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DIRECT INHIBITION OF HISTAMINE RECEPTORS IN THE ANTENNAL LOBE OF MANDUCA SEXTA: PUTATIVE EVIDENCE FOR AN OLFACTORY COROLLARY DISCHARGE CIRCUIT

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Thesis submitted to the Eberly College of Arts and Sciences at West Virginia University in partial fulfillment of the requirements for the degree of

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Abstract

DIRECT INHIBITION OF HISTAMINE RECEPTORS IN THE ANTENNAL LOBE OF MANDUCA SEXTA: PUTATIVE EVIDENCE FOR AN OLFACTORY COROLLARY DISCHARGE CIRCUIT

by Rex Burkland

Animals that plume track have developed navigational strategies that optimize the ability to track an odor to its source. This movement is an active part of the olfactory experience. For vertebrates, sniffing is also an active part of the olfactory experience that controls the speed and regularity of odor interactions with the olfactory receptors. This sniffing is coincident with locomotion throughout the environment and head movement back and forth across an odor plume. Similarly, moths actively beat their wings as they encounter odor plumes. In Manduca sexta, this wing beating influences speed and regularity of olfactory input in such a way that odor processing and perception are enhanced. While it is clear that the antennal lobe (AL) of M. sexta has evolved to process these naturally encountered olfactory stimuli, there may be a source of input that optimizes processing only when odor is sampled through wing beating. In other sensory systems, corollary discharge (CD) mechanisms have evolved to enhance sensory processing when the sensory input is caused by an animal’s own muscle movement, termed reafference. These CD circuits exhibit a functional neural connection, either direct or indirect, between the sensory system and the motor system that caused the reafference. In M. sexta, there is a candidate pair of histamine (HA) immunoreactive neurons that project from the mesothoracic ganglia to the ALs. The goal of this study was to functionally characterize the role of HA signaling within the AL of M. sexta using pharmacological injections and behavioral detection assays. Here we have shown that pharmacological disruption of normal histamine signaling within the AL reduces sensitivity. This provides the first functional characterization of an olfactory CD circuit that uses flight-motor information to mediate olfactory sensitivity.
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INTRODUCTION

Many organisms actively sample their sensory environment using specific behaviors that optimize sensory function. Animals that actively search their olfactory environment for odor sources must maintain odor sensitivity within the turbulent and unpredictable odor plume patterns resulting from environmental factors that impact fluid motion, such as wind and landscape variability (Murlis et al., 2000). Odor plumes are known to be concentrated packets of odor molecules that are patchy in distribution and with variable and dynamic concentration gradients that become more intense near the source of the odor (Murlis and Jones, 1981). Fluid environments vary broadly and comparisons across species that inhabit these different environments suggest that both the sensory processing mechanisms and the odor tracking behaviors they ultimately guide are adapted to the circumstance in which an animal’s sensory array encounters an odor (Weissburg, 2000). For example, moths in search of an odor source will exhibit a casting behavior, zig-zagging across an odor plume (Kennedy, 1983), which provides an essential intermittency to stimulus exposure that is necessary for efficiently locating the source of an odor (Willis and Baker, 1984). The movement of an animal traversing its environment also affects the fine-scale structure of odor plumes around the sensory array. Modeling and anemometry studies of tethered *M. sexta* moths suggest that airflow is furthermore directed from the front to back of the antenna as a result of each downstroke of the wing (Sane, 2006; Sane and Jacobson, 2006). Thus, as the moth crosses into a plume, packets of odor within the plume are experienced as discontinuous pulses that entrain to the wing beat. Accordingly, physiological studies of olfactory processing as well as behavioral studies of olfactory acuity demonstrate that simulating wing beat
effects on airflow enhances olfactory function (Daly et al., 2013; Houot et al., 2014; Tripathy et al., 2010). For example, electroantennogram measures, AL local field potentials and spiking in both LNs and PNs all entrain to olfactory stimuli that match the natural wing beat frequency. Population analysis of the unitary AL responses to pulsed odor shows stronger, more sustained, and more distinctive patterns of neural activity as compared to prolonged continuous stimuli. The use of “flight-driven” intermittent olfactory stimuli ultimately results in an increased ability of the animal to detect and discriminate odors (Daly, 2013).

Furthermore, in flight, the insect is constantly being exposed to the forces of oscillating airflow caused by wing flapping. Interestingly, as with sniffing in mammals (Mainland and Sobel, 2006), the olfactory pathway respond to these “clean-air” mechano-sensory stimuli at wing beat frequencies that are preferentially tracked and processed by the (AL; Houot et al., 2014; Tripathy et al., 2010). Although the mechanism for mechanosensory driven activation of AL neurons has not been identified in insects, in mammals it is the olfactory receptors themselves that transduce the mechanosensory signals (Connelly et al., 2015; Grosmaitre et al., 2007). Given the complexity of airflow imposed by the animal’s own movement and the unavoidable olfactory response that these behaviors drive, it is reasonable to expect that some form of neural signal from motor centers that produce these behaviors would project to the olfactory pathway to account or otherwise facilitate the processing of olfactory signals under the conditions of a constantly oscillating flow.

