was measured for the following second minute. Again, there was no significant change in HR with a longer exposure to dbcAMP (Fig. 11, A and C). When the dbcAMP solution was exchanged with dbcAMP + CNO solution, HR slightly increased in background larvae (Fig. 11, A and C). Application of dbcAMP slightly decreased HR in

Fig. 6. Effect of forskolin plus hM4Di activation on larval heart rate. A: Background, UAS-M4D; n = 7. B: UAS-M4D X 24B-GFP; n = 5. C: Comparison between background (dark grey) and M4D expressing group (light grey). The differences between genotypes are represented by the NS (not significant) or asterisk(s) above the lines. The differences, which are due to the changing one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars. P ≤ 0.02 (**), P ≤ 0.001 (***)
When the dbcAMP solution was exchanged with the dbcAMP + CNO solution, the HR dramatically decreased in the hM4Di expressing larvae (Fig. 11, B and C). dbcAMP did not have a significant difference between two genotypes. Because there was no significant change after application of 300 μM dbcAMP, a higher concentration of dbcAMP (1 mM) was used. Application of dbcAMP at this higher concentration only slightly decreased the HR in the first and second minute (Fig. 12) to the same degree as with the lower concentration (Fig. 11). However, when the dbcAMP solution was exchanged with dbcAMP + CNO, the HR markedly decreased (Fig. 12). Changing dbcAMP + CNO solution with saline alone showed a marked increase in HR (Fig. 12).

DISCUSSION

Drosophila has been established to be a good model for the study of cardiac physiology in part because of malleable genetics and conserved development with mammalian heart (33). Moreover, fly and mammalian hearts use many of the similar signaling pathways during development (39). In mammals, 5-HT receptor dysfunction or overactivation leads to abnormal cardiac development and physiological disturbance (23, 28). Clearly, 5-HT and proper activation of relevant 5-HT receptors is critical for normal cardiac development and function in mammals (5). 5-HT receptors are all G-protein coupled receptors (GPCRs), with the exception of the 5-HT3 receptor that is a ligand gated ion channel (31). Modified DREADD GPCR receptors have been shown to be excellent tools to further understand the function of specific GPCRs, like serotonin receptors, and their signaling pathways in normal physiological processes in both fly and mammals (4, 29). For example, the hM4D1 DRAEDD receptor we have used here has also been used to understand the role of serotonin in respiratory control (34) and to determine key structural components of the native muscarinic M4 acetylcholine receptor itself (27). These pharmacogenetic approaches have opened up new avenues to further understand the roles of GPCR receptors in physiology and are promising tools to obtain additional insight into underlying molecular mechanisms of human diseases.

Here, we initiated studies to elucidate effector pathways underlying HR regulation in the Drosophila heart and to further explore the positive chronotropic role of serotonin on the Drosophila larva heart. Our original hypothesis was that Drosophila larva HR, like mammalian HR, was governed by adenylyl cyclase activity and its downstream effectors and second messengers. Further, that the positive chronotropic effect of 5-HT was due to positive modulation of adenylyl cyclase activity. To address this hypothesis, we used both pharmacological and pharmacogenetic approaches using a combination of drugs and the modified mammalian G-protein...
coupled receptor (hM4Di, coupled to Goi) to provide an insight into potential signaling pathways underlying HR and the effects of 5-HT in *Drosophila* larval heart. We initially observed that activation of hM4Di can slow or stop the heart. Moreover, that hM4Di activation can override the positive chronotropic effect of 5-HT. These observations were consistent with our hypothesis, with the prediction that hM4Di activation would activate Goi, reduce adenylyl cyclase activity, and slow the heart.

It has been shown, however, that forskolin does not noticeably change the HR in P1 pupal stage of *Drosophila* (21). We extended the investigation in larvae and showed, in general, manipulation of adenylyl cyclase activity and cAMP levels with the dbcAMP, SQ 22,536 and dideoxyadenosine did not have a noticeable impact on the HR. In rodents adenosine analogs can retard the positive chronotropic effect of norepinephrine (35). There was a positive effect of forskolin; however, the effect was only slight. Also, dbcAMP slightly decreased the HR. This may be due to activation of targets by cAMP that could bring about ion channel activation, such as K⁺ channels. This might result in depolarization of the pacemaker cell membrane potential. Further, adding dbcAMP is not the same as stimulating AC as there are various isoforms of AC that might be differentially activated by forskolin whereas addition of dbcAMP maintains a high level of cAMP, bypassing activation of AC.

