Disruption of GABA$_A$ in the Insect Antennal Lobe Generally Increases Odor Detection and Discrimination Thresholds

Esther K. Mwilaria*, Chitrita Ghatak and Kevin C. Daly*

Department of Biology, West Virginia University, PO Box 6057, Morgantown, WV, USA

Correspondence to be sent to: Kevin C. Daly, Department of Biology, PO Box 6057, West Virginia University, Morgantown, WV 26506, USA. e-mail: kevin.daly@mail.wvu.edu

*These authors contributed equally to this work.

Abstract

Studies of olfactory function show that disruption of GABA$_A$ receptors within the insect antennal lobe (AL) disrupts discrimination of closely related odors, suggesting that local processing within the AL specifically enhances fine odor discrimination. It remains unclear, however, how extensively AL function has been disrupted in these circumstances. Here we psychophysically characterize the effect of GABA$_A$ blockade in the AL of the moth *Manduca sexta*. We used 2 GABA$_A$ antagonists and 3 Pavlovian-based behavioral assays of olfactory function. In all cases, we used matched saline-injected controls in a blind study. Using a stimulus generalization assay, we found that GABA$_A$ disruption abolished the differential response to related odors, suggesting that local processing mediates fine odor discrimination. We then assessed the effect of GABA$_A$ antagonist on discrimination thresholds. Moths were differentially conditioned to respond to one odor (reinforced conditioned stimulus [CS+]) but not a second (unreinforced conditioning stimulus [CS−]) then tested for a significant differential conditioned response between them across a series of increasing concentrations. Here, GABA$_A$ blockade disrupted discrimination of both similar and dissimilar odor pairs as indicated by generally increased discrimination thresholds. Finally, using a detection threshold assay, we established that GABA$_A$ blockade also increases detection thresholds. Because detection is a prerequisite of discrimination, this later finding suggests that disrupted discrimination may be due to impairment of the ability to detect. We conclude that the loss of ability to detect and subsequently discriminate is attributable to a loss of ability of the AL to provide a clear neural signal from background.

Key words: antennal lobe, detection threshold, discrimination threshold, GABA, *Manduca sexta*, odor encoding, olfactory bulb

Introduction

Behavioral studies of insects have revealed that their olfactory systems can readily discriminate among a wide variety of odors and odor blends (Laska et al. 1999; Daly and Smith 2000; Daly, Durtschi, et al. 2001; Fan and Hansson 2001; Wright et al. 2002; Daly, Wright, et al. 2004; Skiri et al. 2005). These studies typically use stimulus generalization and differential conditioning protocols, which are important behavioral paradigms for investigating the perceptual relatedness of stimuli. Stimulus generalization can be defined as the degree to which a novel stimulus elicits a conditioned response (CR), based on its overall perceptual similarity to the stimulus initially used to condition the animal (Daly, Chandra, et al. 2001). Discrimination on the other hand is the ability to detect differences in odor identity. Typically, experiments, which establish that an animal can discriminate between odors, are based on the product of differential reinforcement of one stimulus (reinforced conditioning stimulus [CS+]), with nonreinforcement of a second (unreinforced conditioning stimulus [CS−]; e.g., Daly and Smith 2000; Daly, Chandra, et al. 2001). Both stimulus generalization and differential conditioning experiments have been used to investigate the ability of animals to discriminate subtly different monomolecular odors based on their physical characteristics, such as carbon chain length (Bhagavan and Smith 1997; Daly, Chandra, et al. 2001; Cleland and Narla 2003). These experiments provide basic information about which stimulus dimensions are relevant to odor coding in olfactory systems.

It is now generally accepted that the ability of animals to detect and discriminate a seemingly limitless number of odors and odor blends is dependent on a relatively limited number of genetically encoded olfactory receptor proteins (Buck and Axel 1991). Each receptor type is expressed more or less individually within a given subset of olfactory

receptor neurons, though this is not strictly the case (Couto et al. 2005; Fishilevich et al. 2005). Nevertheless, all receptor neurons expressing the same profile of receptors converge on an individual glomerulus within the insect antennal lobe (AL; Vosshall et al. 2000). This initial input is then modified by an array of local interneurons (LNs) in insects (Matsumoto and Hildebrand 1981; Christensen et al. 1993) and vertebrates (Shipley and Ennis 1996). In the AL, LNs are primarily GABAergic and inhibitory. LNs tend to have broad multiglomerular ramifications (Matsumoto and Hildebrand 1981; Leitch and Laurent 1996) though other morphologies, which have more restricted ramification patterns, are also present (Christensen et al. 1993). Thus, as with vertebrates, there appear to be several classes of LNs with potentially distinct functional roles. Physiological studies of GABA function within the AL establish that this inhibitory neurotransmitter is involved in restricting spatial patterns of glomerular activation (Sachse and Galizia 2002) as well as generating temporal patterns of activity within the AL that may be critical for representing olfactory information in other central nervous system regions (Laurent and Davidowitz 1994; Laurent et al. 2001; Daly, Wright, et al. 2004).

Behavior pharmacological and physiological studies of honeybee AL function suggested that GABA regulates the specificity of odor representations (Hosler and Smith 2000) by mediating the transient synchronization of distributed AL projection neurons (PNs) on an oscillatory timescale (Stopfer et al. 1997). Oscillatory synchronization of PNs was abolished in these studies by application of picrotoxin (PCT), a competitive $GABA_A$ antagonist. This loss of periodic synchrony lead to impairment of discrimination of molecularly similar but not dissimilar odors in a stimulus generalization paradigm. Unanswered, however, is whether $GABA_A$ blockade affects other measures of olfactory acuity.

For example, the AL's local network also provides a mechanism for distribution of primary olfactory input from one glomerulus to others thereby modulating the output of these secondary glomeruli. This lateral activity is manifest as excitatory responses (Wilson et al. 2004; Olsen et al. 2007), though it remains unclear if this can be attributed to both inhibitory and excitatory lateral activity (Shang et al. 2007). It has also been shown that output from the AL can take an indirect path via $GABA_A$ergic LN activity (Christensen et al. 1998). Thus, in principle, the application of $GABA_A$ antagonists, in addition to disrupting classic lateral inhibition of nonencoding glomeruli and oscillatory synchrony, we hypothesize, could also block indirect output from the AL. Blocking this secondary output could result in decreased information available to downstream systems; this we predict will affect measures of discrimination more generally than previously described (Stopfer et al. 1997; Hosler et al. 2000) and should also impact the ability to detect odors.

Therefore, to assess the effect of impairment of local inhibitory processing within the AL on olfactory function, we first performed comparative behavior pharmacological studies of stimulus generalization in the moth, *Manduca sexta*. *Manduca sexta* is a favorable comparative model system because like the honeybee, this moth readily learns odor–food relationships in a Pavlovian olfactory conditioning paradigm. We used stimulus generalization protocols, where moths were conditioned and tested using high concentration stimuli as previously described in honeybee (Stopfer et al. 1997; Hosler et al. 2000). This first experiment was performed in order to establish whether $GABA_A$ disruption affects discrimination of molecularly similar (S) but not molecularly dissimilar (D) monomolecular odors in a moth species, using similar experimental circumstances. In this case, we also extend previous work by using multiple $GABA_A$ antagonists, PCT, and bicuculline methiodide (BMI), which disrupt the $GABA_A$ pathway in fundamentally different ways. We also used a different combination of odorants to assess the generality of previous studies to different odor combinations.