Neural signals that project from motor pathways to sensory pathways, broadly known as corollary discharge (CD) circuits, have evolved several times across a number
of taxa and sensory systems in order to resolve effects to sensory processing caused by the animal’s own movement, termed “reafference”. These motor-to-sensory circuits have been extensively characterized in the auditory systems of songbirds (Troyer and Doupe, 2000), echolocating bats (Moss and Sinha, 2003), the electro-sensory lateral line in several species of electric fish (Bell and Grant, 1989), and the saccadic eye movement in primates (Thiele et al., 2002). Only lower order CD mechanisms have been observed in invertebrates, such as sensory filtration that prevents sensory habituation/adaptation or otherwise becoming desensitized. Examples of this are seen in the sensorimotor circuits in the crayfish tail-flip/escape response (Krasne and Wine, 1977; Mellon and Christison-Lagay, 2008) and cricket stridulation (Poulet and Hedwig, 2002). In each case, the motor system involved in generating a reafferent signal also had some form of neural circuitry projecting to the sensory pathway that blocks (via direct inhibition) self-generated sensory signals.

Few studies have investigated the presence or importance of potential CD circuits in olfactory systems, despite the known fluctuations in flow (air or water) created by motor systems during active sampling of the olfactory environment, or sniffing (Huston et al., 2015; Koehl, 2006; Loudon & Koehl, 2000; Mainland and Sobel, 2006; Sane & Jacobson, 2006; Vickers, 2000) that drive “olfactory” responses even in the absence of a stimulus. Investigation of these potential CD circuits requires anatomical evidence of neural circuitry that could reliably provide relevant motor signals to the sensory system as well as reliable methods to evaluate its function. In M. sexta, a pair of histamine (HA) immunoreactive neurons ascend ipsilaterally from the mesothoracic ganglia (MsG), a region controlling wing motor generation, and project bilaterally to the antennal
mechanosensory and motor center and to a subset of ventral AL glomeruli as well as a structure of unknown function, the antennal lobe isthmus (Homberg and Hildebrand, 1991; Rind, 1983). As demonstrated by Bradley et al., (2016) this single histamine immunoreactive cell pair represents the only source of histamine (HA) immunoreactivity in the AL. The receptors for HA in the AL (MsHisClB) are expressed by a subset of local interneurons (LNs) that are GABAergic with a limited subset also expressing FMRFamide, and allatotropin (Bradley et al., 2016). The LNs within the AL, which have broad extensions into all olfactory glomeruli, belong to morphologically and functionally distinct classes (Chou et al., 2010; Seki et al., 2010). From a neural coding perspective, the role of HA in odor processing is difficult to establish given the complexity and potential scope of its influence, however, given that histamine receptors act as ligand gated chloride channels (McClintock and Ache, 1989) and are themselves expressed on inhibitory LNs, the role is disinhibitory in nature. The purpose of this study was to functionally characterize the role of HA in odor processing at the level of sensory perception using behavioral pharmacology. Additionally, we sought to determine HA’s influence on AL processing using intermittent stimuli that would be encountered during odor guided flight, which should improve detection. The morphology of the HA neurons from the MsG provides putative evidence for a CD circuit that integrates flight sensory-motor information with olfactory processing, thus disrupting histamine function in the AL should result in disrupted sensitivity in a detection threshold assay.

METHODS

Subjects
Male and female *M. sexta* were reared in house using documented methods (Bell and Joachim, 1976). At pupal stage 17, individual moths were isolated into brown paper bags and placed in an incubator that maintains a reverse 16/8 light/dark cycle, at 25°C, and a relative humidity of 75%. The paper bags were checked daily; bags with newly eclosed moths were dated to record relative age post-eclosion. All subjects were kept in the incubator 5-7 days post-eclosion prior to use to increase feeding motivation. On day 1 of experiments male and female moths were selected in approximately equal numbers and were assigned to experimental groups.

*Preparation*

Moths were inserted head first into 4 cm long modified plastic centrifuge tubes (1.2 cm ID) with the head protruding out and above a ~1 cm square tab at the end of the tube. The head was then immobilized by placing a piece of tape over the exposed back of the moth’s body, securely fastening the ends of the tape to the tab at the front of the tube. The head was immobilized for the purpose of stabilizing the subject during the surgery and injection procedure. Scales on the head capsule were removed with forceps and compressed air. Next, a small opening was made in the cuticle of the head capsule to the right side of the sagittal midline using the sharp end of a dissection pin. A small opening was also made on the anterior edge of the contralateral eye. A Teflon coated silver wire electromyographic (EMG) electrode was then placed through the openings in the cuticle just above the cibarial pump muscle (the moth’s primary feeding muscle) and the contralateral eye. These electrodes were attached to wire leads attached to the tube that could be connected to an amplifier (DAM 50, WPI Inc.). The amplified signal was output
to a loudspeaker and oscilloscope that could be used to monitor feeding muscle activity. A small amount of adhesive was applied to each electrode in order to keep the electrodes in place during the surgery and injection process. The impedance of the electrode circuit was tested using an FHC low voltage impedance meter in order to confirm adequate electrode placement; this was indicated by a circuit impedance in the range of 0.1-0.9 M ohms. To allow for consistent feeding trials during the training protocol, the proboscis was then threaded through a 4 cm long piece of Tygon brand tubing (1.27mm ID) leaving the distal end of the proboscis exposed and unrestrained. The tubing was then attached to the plastic tube using a small amount of soft dental wax.