Our findings indicate that cAMP signaling does not contribute to regulation of HR in *Drosophila* larvae as it does in mammals, and that CNO is not a component of the positive chronotropic effects of 5-HT in the fly heart. Supporting this conclusion is that 5-HT is still able to produce a large increase in HR in the presence of AC inhibitors. As an additional tool to probe mechanisms underlying HR, we employed the designer receptor hM4Di, which is positively coupled to Goi signaling in both mammals and the fly, as well as the G-coupled inwardly rectifying potassium channel (GIRK) to produce silencing. For example, CNO activation of hM4Di in mammalian systems results in the hyperpolarization and electrical silencing of hippocampal neurons in culture (1). We previously observed that CNO significantly slowed or stopped the heart when applied to *Drosophila* hearts expressing hM4Di receptors (4) and observe the same effect here. Significantly, pharmacological manipulation of AC or cAMP levels in the presence of activated hM4Di had no effect on the negative chronotropic effects of activated hM4Di. Forskolin was unable to override the suppressing effects of CNO. Together, the responses likely indicate that the main effects of hM4Di activation on HR are probably mediated through Gβγ activation of GIRK channels with subsequent hyperpolarization and silencing of cardiomyocytes rather than through negative modulation of cAMP levels through Goi signaling.

When we applied 5-HT to the larvae preparations, we observed that 5-HT markedly increased the HR, in agreement

![Fig. 9. A: Effect of dideoxyadenosine and dideoxyadenosine + 5-HT combination on larval heart rate (UAS-M4D X 24B-GFP), n = 8. B: The difference in heart rate before and after change of solution. P ≤ 0.02 (**), P ≤ 0.001 (***)](http://www.jappl.org/)

![Fig. 10. A: Effect of a high concentration of dideoxyadenosine and dideoxyadenosine + 5-HT combination on larval heart rate (UAS-M4D X 24B-GFP), n = 10. B: The difference in heart rate before and after change of solution. P ≤ 0.05 (*), P ≤ 0.001 (***)](http://www.jappl.org/)
with our earlier studies. Our original hypothesis was that 5-HT activates AC, which in turn lead to the activation of PKA and phosphorylation of Ca\textsuperscript{2+} channels that lead to inward Ca\textsuperscript{2+} currents and contraction. If this were correct, then the positive chronotropic effects of 5-HT action could be blocked by decreasing AC activity or levels of cAMP. Since our multiple attempts at manipulation of AC and cAMP levels had no or little effect on HR indicate that HR in the fly is in fact not substantially mediated by cAMP. The inhibitory effect of activation of hM4Di we observed is therefore likely mediated through G\textsubscript{i} activation of GIRK channels and silencing of cardiomyocytes, rather than inhibition of AC through G\textsubscript{i} it o
decrease cAMP levels and PKA activity.

Toward understanding the mechanism of action of serotonin on the heart, it needs to be determined if there may be different 5-HT receptors on heart muscle fibers acting through multiple signaling mechanisms. The known fly 5-HT receptors are all G-protein coupled receptors: 5-HT1ADro and 1BDro inhibit adenylyl cyclase through activation of G\textsubscript{i}; 5-HT7Dro activates adenylyl cyclase through G\textsubscript{s} activation; 5-HT2Dro signaling pathway has not been identified yet (6, 32, 36). A remaining possibility for the positive chronotropic effect of 5-HT could be that 5-HT2Dro receptors are mediating HR. 5-HT2 receptors couple with G\textsubscript{q} and activation of phospholipase C (PLC). PLC cleaves PIP\textsubscript{2} to IP\textsubscript{3} and diacylglycerol (DAG). IP\textsubscript{3} leads to increases in intracellular Ca\textsuperscript{2+} (16), which could lead to contraction. However, we previously demonstrated that mutation or misexpression of the 5-HT2Dro receptor does not have a dramatic effect on Drosophila larval HR (12). A second 5-HT2Dro receptor has recently been reported (17), and it may be this receptor mediating the effects if
Goq pathways are involved. Recently, a pharmacological study has shown that activation of 5-HT2 receptor by 5-HT2 agonist increases HR and ketanserin, which is 5-HT2 antagonist, markedly decreases the action of 5-HT on HR in Drosophila larvae (Majeed ZR, Stacy A, Cooper RL, unpublished observations). Therefore, we speculate that a PLC pathway might mediate the positive chronotropic effect of 5-HT. However, to confirm this suspicion, various pharmacological agents should be used to manipulate the PLC pathway to observe how it effects 5-HT action related to HR. Another possibility is that 5-HT1ADro, 5-HT1BDro, and 5-HT7Dro receptors are mediating HR through coupling to other effector pathways than adenyl cyclase. GPCRs couple to a wide range of effectors, and certain drugs and natural ligands can activate these pathways differentially through functional selectivity (38). In this scenario the 5-HT1ADro or 5-HT7Dro receptors could mediate HR through noncanonical pathways yet to be determined. In summary, we present evidence here that the positive chronotropic effect of 5-HT in the Drosophila larva HR is not governed by the adenyl cyclase signaling pathway, and that the negative chronotropic effects of hM4Di activation are likely due to electrical silencing rather than negative modulation of AC.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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