We were also interested in whether other measures of olfactory function were affected by $GABA_A$ disruption. Thus, we have developed psychophysical methods that characterize odor detection thresholds and establish concentration-response functions for individual odors (Daly et al. 2007). We have also developed methods that characterize discrimination thresholds and differential concentration-response functions (the rate at which a CS+ and CS− come to elicit a differential CR as a function of increasing concentration) for pairs of odors (Daly et al. forthcoming). Results of these studies are consistent with similar studies in vertebrates (Cleland and Narla 2003). Using these new methods, we establish that $GABA_A$ disruption more broadly affects olfactory function than previously described.

Materials and methods

Subjects

Male and female *M. sexta* were obtained at or near stage 18 of pupal development from Division of Neurobiology, Arizona Research Labs (Tucson, AZ) via overnight delivery. Upon arrival, pupae were isolated in brown paper bags where they remained undisturbed until used. Bags were placed in an incubator that holds temperature at 25 °C, relative humidity at 75%, and a reverse 16/8 light/dark cycle. Eclosion dates were recorded once daily on bags in which newly emerged adults were found. Age at initiation of conditioning was between 5 and 7 days posteclosion to increase motivation to feed (Daly and Smith 2000). Experiments were performed during the dark period of the light/dark cycle. Subjects were randomly assigned, in approximately equal numbers of males and females, to one experimental group and used only once.

Preparation

Moths were inserted head first into a snugly fitting 2.54-cm internal diameter (ID) plastic tube with the head protruding

out and over a 0.75 cm^2 raised tab at the end of the tube. The exposed back of the moth's body up to the head was then firmly shackled to the tab with a piece of tape. This method immobilized the insect and provided a secure platform for the head in preparation for dissection. After removing all scales from the head, a single-ended electromyographic (EMG) electrode was placed through the cuticle just above the left cibarial pump muscle (a large muscle involved in feeding behavior; Eaton 1971), and a reference electrode was placed in the contralateral eye. Electrode impedance was tested using an FHC low-voltage impendence meter to confirm electrode circuit quality with the cibarial pump muscle. The proboscis was threaded through a 5-cm (0.5 mm ID) length of Tygon tubing and affixed to the tube containing the restrained moth, with a piece of soft wax. At this point, the moth was ready for the conditioning phase of the experiment.

Stimulus delivery

The conditioning and testing stage consisted of an odor delivery system and an odor evacuation vent. Naive moths were placed into the threshold of the evacuation vent where a steady stream of air flowed by the animals at a rate of 0.2–0.3 m/s. Airflow was measured by a Fisher hotwire anemometer. An odor cartridge was placed 10 cm upwind and aimed directly at the moth's head. Distance from the cartridge to the moth ensured adequate dispersion over both antennae; this has been confirmed with titanium–tetrachloride (liquid smoke) tests. Airflow through the odor cartridge, as well as conditioned stimulus (CS)/unconditioned stimulus (US) timing was controlled by a programmable logic chip (PLC). Filtered air was supplied via a central air line. Air was first passed through a 500-cc Drierite brand cartridge to extract moisture and then passed through a 500-cc active charcoal filter. Output from the filter array then passed through a flow meter, which was set at 250 ml/min, and into a Lee brand 3-way valve, which was controlled by the PLC. The final velocity of effluent from the nozzle was measured at less than 4 ms via a hotwire anemometer. Air flowed into the input port on the valve then immediately out a second, normally open exit port. When the valve was activated, the output was shunted to the third, normally closed exit port, which was connected via Tygon brand tubing to the odor cartridge. Though difficult to measure, liquid smoke tests suggested that the flow from the nozzle decelerated to approximate the exhaust flow as it passed by the moth; hotwire anemometry did not indicate a measurable difference in flow at the position of the moth while the valve was activated.

Odor cartridges were fashioned from glass tubing (6 mm ID) cut to a length of 7 cm. Cole-Parmer brand nylon lure fittings were inserted into either end of the glass tube. The internal volume of this cartridge was 1.5 ml after the fittings were inserted. Given this cartridge size and a flow rate of 250 ml/min, it should take an estimated 0.36 s for the initial air volume of the cartridge to be replaced assuming no mixing.

Four monomolecular odors were used in the current study: racemic linalool (LOL), methyl salicylate (MES), 2-hexanone (HEX), and 2-octanone (OCT). The odors were picked so that we had pairs of closely related and different monomolecular odors (Stopfer et al. 1997). Detection thresholds for these odors have been previously characterized (Daly et al. 2007). All odors were 97% pure or better. A 5-log-step range of concentrations was established (0.0005 μg/μl, 0.005 μg/μl, 0.05 μg/μl, 0.5 μg/μl, and 5 μg/μl) based on dilution in mineral oil. When undiluted odors were used (for conditioning), an approximately 3-μl aliquot of odor was placed on a strip of Whitman brand No. 3 white filter paper, which was then placed inside the glass cartridge. This insured that a repeatable, high concentration stimulus could be delivered using a single cartridge. When dilutions were used, approximately 2-μl aliquot was applied to the filter paper. This total dose was used in order to closely match the current study with our previously published work (Daly et al. 2007, forthcoming).

Conditioning protocols

We used 2 basic Pavlovian conditioning protocols in the current study that were based on standard methods (Daly and Smith 2000). For experiments 1 and 3, a single odor was simply forward paired with food. Each animal received 6 forward paired conditioning trials of the undiluted conditioning odor (CS) followed by application of sucrose solution (US; 5 ml of 0.75 M) to the proboscis. Undiluted odor was used as the CS during conditioning to insure that salience-dependent effects on learning were minimized. During each conditioning trial, a 4-s puff of the CS was blown over the antennae followed by a 4-s presentation of the US upon the proboscis. The timing of CS and US presentation was overlapped by 1 s. There was a 6-min interval between conditioning trials. After conditioning, animals were placed back into the environmental control chambers for 24 h prior to testing.

For Experiment 2, we used differential conditioning protocols. Moths in this experiment received 6 forward pairings of the conditioning odor (CS+) followed by sucrose and 6 unreinforced trials of a second odor (CS−). Odor stimuli were again undiluted during conditioning. The odor of a pair that was reinforced was counterbalanced. That is, each odor of a given pair was treated as the CS+ for half of the moths in a group and CS− for the other half. This reinforcement counterbalance methodologically controls for salience-dependent differences in subsequent estimates of discrimination thresholds (Daly et al. forthcoming). During conditioning, CS+ and CS− trials were presented in a pseudorandom manner using one of the following 2-trial sequences: "−++−+−−+−++−" or "+−−+−++−+−−+," (where "+" = CS+ and "−" = CS− trials). This ensured the CS+ both preceded and followed the CS− equally often. Six minute intertrial intervals were again used. During CS+ presentation, the CS and US were presented as previously described for single-odor learning. The CS− was presented

for 4 s in separate trials and was not followed by the US. Following conditioning, animals were placed back into the environmental control chamber for 24 h prior to testing.

Surgery and injection procedures

Approximately 10 min prior to testing, the caudal end of the head capsule of each conditioned moth was opened, thus exposing both ALs for injection without having to remove the proboscis and associated musculature. However, it was necessary to reposition the cibarial pump muscle forward for clearer access to the AL. The patch of cuticle with the muscle attached was simply sectioned, then moved forward into the previously opened area, and readhered to the still intact head capsule with super glue. This procedure has been successfully used in previous studies and has no overt effect on the animal's ability to elicit normal feeding behavior (Daly, Christensen, et al. 2004).