Stimulus Delivery System

All moths were trained and tested using a custom standardized odor delivery and exhaust system. Briefly, the olfactometer was fed with compressed air from a centralized supply line. Moisture was removed from the air by passing it through a 500cc Drierite cartridge (Indicating Drierite, mesh 8; Drierite: 23025) then purified using a charcoal filter made from a 500cc Drierite cartridge containing granular 20-60 mesh activated charcoal (Sigma-Aldrich: C3014). The airflow rate was controlled using a 150-mm direct reading flowmeter with an aluminum/sapphire float (Cole-Parmer: 1-010293). Filtered air was then passed through a 3-way valve controlled by a programmable log chip (PLC). Filtered air normally enters the valve and passes out of an exit port. Upon activation from the PLC, air is shunted towards a port that directs the airflow to the odor cartridge. Odor cartridges were made from borosilicate glass attached to luer fittings for an approximate internal volume of 1.7 ml. Odors were applied to small piece of Whatman No. 1 filter
paper and inserted into the cartridge. After each stimulus delivery odors were removed from the air by a 13 cm x 13 cm exhaust system positioned behind the odor delivery stage. The exhaust produced an ambient airflow of .3 m/s, which was calibrated using a hotwire anemometer (Fisher Scientific).

**Conditioning Protocol**

All moths were conditioned using a continuous stream of undiluted odor directed at the moths antennae using an airflow rate of 275 ml/min. Subjects were placed ~9 cm from the nozzle of the odor delivery cartridge and 1 cm in front of the exhaust vent. In all experiments, 3 µl of undiluted 2-hexanone (Sigma, 98% pure) was forward paired with a .75 M sucrose reward across 6 successive conditioning trials. The conditioning stimulus (CS), 2-hexanone, was delivered for a total of 4 s. During the final 1 s of CS presentation a small drop of the sucrose solution, the unconditioned stimulus (US), was applied to the distal tip of the proboscis for a total of 4 s. This resulted in 3 s of odor presentation, 1 s of overlap between the odor delivery and sucrose presentation, followed by 3 s of only sucrose application. This method of conditioning allows the animals to learn an odor-food relationship (Daly and Smith, 2000), where the conditioned response (CR), feeding activity, can be used to assess behavioral detection of the target odor (Daly et al., 2007). A trained observer scored the presence or absence of feeding activity in response to CS presentation; positive responses were recorded based on either movement of the proboscis or an increase in activity on the oscilloscope and loudspeaker. During conditioning trials, responses were only recorded if feeding activity occurred during the first 3 s of odor presentation, prior to application of sucrose.
Surgery and Injection

Prior to injection both ALs were surgically exposed. Surgeries were performed under a dissection microscope set on 20x magnification. First, a \( \sim 2 \text{ mm}^2 \) piece of cuticle was removed from the head capsule just above the brain. The patch of cuticle with the cibarial pump muscle attached was sectioned and moved forward into the area where cuticle had been previously removed in order to expose the ALs. A small drop of adhesive was used to hold the repositioned muscle and cuticle in place. Some minor trachea that obstructs direct access to the antennal lobes was also carefully removed.

During surgery and post injection, the moths were periodically given 3-4 droplets from a syringe of pH buffered *M. sexta* physiological saline (Heinbockel et al., 1998) to clear hemolymph and keep the brain hydrated.

The injection procedure was modified from previously successful experiments using precision pressure injections (Mwilaria et al., 2008). A sharp quartz intracellular electrode was pulled using a Sutter Instruments P2000 microelectrode puller to produce a slow-tapering injection probe. This allows for a minimal damage to the AL while providing consistent calibration across uses. The tips of the probes were removed under a dissection scope by slowly bringing the electrodes into contact with the surface of a steel single edge razor blade. The resultant opening is \( \sim 10 \mu m \) in diameter. The injection probe was filled with the desired solution and was then fitted to a line from a General Valve Pico Spritzer II and used to pierce and pressure-inject the AL. The fitting for the injection probe was attached to a micromanipulator to aid in maneuvering the electrode within the moth’s head capsule. Each probe was calibrated to produce a droplet of
approximately 1 nl. This was accomplished by setting the picospritzer to a constant
pressure of 20 psi and varying the amount of time the valve is open. Calibration was
accomplished by dispensing injection droplets into a pool of mineral oil positioned under
the dissection scope. A scale in the ocular of the scope was used to measure the droplets
diameter, from which its volume could be calculated. The injection droplet size
calibration was performed before and after every injection in order to ensure proper
treatment delivery. If an electrode was found to be clogged or broken, the animal that was
just injected with that probe was discarded and the probe was replaced.

Moths from control groups were injected with pH buffered *M. sexta* physiological
saline. Moths in separate drug treatment groups were injected with one of a series of
dilutions of either histamine (HA; 1 mM, 0.1 mM, 0.01 mM; Sigma-Aldrich) or one of
two histamine h-2 receptor antagonists: cimetidine (CIM; 50 μM, 5 μM, 0.5 μM; Sigma-
Aldrich) or ranitidine hydrochloride (RAN; 50 μM, 5 μM, 0.5 μM; Sigma-Aldrich). The
human H-2 receptor antagonists were selected due to their effectiveness on arthropod
histamine receptors, which are ligand gated chloride channels (McCIntock and Ache,
1989). The antagonist dilution ranges were selected to minimize secondary effects that
may have interfered with the signaling of other biogenic amine pathways (Cannon et al.,
2004). All dilutions were prepared by dissolving the drugs in saline solution at room
temperature. In all cases, the individual testing the moths for behavioral responses to odor
was blind to the injection contents to avoid experimenter bias.

*Testing Protocol: Experiment 1*

Testing was conducted 15 minutes post injection. This time allows the moth to recover
from the surgery (Mwilaria et al., 2008) and gives the pharmacological agents enough
time to reach effect (unpublished multi-electrode recordings using bath application, Daly lab). Upon preparation, moths were assigned to one of 12 treatment groups (n = 60/group or 720 total moths). In addition each concentration of all pharmacological agents (HA, CIM, RAN) had an independently matched saline control group that was randomly collected in tandem (n = 60/group or 720 total moths). All moths were tested across a log-step dilution series of the CS (2-hexanone) from 0.001 - 10 µg/2 µl, calculated based on density. In this experiment, a single 4 s continuous stream of odor was directed at the moth’s antennae 9 cm from the tip of the nozzle and 10 cm from the exhaust system. Air flow rate was set to 275 ml/min. Prior to the presentation of odor, an odorless blank was directed at the moth’s antennae. Odor dilutions were then presented once each in order of increasing concentration. Again, responses were scored based on increased activity from the feeding muscle as observed on the oscilloscope or extension of the proboscis. During testing the time allotted for a positive response is 7 s. After testing, each test subject will receive a small drop of .75 M sucrose solution to confirm that the animal was still able to elicit a feeding response. This ensures that a lack of response response was not attributable to the effects of the pharmacological agent or injection procedures.

Testing Protocol: Experiment 2

As in experiment 1, animals were tested with the CS (2-hexanone) 15 minutes post injection of both ALs. Again, an odor dilution series from 0.001 - 10 µg/2 µl mineral oil was delivered in sequential steps of increasing concentration following presentation of an odorless blank stimulus. Each concentration was only delivered once. However, for this experiment odor was interleaved into clean air and delivered as a 20 Hz pulse train for a
total of 4 s. Each successive pulse of odor within the train was 10 ms, followed by 40 ms of clean air. Additionally, a 3-way barbed T-fitting was used to deliver the odor at the antenna. One opening of the fitting was constantly supplied with a continuous stream of clean air, another opening was attached to odor cartridge, and the third opening of the fitting acted as the odor delivery nozzle. The odor delivery nozzle was positioned approximately halfway up the length of a single antenna with tip of the T-fitting at a distance of ~3mm. A continuous stream of clean air was constantly leaving the end of the nozzle; upon actuation of the PLC controlled valve air flow was shunted into the air line that supplied the odor cartridge. Air velocity at the odor delivery nozzle was lowered to 30 cm/s such that potential mechanosensory stimulation of the olfactory receptors that could result from “wind” artifacts created by the olfactometer would be normalized to the approximate amplitude produced by the beating wings (Sane and Jacobson, 2006).

Confirmation that the olfactometer produced the approximate amplitude of flow variation as wing beating has been established (Daly et al., 2013). After the surgery and injection procedure, the antenna was isolated by threading it through a small spring (0.3 cm in length) fitted to a blunted syringe needle that could be attached to the plastic tube. Responses were scored the same as the testing phase of experiment 1. In this experiment, behavioral detection of pulsed odor was assessed after delivering injections to both ALs, using select concentrations of pharmacological solutions from experiment 1. Due to the proximity of the odor delivery nozzle to the exhaust vent, each successive change of the odor cartridge was performed outside of the direct path of the exhaust system’s ambient airflow to prevent unintended odor exposure. Upon preparation, moths were assigned to one of 3 treatment groups: 0.01 mM HA, 50 µM CIM, or a saline control (n=60 for each).
Once again, the animal was fed with a droplet of 0.75 M sucrose solution after all odor had been delivered to ensure that the ability to make a CR was not prevented by the surgery and injection procedure.