The injection procedures are an enhancement of the methods described by Stopfer et al. (1997) and Hosler et al. (2000). As opposed to spritzing topically upon the exposed AL, a sharp quartz intracellular electrode was used to produce a wispy slow-tapering injection probe. The tips of these probes were sheared using fine forceps to produce a relatively larger 10-μm diameter opening. By comparison, PN and LN axons and primary dendrites have diameters of about 3–10 μm (Staudacher EM, unpublished data). The finished injection probe was used to pierce directly into the center of the AL and pressure inject using a General Valve Pico SpritzerII.

The use of relatively sturdier quartz glass made it possible to pierce the protein sheath surrounding the AL without removing it. The narrow tip minimized damage to the AL, whereas the slow-tapering shaft provided consistent calibration across repeated uses, even when minor chipping of the tip occurred. Each probe was calibrated to produce a standard droplet volume estimated at approximately 2 nl, using consistent injection pressure of 20 psi and varying injection time. Calibration was accomplished by injecting into a mineral oil pool and measuring the diameter of the droplet sphere under a dissecting scope. Additionally, after the injection of each animal, the probe was again tested in this manner to confirm calibration. If an injection probe was found to be clogged or out of calibration, that animal was disqualified from the study and the probe replaced. It should be noted that this method also differs from Waldrop et al. (1987) and Christensen et al. (1998) who superfused at 13 psi for up to 5 min in desheathed and isolated brains. These studies also used an approximately 60-μm diameter multibarrel pipette with individual barrel inner diameters of approximately 20 μm. Thus, the method implemented here should be less intrusive because it produced a far smaller entrance hole, it leaves the protective sheath intact, and it delivered a far smaller bolus.

Control moths were injected with physiological saline solution containing (in mM): 150 NaCl, 3 CaCl$_2$, 3 KCl, 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid buffer, and 25 sucrose, pH 6.9 (Christensen et al. 1993). Treatment groups were injected with either 100 μM PCT or 2000 μM BMI diluted in this same stock physiological saline. This PCT concentration was chosen because it has been shown to abolish inhibitory GABAergic feedback in locusts as well as honey bees (Stopfer et al. 1997). On the other hand, the BMI concentration used was one order of magnitude above the concentration used in previous *M. sexta* studies (Christensen et al. 1998). Here we injected a far smaller volume at higher concentration in an effort to optimize the effect of BMI while minimizing the effects of the injection procedure.

Experiment 1: The effect of GABA$_A$ blockade on the generalization of a CR

The general sequence of procedures for Experiment 1 is depicted in Figure 1A and a schematic of the experimental design is shown in Figure 1B. Experimental and control groups were conditioned, using the single-odor learning protocols, 24 h prior to testing. For this experiment, OCT was used as the CS. Prior to testing, the ALs were surgically exposed and injected with either saline or a combination of saline and drug, then tested with undiluted CS (OCT), a molecularly similar odor (S; HEX), and a dissimilar odor (D; MES). Odors were presented in random sequences; this general method is consistent with prior published reports (Hosler et al. 2000; see also Stopfer et al. 1997).

A total of 240 moths were used in the current experiment. As shown in Figure 1B, 120 moths were used for each drug experiment; half were injected with saline and the other half with drug (either BMI or PCT). Injection and testing were performed by 2 researchers, one performed the injections and the other performed testing. The tester was blind to the specific injection treatment that the moths received.

Experiment 2: The effect of GABA$_A$ blockade on discrimination thresholds

The conditioning and injection protocols for Experiment 2 are depicted in Figure 2A and based on the differential conditioning protocols. Testing in this case was with both CS+ and CS− odors in separate test trials. Whereas moths were conditioned using undiluted odors to optimize learning, during testing the dilution series of the CS+ and CS− were used in order to identify the lowest concentration in the series that could elicit a differential CR to the CS+ and CS− and to establish differential concentration-response functions. The CS+ and CS− odors were randomly presented at each concentration in the series; however, concentrations were always systematically presented from low to high to minimize extinction and adaptation effects.

A total of 720 moths were used in this experiment. Figure 2A is a schematic of the basic experimental design and Figure 2B shows the distribution of moths across conditions. For
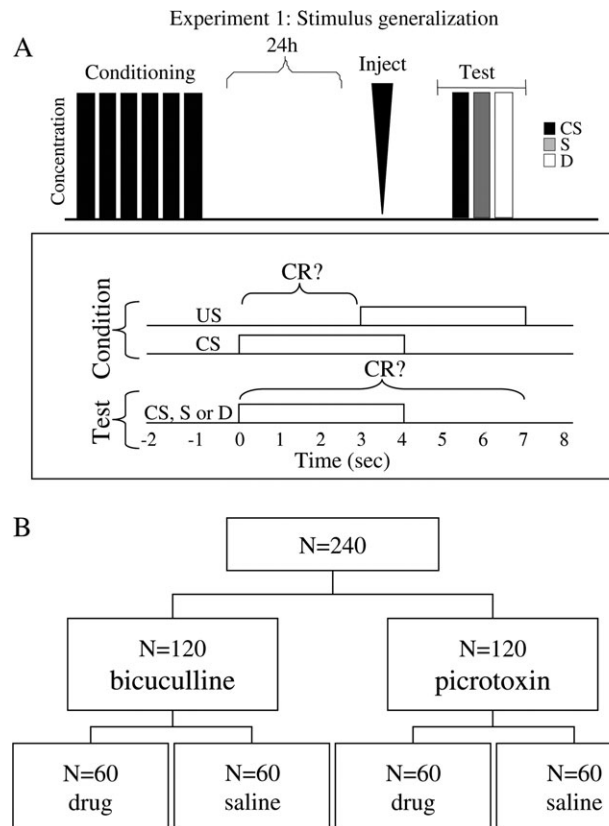
Figure 1 **(A)** Schematic of the experimental protocols used for Experiment 1. Moths were conditioned across 6 trials using undiluted odor, held for 24 h, injected, and tested with the CS, S, and D odors. Inset (below) shows the temporal sequence of the CS and US for each conditioning (top) and test (bottom) trial. Note that the amount of time available for the moth to elicit a CR varied between conditioning (3 s) and test (7 s) trials. Also note that the test sequence of CS, S, and D was randomized across individuals. **(B)** A flow chart that describes the design of Experiment 1. Listed for each treatment are the numbers of moths used (*N*), the drug used, and the type of injection (i.e., whether they were injected with the drug in the saline vehicle or just the saline vehicle).

each of 3 odor pair and drug treatment combinations, we used 240 moths. The first 2 groups were conditioned and tested with OCT/HEX (molecularly similar odors) or LOL/MES (molecularly dissimilar odors). In these first 2 groups, those injected with drug received BMI. To confirm the effects of BMI, a third group was differentially conditioned with LOL/MES and injected with PCT. In this case, we wanted to confirm effects on discrimination of molecularly different odors specifically. Of the 240 moths in each of the 3 conditions, 120 were injected with saline alone and the other 120 with injected with drug (either BMI or PCT). In addition, 60 of each group of 120 moths were conditioned with one of the 2 odors of a pair as CS+ and the second half were conditioned with the second odor as the CS+. Thus, this provided a reinforcement counterbalance so that all odors used in this experiment were treated equally as the CS+ and CS−. Finally, the tester was always blind to the specific injection treatment that each moth received.

Experiment 3: The effect of GABA$_A$ blockade on detection thresholds

The protocols for the third experiment are described in Figure 3A. Here, the single-odor learning protocols were

used as described above, and moths were again held for 24 h prior to surgery, injection, and testing. Whereas in Experiment 1, moths were tested with the CS, S, and D odors in a randomized manner, here moths were tested with a log step increase in concentration of the CS beginning with blank and sequentially increasing the concentration. This test procedure makes it possible to establish an estimate of the detection threshold for a given odor (Daly et al. 2007). A total of 720 moths were used in the experimental design shown in Figure 3B. For each of the 4 odors (OCT, HEX, LOL, MES), 120 moths were used in combination with BMI injection. In addition, for comparative purposes we replicated 2 of the odor groups (LOL and MES), this time injecting PCT. As before, 60 of each group were injected with saline, whereas the other 60 were injected with drug. As before, the tester was always blind to the treatment given.

Posttest assessment of feeding response

Finally, a total of 90 moths each from the above saline, BMI, and PCT groups were presented with sucrose upon the proboscis immediately after odor testing to quantify the presence of an unconditioned feeding response. This allowed us to
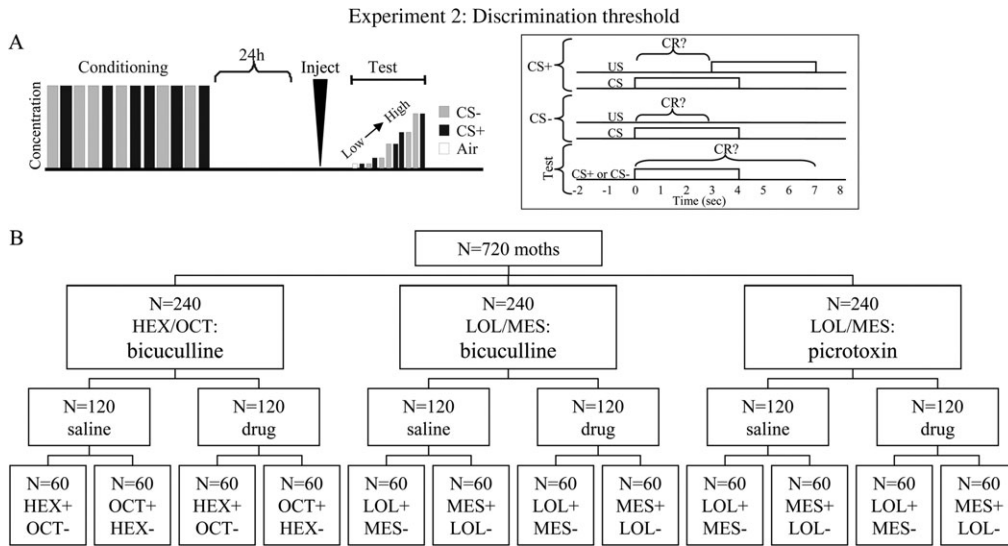
Figure 2 **(A)** Schematic of the experimental protocols used for Experiment 2. Moths were differentially conditioned across 6 CS+ and 6 CS− trials using undiluted odor stimuli, held for 24 h, injected, and tested across a 5 log step dilution series of both the CS+ and CS−. Inset (right) shows the temporal sequence of the CS and US for the CS+ conditioning (top), CS− conditioning (middle), and test (bottom) trials. Note that the amount of time available for the moth to elicit a CR varied between conditioning (3 s) and test (7 s) trials. The test sequence of CS+ and CS− within a concentration was randomized across individuals, but concentration was always presented from low to high to minimize extinction and adaptation effects. **(B)** A flow chart that describes the design of Experiment 2. Listed for each treatment are the numbers of moths used (N), the odor combination, the drug used, and the type of injection (i.e., whether they were injected with the drug in the saline vehicle or just the saline vehicle). In addition, whether the odor was reinforced or not reinforced is indicated by + or −, respectively.
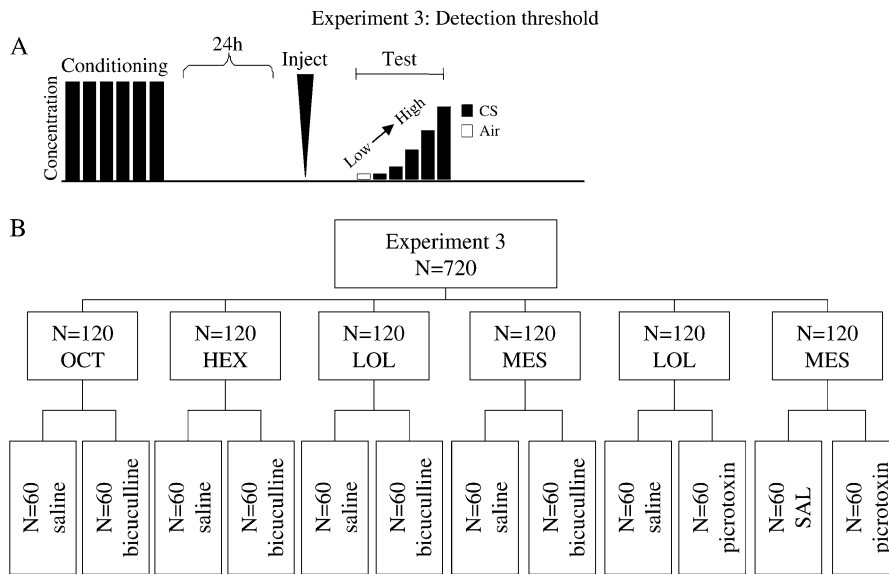

Figure 3 **(A)** Schematic of the experimental protocols used for Experiment 3. Moths were conditioned across 6 trials using high concentration, held for 24 h, injected, and tested across a 5-log step dilution series of the CS. Concentration was always presented from low to high to minimize extinction effects. Note that the sequence of events for conditioning and testing trials was the same as described in the inset for Figure 1A. **(B)** A flow chart that describes the design of Experiment 2. Listed for each treatment are the numbers of moths used (N), the odor combination, the drug used, and the type of injection (i.e., whether they were injected with the drug in the saline vehicle or just the saline vehicle). In addition, whether the odor was reinforced or not reinforced is indicated by + or , respectively.

establish whether the effects of PCT and BMI were attributable to an effect on the ability to elicit a feeding response.

Response measures

Behavioral response measures used here have been established and detailed elsewhere (Daly and Smith 2000; King et al. 2004). Briefly, measures were based on changes in the rate of EMG activity from the feeding muscle and/or extension of the proboscis. The EMG signal of the activated cibarial pump and associated feeding muscles typically ranges up to approximately 6 mV. This size signal is easily amplified and discernable from background noise. Typically,

there is little or no spontaneous EMG signal prior to conditioning or testing. Change in EMG is indicated visually via an oscilloscope and by an audible change in amplified EMG signal output through a loud speaker. Subjects were scored based on an increase in feeding behavior upon presentation of the odor. During conditioning trials, any increased feeding activity (as indicated by an increase in EMG activity or extension of the proboscis) during CS presentation but prior to US presentation was recorded as a CR for that trial; this was used to index acquisition of the CR. During test trials, a 7-s period was used to score behavioral responses; these data were used to assess the effects of treatment on generalization, discrimination, and detection thresholds. In the final control experiment, any EMG activity and/or proboscis extension during sucrose application was recorded as a response.