Data Analysis

Behavioral data were analyzed in SAS using the general linear modeling (GLM) procedure. This procedure is particularly well suited for both continuous and categorical independent variables, along with any interactions and nested effects. Additionally, GLM has the advantage of hierarchically partitioning variance components, thus providing a more stringent test. Each drug treatment group was compared individually against a methodologically matched control group. Here, we tested the main effect of odor concentration, treatment type, age, and sex as well as their 2 and 3 way interactions with a significance value of $p < .05$. Note that we were not interested in age and sex effects but removed variance attributed to them prior to our variables of interest. Post-hoc analysis was performed on individual groups where significant main effects and treatment by odor concentration interactions had occurred. Additionally, one-tailed paired t-tests were used to statistically define detection thresholds. Here detection threshold is defined as the concentration of odor that caused a significant increase in conditioned response probability over the response to blanks ($p < .01$). Acquisition of the conditioned response during conditioning trials was normalized by subtracting the average response to the CS during the first trial for each group.

RESULTS

The goal of this study was to determine the role of histamine in olfactory function using psychopharmacological techniques. Our approach was to condition animals to a test
odorant, pharmacologically manipulate histamine function using focal injections, and
then compare detection thresholds as a function of pharmacological treatment. In our first
series of experiments we conditioned four groups of moths to respond to 2-hexanone.
Figure 1A displays acquisition curves for these groups, which include three
concentrations of cimetidine groups and a saline-vehicle injected control group. Mean
values were normalized to the initial responsiveness for each group. Note that inset
acquisition regression lines indicate that all moths learn the odor-food relationship at
approximately the same rate.

The statistical model explaining variance in conditioned response probability
among cimetidine and saline treatment groups was significant (p<.0001) and explained
approximately 35% of the variance. The main effect of drug dose was not significant
(Figure 1B; p=.2753). However, there was a significant treatment by odor concentration
interaction (p<.05). Post hoc analysis of this effect indicates that the .5 and 5 µM doses
(p=.09 and p=.14, respectively) of drug did not produce response functions different from
the control group, which received saline vehicle only. However, the highest dose, 50 µM,
produced significantly lower CR response probabilities in response to some
concentrations of odor relative to the controls (Figure 1C). We next independently
statistically compared each treatment dose versus the saline-vehicle control to assess the
treatment by concentration interaction. This effect, if significant, indicates a different
concentration response function, and hence detection threshold, for the saline versus drug
treated groups. As shown in Figure 1D and 1E, corresponding to the non-significant
interactions, statistical assessment of detection threshold found no difference as well.
However, the treatment by concentration was significant for the highest dose

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concentration (p<.05). Figure 1C highlights this significant interaction which results in an increased detection threshold for the drug treatment group (p<.01). Thus, in all cases the detection threshold was determined to be 1 µg/2µL, except for the highest concentration dose of the histamine receptor antagonist, cimetidine.

The next set of experiments sought to confirm the effect of competitive histamine h-2 receptor antagonists on olfactory sensitivity. Figure 2A displays acquisition curves for 3 concentrations of ranitidine-injected groups along with a matched saline vehicle injected control group; again responses were normalized to the initial responsiveness for each group. Inset regression lines indicate that all moths acquisition at approximately the same conditioning trial. The statistical model explaining variation in conditioned response probability between groups was significant (p<.0001) and explained approximately 37% of the variance. The main effect of drug dose was not significant (Figure 2B; p=.9745). Importantly, there was a significant interaction of treatment by odor concentration (p<.05). Post hoc comparison of individual drug injected groups with saline vehicle controls revealed significant effects for moths receiving the .5 and 50 µM drug dose (Figure 2E and 2C, respectively; p<.05). The treatment group receiving the 5 µM dose did not produce a treatment by odor concentration interaction (p=.8538). We then independently compared detection thresholds for the odor concentration response functions belonging to each treatment group to further assess the treatment by odor concentration interaction. Figure 2C displays this significant interaction which results in an increased detection threshold for the 50 µM treatment group (p<.01). While GLM did not suggest a significant interaction for the 5 µM treatment group, detection threshold analysis indicates a shift in the concentration response function where the 5 µM injected
moth required a higher concentration of odor for initial behavioral detection (Figure 2D; p<.01). Conversely, the GLM indicated a significant interaction for the lowest drug dose. However, both the control and .5 µM treatment groups showed the same detection threshold of 1 µg/ 2µL, which suggests that any differences detected by the GLM were likely spurious effects below the behavioral detectable range (Figure 2E). Taken together with the first experiment there is a generalized disruption of olfactory function when injecting competitive histamine h-2 receptor antagonists into the AL. Thus histamine signaling is necessary for normal AL function.