Analysis

In Experiment 1, our goal was to quantify the relative differences in percentage of moths eliciting a CR when tested with the CS versus the S and D odors as a function of drug treatment. To replicate previously published analysis in honeybee (Hosler et al. 2000; Stopfer et al. 1997), we used 1-tailed paired t-tests, performed in Excel, specifically to compare differences in the percentage of moths eliciting a CR between CS and S and CS and D under saline, versus drug treatments. In this case, because only a limited number of comparisons were made (4 per drug group), a significance level of $P < 0.05$ was used for individual comparisons.

In Experiment 2, the goal was to measure the effect of $GABA_A$ blockade on discrimination thresholds. To achieve this, we first established a number of variables to explain the variation in the percentage of moths eliciting a CR. The most important variable in any study of odor discrimination is the effect of differential reinforcement. Here reinforcement was treated as a categorical variable to indicate whether an odor was used as the CS+ or the CS−. Next, concentration was treated as a categorical variable ranging from 0 (air only) to 10 μg/2 μl. Odor pair was also a categorical variable indicating the molecular similarity of the odors used within a given group, similar (OCT/HEX) or dissimilar (LOL/MES); note that the odor effect was nested within odor pairs in the analysis. Finally, treatment (drug vs. saline) and drug (BMI vs. PCT) were also treated as categorical variables. General linear modeling (GLM) analysis was used to analyze variation in the percentage of moths eliciting a CR as a function of the variables described above. The significance threshold for all effects in the model was set to ($P < 0.01$) to reduce experimentwise error rate and at the same time reduce the number of significant effects that explain a nominally small amount of variance. However, given the number of post hoc comparisons in this experiment, a Tukey's HSD post hoc analysis was implemented to adjust the overall significance level to $P < 0.05$.

The goal of Experiment 3 was to establish whether detection thresholds were affected by $GABA_A$ blockade. Here again concentration, odor, and drug treatment were categorical independent variables that were coded in the same manner as in Experiment 2. GLM was also used to analyze variation in percentage of moths eliciting a CR as a function of these variables.

In the final control experiment where we assessed differences in unconditioned response as a function of treatment, 1-tailed paired t-tests were performed in Excel, specifically to compare differences in feeding response between saline, BMI, and PCT treatments. Finally, in all experiments, all possible 2- and 3-way interactions were tested. As in Experiment 2, significance thresholds for all effects in all models were set to $P < 0.01$ and Tukey's HSD post hoc analysis was implemented with a $P < 0.05$.

Results

Experiment 1: the effect of $GABA_A$ blockade on stimulus generalization

As mentioned previously, honeybee studies have demonstrated that $GABA_A$ blockade increases generalization from CS to S but not D odors. The aim of Experiment 1 was to assess the generality of these findings using a comparative model (*M. sexta*) while at the same time expanding the number of $GABA_A$ antagonists and the number of odor combinations for which this effect has been documented.

Figure 4A displays the percentage of moths eliciting a CR in the 3 s prior to US presentation during the conditioning phase of the experiment (i.e., prior to injection). Data are presented for both the saline control groups and the drug-treated groups. Results are averaged across BMI and PCT treatments (see Figure 1B). In both cases, saline- and drug-treated groups acquired CR's at the same rate across the 6 conditioning trials. Figures 4B and C display the percentage of moths eliciting a CR to the CS, S, and D odors in the postinjection test phase for the PCT and BMI drug treatments, respectively. Also displayed are the responses from the matched saline controls for each drug treatment. Results of the specific statistical comparisons are inset to highlight the pattern of effects. In general, saline-treated moths elicited a CR significantly more to CS than they did to S and D odor ($P < 0.05$), suggesting that they perceived both the S and D odors as distinct from the CS. On the other hand, PCT- (Figure 4B) and BMI (Figure 4C)-treated moths failed to differentially respond to the CS and S odors, as indicated by a nonsignificant difference between mean CR for these stimuli ($P > 0.05$). This suggests that discrimination of molecularly similar odors had been disrupted. By comparison, generalization from the CS to D was still significant in both PCT- (Figure 4B) and BMI (Figure 4C)-treated groups ($P < 0.05$). This later result suggests that moths are still able to discriminate molecularly different odors when $GABA_A$ function is blocked.
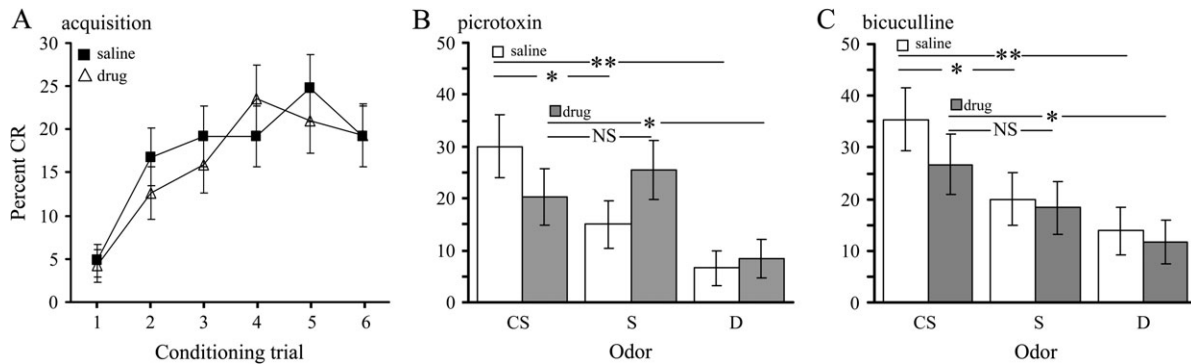
Figure 4 Results of Experiment 1: CS generalization. **(A)** Acquisition of a CR to the CS across the 6 conditioning trials. **(B, C)** Mean percentage of moths eliciting a CR as a function of odor (CS, S, and D), saline versus drug injection. Results separated by PCT (B) versus BMI (C). Results of specific post hoc comparisons using 1-tailed paired t-tests are inset (* $P < 0.05$; ** $P < 0.01$; NS, $P > 0.05$). Error bars indicate standard error. In both (B, C), the response probability for saline-treated moths decreases as the similarity of the test odor to CS decreases. Note that difference in the percentage of moths eliciting a CR for only CS to S becomes insignificant as a result of PCT and BMI treatments, respectively.

Experiment 2: the effect of GABA$_A$ blockade on discrimination thresholds

Experiment 1 results suggest that GABA$_A$ blockade affects discrimination between similar but not dissimilar odors. This leads to the expectation that when GABA$_A$ is blocked, moths that have been differentially conditioned to a CS+ and a CS− odor should only be able to produce a differential CR to these odors when they are molecularly unrelated.

Figure 5 displays the acquisition of the differential CR across the differential conditioning phase of the experiment for the BMI-injected and associated control groups. Data are presented for similar (Figure 5A$_i$ and A$_{ii}$) and dissimilar (Figure 5B$_i$ and B$_{ii}$) odor pairs. Note first that the percentage of moths eliciting a CR to the CS+ tends to increase across trials, whereas in response to the CS− the percentage trends downward; this pattern is consistent with the interpretation that the moths are learning to differentially respond to the CS+ and CS−. Second, note that corresponding drug and saline groups perform approximately equivalently prior to actual injection. Finally, the degree to which the CS+ and CS− elicit a differential CR is clearly greater for the dissimilar odors (Figure 5B$_i$ and B$_{ii}$) relative to the similar odors (Figure 5A$_i$ and A$_{ii}$). This indicates that the dissimilar odors were relatively more easily discriminated.