The goal for the third experiment was to determine if introducing histamine into the ALs could positively impact olfactory sensitivity. Figure 3A shows acquisition curves for 3 concentrations of histamine-injected groups and a matched saline vehicle injected control group; groups acquisitioned at approximately the same rate. The statistical model explaining variation in CR probabilities for saline versus histamine-injected moths was significant (p<.0001) and explained approximately 35% of the variance. Both the main effect of treatment and the interactive effect of treatment by odor concentration were not significant (p=.9785 and p=.8994; Figures 6B-E). Furthermore, when groups were compared independently to the control none of the three doses of histamine was able to generate a significant effect on CR probability. Thus, the histamine injection concentrations used in this experiment were not enough to enhance or disrupt AL function.

The first three experiments used a continuous odor stream for test stimulus presentation. In contrast, the goal for the final experiment was to examine how manipulation of histamine within the AL effects odor detection when odor is presented as
a discontinuous pulse train. Pulsing the odor more accurately represents the airflow dynamics around the antenna of a flying moth (Sane and Jacobson, 2006) and has been shown to enhance AL function (Houot et al, 2014) and odor detection (Daly et al, 2013). Figure 4A displays acquisition curves for two drug treatment groups (.01 mM histamine and 50 µM cimetidine) along with a saline vehicle injected control group. Separate statistical models were used for cimetidine and histamine with the expectation that they exert opposing effects. The model explaining variation between histamine and control injected moths was significant (p<.0001) and explained 43% of the variation; however, no significant main or interactive effects were produced (Figures 4B and C, respectively). The model explaining variation in CR probabilities for cimetidine versus control injected moths was significant (p<.0001) and explained approximately 38% of the variance. No significant main effects were generated, but the treatment by odor concentration interaction was significant (p<.05), which resulted in a reduced CR probability for cimetidine-injected moths. Additionally, independent statistical comparisons of detection thresholds for each of the odor concentration response functions were generated. Figure 4C shows that cimetidine injections increase the detection threshold relative to the controls, reducing olfactory sensitivity. Interestingly, injection of the same antagonist at the same concentration and volume produced detection threshold shifts of different magnitudes in pulsed and continuous odor experiments. The 50 µM cimetidine injections increased the detection threshold 2 orders of magnitude in odor concentration when odor was pulsed (Figure 4C), as compared with the detection threshold increase of 1 order of magnitude when odor was presented continuously (Figure 1C). To highlight this comparison, the detection thresholds from first set of cimetidine experiments where
continuous odor stimulation was used, are inset in red for the 50 µM cimetidine injected and control groups (Figure 4C). While no direct statistical comparison can be made, note that detection thresholds are lower for pulsed stimulation versus continuous. Furthermore, blocking HA activity when odor was pulsed not only decreased sensitivity, as it did when odor stimulation was continuous, but also decreased the impact that pulsed stimulation has on olfactory sensitivity. This suggests that HA mediates odor sensitivity and that odor sensitivity is enhanced when the odor is pulsed.

DISCUSSION

Sensory systems of all animals must process details of the environment under ever changing conditions. Often, changes in the sensory environment are the result of fluctuating environmental conditions such as wind, temperature, humidity, sunlight, and season. To accommodate, sensory processing is modulated by a variety of neural mechanisms using a variety of neurotransmitters and neuropeptides in response to the dynamic variation within the environment. As the animal’s own behavior is inextricably part of their sensory environment, sensory systems must also be able to adapt to a variety of behaviors, especially those that change, or otherwise drive, the way in which a stimulus is encountered. Modulatory input from a variety of sources converge in the AL and the overall contribution to processing is complex and largely state-dependent. Although HA is a neuromodulator, it acts as a neurotransmitter in the nervous system of insects (Hardie, 1989). The goal of this study was to evaluate HA’s contribution to odor processing within the AL. Furthermore, we sought to assess HA’s impact on odor processing while using fine-scale temporally structured stimuli, similar to those that
would be encountered during odor-guided flight. In all cases, our results show that blocking HA activity using sufficient doses of HA receptor antagonists increased detection thresholds, reducing olfactory acuity. Our data suggests that histamine plays a vital role in odor processing necessary for maintaining sensitivity to odor during flight. This statement only assumes that the histamine cells become active (or more active) during flight.

Indeed the *M. sexta* HA neurons (MDHns) receive inputs from multiple regions of the MsG (Homberg and Hildebrand, 1991), which contain various leg and wing motor neurons (Rind, 1983). Interestingly, the LNs in the AL, which express the HA receptor, are not present during the animal’s flightless larval stage (Bradley et al., 2016), suggesting that the information projected to the AL is specific to flight behavior. Collectively, the activity of the MDHns is likely driven by neurons within the flight-motor pathway. MDHns are the sole source of HA input in the AL of *M. sexta*. The neurons that express MsHisClB belong to a subset of the ~360 LNs known to exist within the AL (Homberg et al., 1988). HA receptors within the arthropod olfactory system are known to be ligand gated chloride channels (McClintock and Ache, 1989), which suggests that the effect of HA on any of its target LNs would be inhibitory. The GABAergic neurons within the AL are known to be inhibitory LNs that contribute to odor detection and discrimination (Mwilaria, 2008) through mechanisms of contrast enhancement (Lei et al., 2009) and gain control (Olsen and Wilson, 2008). Therefore, it is perhaps not surprising that disruption of HA activity on the subset of these GABAergic LNs that express MsHisClB accordingly reduces the ability of the animal to detect the odor, as we have shown above. While the exact mechanism through which HA is exerting
its influence is unclear, this provides us with a putative explanation for diminished olfactory acuity. Though it is likely that the MDHn causes disinhibition, a mechanism proposed to be involved in olfactory processing (Christensen et al., 1993). In addition, some of the LNs expressing MsHisClB are peptidergic, releasing either allatotropin or FMRF-amide. While it is certain that these neuropeptides contribute to olfactory processing (Hildebrand et al., 1986) it is unclear how they do so. Additionally, classical transmitters like acetylcholine and GABA are known to be colocalized with these neuropeptides, potentially acting as co-transmitters or neuromodulators (Berg et al., 2009; Fusca, 2015). The diversity in neurotransmitter content of the LNs that express MsHisClB further adds to the complexity of the proposed circuit.

Input from sensory neurons along with lateral inhibition from LNs shapes AL output. However, AL processing is modulated in ways that enhance olfactory function, thus improving behavioral responses. In M. sexta, there is a single neuron that releases increased amounts of serotonin during the animal’s “wake cycle” which allows the animal to mediate sensitivity at different phases of its circadian rhythm (Dacks et al., 2008; Kloppenburg et al., 1999). This type of context specific modulation can allow for targeted optimization of sensory processing. In addition, modulation can enhance sensory output used for learning of odor-food relationships (Dacks et al., 2012). The proposed input from wing motor regions to the centers of olfactory processing could serve as a mechanism of sensorimotor integration, thereby enhancing the olfactory percept when the wings are used to introduce odor-laden air into the sensillar array.

While blocking HA activity resulted in a decrease of olfactory function, injections of HA into the AL did not provide any added sensitivity, which could be for several
reasons. First, HA neuron activity may have been ongoing during the experimental protocol. Indeed, anecdotally it is common to observe the moths actively moving the wing joint while restrained. While it is uncertain what directly influences histamine release, the MDHn is either phasically or tonically active as a result of ongoing wing beating activity. In any case, there is presumably some basal amount of HA present that would be contributing to signaling within the AL. Furthermore, we can only speculate as to how much HA is being released onto the LNs and the timing scale of the MDHn’s input. Here we provide a pharmacological characterization of HA signaling within the AL and propose that it impacts olfactory sensitivity within the context of active sensing.

Previous studies have suggested that the intermittency imposed by casting or counter-turning behavior is analogous to human sniffing (Kennedy, 1983; Willis and Baker, 1984) it is likely that any “sniff” related olfactory information would be conveyed by the motor system that drives its input (Wachowiak, 2011). The casting and counter-turning behaviors are performed within the typical behavioral movements during odor-guided search behaviors, sterotypical among most animals in search of odor cues (Porter et al, 2007; Vickers et al, 2000). However, the airflow through the antennae is driven, primarily, by the beating of the wings during flight (Koehl, 2006; Sane and Jacobson, 2006). It is more plausible, perhaps, that any sniff like benefit would originate in the centers of flight control, as they would for inhalation of air into the lungs. Presumptively, that would originate from feed forward CD input in a connection from the wings to the AL via the MDHns.

Potential for the existence of CD circuits are likely widespread. Although, any “sniff-like” CD signaling would occur within a narrower context of the behavior that
controls the intermittency, such as antennal flicking in crustaceans and locusts (Huston et al., 2015; Koehl et al., 2001). In other sensory systems, CD serves to provide motor information to enhance the sensory percept through direct influence or modulation of signaling. Auditory systems of bats and songbirds have co-evolved CD mechanisms that act as a functional motor signal copy that is used for comparison in downstream processing, albeit for distinct sensory tasks (Moss and Sinha, 2003; Troyer and Doupe, 2000). Bats use CD signals to compare self-generated calls with its echo in order to derive information about distance, while songbirds project feed-forward vocalization motor signals that are essential for proper learning of song patterns. Lower order CD systems, such as those typically seen in invertebrates, have a more prominent role in providing sensory filtration that counters the effects of reafference, such as those in the crayfish tail-flip escape pathway and in the cricket auditory system (Mellon and Christison-Lagay, 2008; Poulet and Hedwig, 2002). These mechanisms enhance behavioral responses through direct inhibition of self-generated sensory input. In the olfactory system, CD circuits have yet to be explored more broadly. Previous studies have generally focus on a connection between motor driven sniff activity along with its contribution to the timing and effectiveness of olfactory processing, however they lack a distinct morphological and physiological characterization of the underlying circuit. Investigation of the processes involved in potential olfactory CD circuits is essential to our understanding of olfactory processing. Furthermore, the loss of CD input that predominates in patients with Parkinson’s disease and various mental illness (primarily schizophrenia) necessitates a broader understanding of their impact in order better understand their underlying causes (Ford et al, 2001; Moore, 1987). Our results provide
initial evidence for the MDHn as a candidate for an olfactory CD circuit, where motor information is utilized in a flight-specific sensory context in a manner that enhances the olfactory experience.
Figure 1