The overall statistical model explaining variation in the percentage of moths eliciting a CR for 24 h postconditioning test phase of Experiment 2 was significant ($P < 0.0001$). The main effects of CS and odor concentration were significant ($P < 0.0001$). Furthermore, their interaction was also significant ($P < 0.0001$). This significant interaction indicates that the ability to differentially respond to the CS+ and CS− was concentration dependent. Figure 6A shows the percentage of moths eliciting a CR as a function of differential reinforcement and concentration. This figure indicates that at lower concentrations there was no difference in the percentage of moths eliciting a CR in response to the CS+ and CS−. However, as concentration increased, response to CS+ increased

significantly over that of CS−; we term this systematic divergence between the CS+ and CS− the differential concentration-response function (Daly et al. forthcoming).

In addition, we found a significant main effect of treatment ($P < 0.0001$), which was also dependent on concentration as indicated by the significant treatment by odor concentration interaction ($P = 0.0006$). Figure 6B displays the percentage of moths responding to odor with a CR as a function of odor concentration and whether the moths were injected with drug or saline. This figure indicates that moths were more likely to elicit a CR when injected with saline than when injected with BMI or PCT, but this was only at odor concentrations at or above 0.1 µg/2 µl. These results suggest that the effect of GABA$_A$ blockade may in part be a disruption in ability to detect odor. This effect may also indicate a possible disruption of the moths' ability to elicit a feeding response. These possibilities are assessed in Experiment 3.

There was also a significant 3-way interaction of reinforcement by concentration by treatment ($P < 0.0001$). Figure 7 displays differential concentration-response functions by drug treatment. Figure 7A, for example, establishes a baseline differential concentration-response function in the saline-injected moths that can be compared with the drug-injected moths (Figure 7B). Note here that the degree to which the CS+ and CS− diverge as a function increasing concentration is far greater in the saline-injected group relative to the drug-injected group. This indicates specifically that the drug treatment has disrupted discrimination. Note that moths begin to significantly differentially respond to the CS+ and CS− at 0.1 µg/2 µl when saline injected, whereas in the drug-treated moths, a significant differential response does not occur until the highest concentration in the range.

Although the above effects indicate that GABA$_A$ blockade increased the concentration required to discriminate between odors, the main effect of which drug was injected was not significant ($P = 0.2075$). This indicates that both BMI and PCT had the same effects on this measurement of
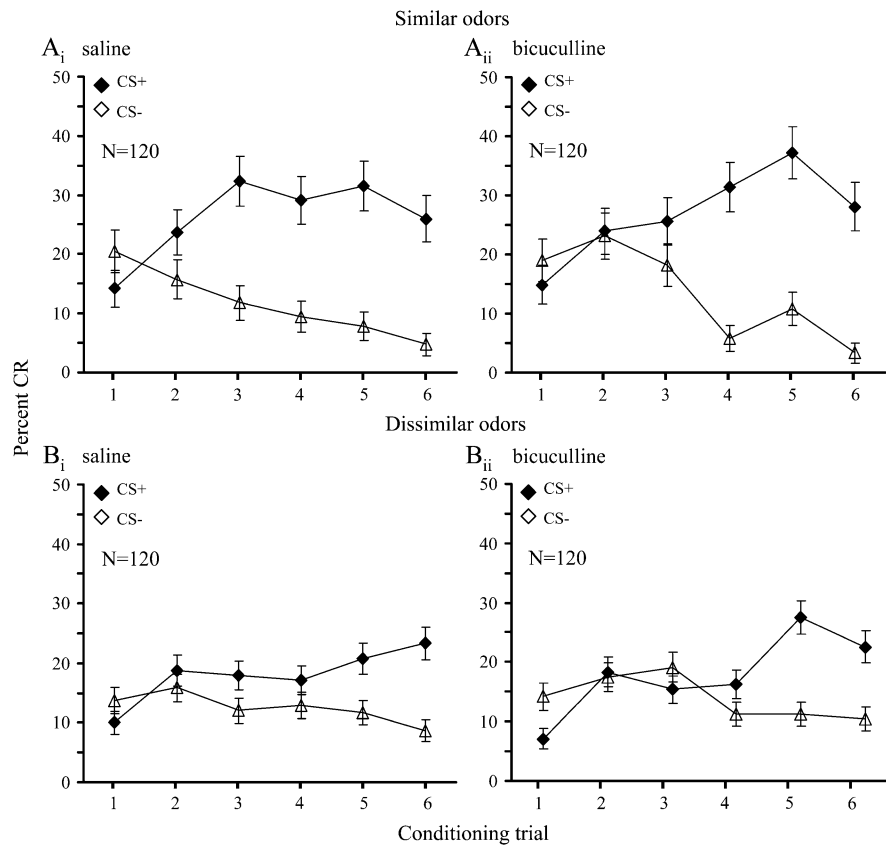
Figure 5 Acquisition of a differential CR to the CS+ and CS− odors across successive conditioning trials. Results are separated by both molecularly similar **(A)** and different **(B)** odor pairs and by saline- (i) versus BMI-injected (ii) groups. Error bars indicate standard error.
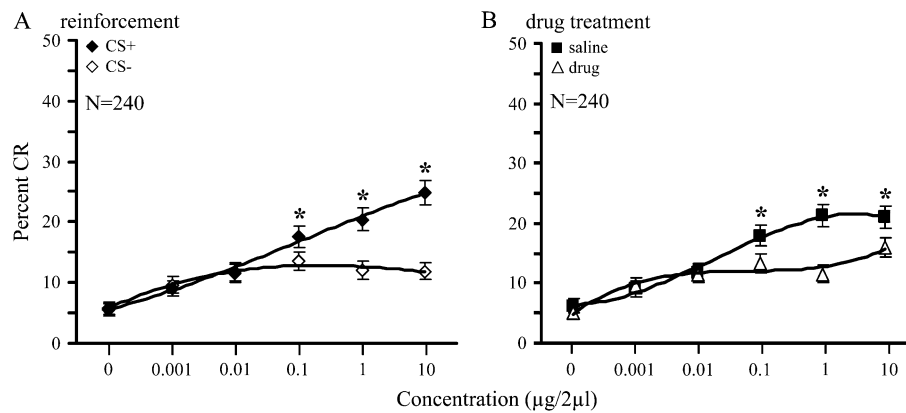

Figure 6 Mean percentage of moths eliciting a CR as a function of the 2-way interaction of concentration by reinforcement **(A)** and concentration by treatment (saline vs. drug injection; **B)**. (A) Data are averaged across treatment (saline and drug) and drug group (BMI and PCT). Note that as concentration increases, there is an increase in the percentage of moths eliciting a CR in response to the CS+ relative to the CS−. (B) Data are averaged across reinforcement (CS+ and CS−) and drug group (BMI and PCT) to show differences between saline-injected versus drug-injected moths. Significant post hoc comparisons are inset (*$P <$ 0.05). Error bars indicate standard error. Note that as concentration increases, saline-treated moths elicit a significantly higher percentage of moths eliciting a CR than drug-treated moths.

discrimination. In fact, there were no significant interactions between which drug was used and any other main effect. This indicates that BMI and PCT had essentially the same impact on olfactory function.

Finally, neither the main effect of the odor pair nor the interaction of odor pair with the other effects in the model was significant ($P >$ 0.05; note that this was the ad hoc significance threshold). In particular, the lack of a significant
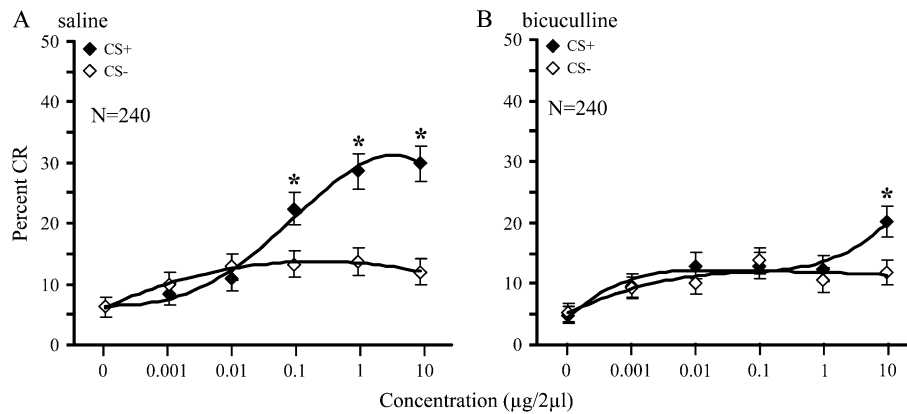
Figure 7 Mean percentage of moths eliciting a CR as a function of the 3-way interaction of reinforcement by concentration by drug treatment. Results are averaged across drug group (BMI and PCT) and separated by saline **(A)** and drug injected **(B)**. Significant post hoc comparisons are inset (*$P < 0.05$), and error bars indicate standard error. Note that the 3-way interaction is evidenced by the distinct divergence of CS+ and CS− beginning at 0.1 μg/μl in saline-treated moths in the saline-injected moths (A) that does not occur until the highest concentration in drug-treated moths (B).

4-way interaction of reinforcement by concentration by treatment by odor pair ($P = 0.8852$) indicates that while GABA$_A$ disruption affected discrimination, it did so equally for both similar and dissimilar odor pairs, this is shown in Figure 8. Figure 8 displays differential concentration-response functions for BMI- versus saline-injected groups. These differential concentration-response functions are also separated by molecularly similar (Figure 8A$_i$ and A$_{ii}$) or different (Figure 8B$_i$ and B$_{ii}$) odor pairs. Notice that for both similar and different odor pairs, when saline was injected, the discrimination thresholds were identified statistically at 0.1 μg/μl. In the BMI-injected moths, however, a discrimination threshold was not observed until the highest concentration. This 2 log step increase in discrimination threshold was present for both similar and different odor pairs, indicating that discrimination thresholds increased by roughly equal amounts for both. This is in contrast to Experiment 1 where drug injection resulted in the loss of differential CR between the CS to the S odors only.

To confirm that discrimination of molecularly different odors was affected equally using a second GABA$_A$ antagonist, we replicated the results for the molecularly different odors, this time using PCT. These results are shown in Figure 9 in the same format as Figure 8 and are entirely consistent with the BMI results.

Experiment 3: detection thresholds

The significant interaction of concentration by treatment in Experiment 2 (see Figure 6B) suggested that detection thresholds might have increased as a function of GABA$_A$ disruption. Subsequently, a drug-mediated increase in detection threshold may result in increased discrimination thresholds. Previous studies have established that discrimination thresholds occur at least one order of magnitude above detection thresholds (Daly et al. 2007, forthcoming). Thus, if detection thresholds increased as a function of GABA$_A$

blockade, then it stands to reason that discrimination thresholds should increase as well. We, therefore, quantified the effects of BMI and PCT on detection thresholds and concentration-response functions.

Figure 10A displays the acquisition of a CR for the saline-versus drug-injected animals and establishes that both groups learned equally well. The overall statistical model explaining variation in the percentage of moths eliciting a CR for Experiment 3 was significant ($P < 0.0001$). We found that there were significant main effects of concentration and treatment ($P < 0.0001$) but no significant effect of which odor ($P = 0.3977$) or drug ($P = 0.7428$) was used. Importantly, the interaction of concentration by treatment was significant ($P < 0.0001$). Figure 10B displays concentration-response functions (averaged across all 4 odors), for both saline- and drug-treated moths (averaged across both drugs). This figure indicates that across all odors, GABA$_A$ blockade (either BMI or PCT) increases detection thresholds relative to saline controls.

An alternative hypothesis is that GABA$_A$ blockade somehow disrupted the ability of the moths to produce a behavioral response. To test this hypothesis, we provided sucrose to the proboscis immediately following the final odor test for a total of 90 moths per condition. If moths were less able to respond to sucrose, this would support the alternative hypothesis that underlying the increased detection threshold measure was an inability to produce the behavior and thus not attributable to changes in detection per se. Figure 10C displays the percentage of moths eliciting an unconditioned response to sucrose presentation upon the proboscis. t-tests of differences between the saline and each drug treatment indicate that PCT and BMI had no significant effect on the moths' ability to feed/respond ($P > 0.05$).

Discussion

As we learn more about primary olfactory networks in both vertebrates and invertebrates, it is becoming increasingly
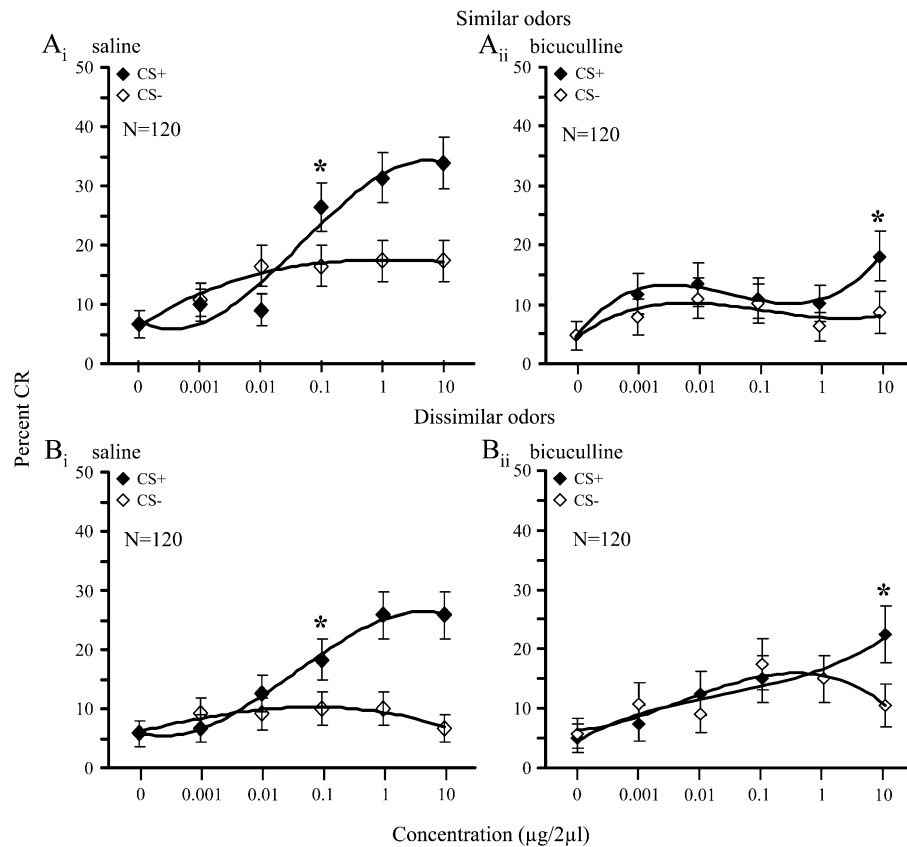
Figure 8 Mean percentage of moths eliciting a CR as a function of the 3-way interaction of reinforcement by concentration by treatment for BMI data only. Data are separated into 2 rows representing molecularly similar (OCT/HEX; **A)** and different (LOL/MES; **B)** and columns representing saline (i) versus BMI (ii) injection groups. Discrimination thresholds, the lowest concentration at which the CS+ and CS− elicit a significantly different CR ($P < 0.05$) are indicated by the inset asterisk. Error bars indicate standard error. Note that the CS+ and CS− statistically diverge at 0.1 μg/μl for saline-injected moths (A_i, B_i) and at 10 μg/μl for the BMI-injected moths (A_{ii}, B_{ii}). This effect was consistent for similar and dissimilar odors.