A. CR Probability vs Conditioning Trial

B. CR Probability vs CIM Dose (µM)

C. CR Probability vs Concentration (µg/2nL) for 50 µM CIM

D. CR Probability vs Concentration (µg/2nL) for 5 µM CIM

E. CR Probability vs Concentration (µg/2nL) for .5 µM CIM

Legend:
- Control (saline)
- 50 µM CIM
- 5 µM CIM
- 0.5 µM CIM
Figure 2

A. CR Probability vs Conditioning Trial

B. CR Probability vs RAN Dose (µM)

C. CR Probability vs Concentration (µg/2nL) for 50 µM RAN

D. CR Probability vs Concentration (µg/2nL) for 5 µM RAN

E. CR Probability vs Concentration (µg/2nL) for 0.5 µM RAN

Legend:
- ○ Control (saline)
- ● 50 µM RAN
- ▲ 5 µM RAN
- ♦ 0.5 µM RAN

* and ^ symbols indicate statistical significance.
Figure 3

A. CR Probability vs. Conditioning Trial

B. CR Probability vs. HA Dose (µM)

C. 1 mM HA

D. .1 mM HA

E. .01 mM HA

- ○ Control (saline)
- ● 1 mM HA
- ▲ 0.1 mM HA
- ▣ 0.01 mM HA

Concentration (µg/2nL)
Figure 4

A. CR Probability vs Conditioning Trial

B. Pulsed Treatment: CIM, HA, Control

C. CR Probability vs Concentration (µg/2nL) for 50 µM CIM

D. CR Probability vs Concentration (µg/2nL) for 1 mM HA

Legend:
- ○ Control (saline)
- • 50 µM CIM
- ▲ 0.1 mM HA
- * Significant difference
- ^ Trend significance

Concentration (µg/2nL) range: 0.001 to 10
Figure 1  (A) CR acquisition curves across 6 conditioning trials, inset logarithmic regression lines for each treatment group. For clarity, only error bars for the control group are shown. (B) Average percentages of moths responding across all test trials, inset linear trendline. (C, D, E) CR probabilities as a function of odor concentration for cimetidine-injected versus saline-injected moths. Inset 3rd order polynomial regression lines describe concentration response functions. Post hoc comparisons for significant treatment by odor concentration are inset; different lower case letters denote significantly different odor response functions (p< .05). Results from detection threshold analysis are inset (p < .01; * control treatment detection threshold; ^ cimetidine treatment detection threshold). All error bars are ±1 SE.

Figure 2  (A) CR acquisition curves across 6 conditioning trials, inset logarithmic regression lines for each treatment group. For clarity, only error bars for the control group are shown. (B) Average percentages of moths responding across all test trials, inset linear trendline. (C, D, E) CR probabilities as a function of odor concentration for ranitidine-injected versus saline-injected moths. Inset 3rd order polynomial regression lines describe concentration response functions. Post hoc comparisons for significant treatment by odor concentration are inset; different lower case letters denote significantly different odor response functions (p< .05). Results from detection threshold analysis are inset (p < .01; * control treatment detection threshold; ^ ranitidine treatment detection threshold). All error bars are ±1 SE.

Figure 3  (A) CR acquisition curves across 6 conditioning trials, inset logarithmic regression lines for each treatment group. For clarity, only error bars for the control group are shown. (B) Average percentages of moths responding across all test trials, inset linear trendline. (C, D, E) CR probabilities as a function of odor concentration for histamine-injected versus saline-injected moths. Inset 3rd order polynomial regression lines describe concentration response functions. Post hoc comparisons for significant treatment by odor concentration are inset; different lower case letters denote significantly different odor response functions (p< .05). Results from the detection threshold analysis are inset (p < .01; * control treatment detection threshold; ^ histamine treatment detection threshold). All error bars are ±1 SE.

Figure 4  (A) CR acquisition curves across 6 conditioning trials, inset logarithmic regression lines for each treatment group. For clarity, only error bars for the control group are shown. (B) Average percentages of moths responding across all test trials. (C, D) CR probabilities as a function of odor concentration for drug-injected versus saline-injected moths. Inset 3rd order polynomial regression lines describe concentration response functions. Post hoc comparisons for significant treatment by odor concentration are inset; different lower case letters denote significantly different odor response functions (p< .05). Results from the detection threshold analysis are inset in black (p < .01; * control treatment detection...
threshold; ^ drug treatment detection threshold). To highlight the comparison of detection thresholds for pulsed versus continuous odor stimulation, inset in red are the detection thresholds from Figure 1C, for 50 µM cimetidine injected and saline-injected control groups. All error bars are ±1 SE.
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