clear that the complexity of the output of these systems is far greater than a simple linear transformation of olfactory input. In this purely linear view, local processing would serve only to sharpen the olfactory input–output pathway via a contrast enhancement mechanism (Shepherd and Firestein 1991). Given this changing perspective, it should not be surprising that the disruption of the local GABAergic network within the AL has multiple effects at the level of sensory perception. In agreement with previous research, we have shown that fine odor discrimination is disrupted across two GABA$_A$ antagonists and a different combination of closely related and unrelated odors as measured in a stimulus generalization paradigm. In addition, when a more comprehensive set of measures of olfactory function was implemented, it became clear that GABA$_A$ disruption affects not only discrimination of molecularly closely related odors, rather discrimination was generally disrupted.

We furthermore observed that odor detection thresholds were increased, meaning that moths had a more difficult time detecting and odor signal. These effects were consistent across 4 different odors and 2 GABA$_A$ antagonists, which affect GABA$_A$ function via different mechanisms.

GABA$_A$ blockade increases CR generalization to odors that were similar but not dissimilar to the CS

In agreement with previous studies, we find that when implementing a stimulus generalization paradigm, generalization of a CR is increased to molecularly closely related odors only when GABA$_A$ blockers are applied to the AL. In a previous report by Stopfer et al. (1997), topical application of PCT to honeybee ALs prior to conditioning produced a disrupted memory template of the CS odor. This resulted in increased generalization of closely related odors. In another study by Hosler et al. (2000), it was established that PCT can be applied either before conditioning or before testing and the same results occur. This suggests, in both cases, whether the memory template is accurate, but the test odor images are disrupted or vice versa, the end result is the same, only fine odor discrimination is disrupted.

GABA$_A$ blockade impairs discrimination of similar and dissimilar odor pairs in a discrimination threshold paradigm

We have previously established methods for psychophysically quantifying discrimination thresholds in *M. sexta* as
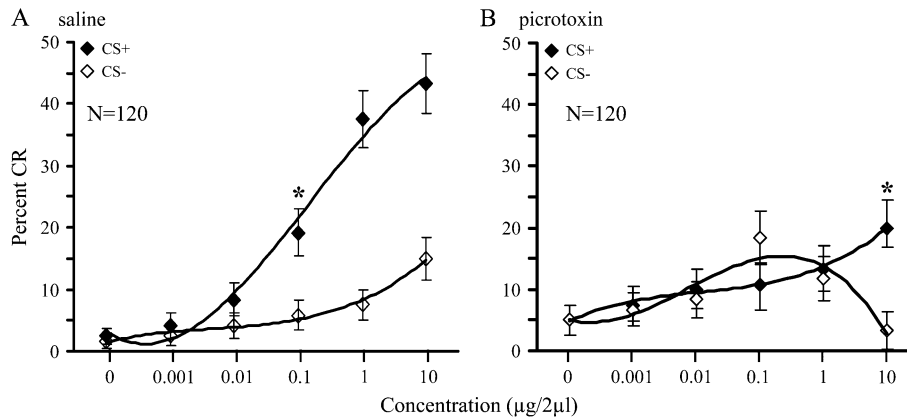
Figure 9 Mean percentage of moths eliciting a CR as a function of the 3-way interaction of reinforcement by concentration by treatment for saline **(A)**-versus PCT-injected **(B)** moths. Note, in this case, we only assessed effects on molecularly different odors (LOL/MES) to confirm that discrimination of different odors was also affected. Discrimination thresholds, the lowest concentration at which the CS+ and CS− elicit a significantly different CR ($P < 0.05$), are indicated by the inset asterisk. Error bars indicate standard error. Consistent with BMI data (see Figure 8), there is a lack of divergence in the percentage of moths eliciting a CR between the CS+ and CS− as concentration increases for PCT-injected moths indicating a disruption in ability to discriminate these odors.
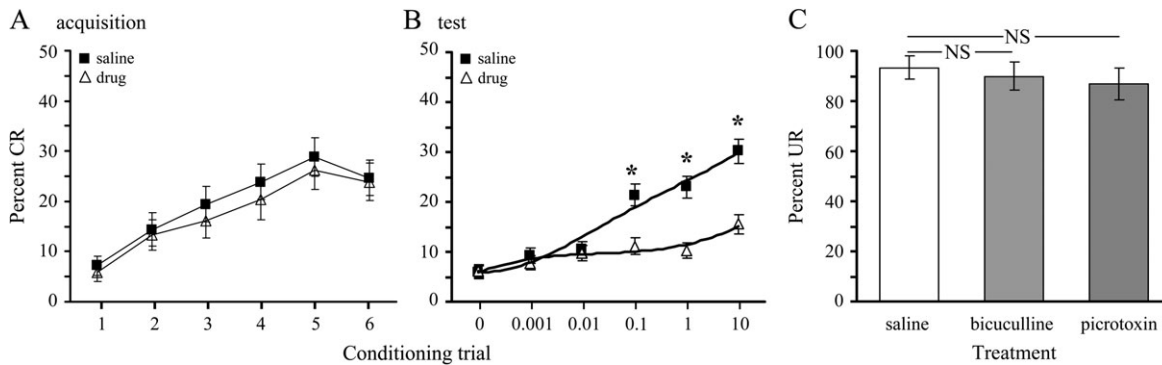

Figure 10 Acquisition and posttest results for the detection threshold experiments. **(A)** The percentage of moths eliciting a CR during the acquisition phase of the experiment for both saline- and drug-injected groups. Data are averaged across all odors because there were no significant differences in acquisition between odor groups ($P > 0.05$). **(B)** Mean percentage of moths eliciting a CR as a function of the 2-way interaction of concentration by treatment. These data are averaged across drug group (BMI and PCT) and odor (OCT, HEX, LOL, and MES), again because their variables did not yield significant effects ($P > 0.05$). Significant post hoc comparisons between saline and drug treatments for each concentration are inset (*$P < 0.05$). Error bars indicate standard error. Note that as concentration increases, the percentage of moths eliciting a CR increases in saline- relative to drug-treated moths. **(C)** Mean posttest percent unconditioned feeding response upon presentation of sucrose solution (US) to the proboscis. Results of specific post hoc comparisons using 1-tailed paired t-tests are inset and indicated by (NS) for nonsignificant effects ($P > 0.05$). Error bars indicate standard error.

well as differential concentration-response functions (Daly et al. forthcoming). Differential concentration-response functions describe the rate at which the differential response to the CS+ and CS− diverge as a function of increasing concentration. Using this method, we can determine with relative precision the degree to which moths can discriminate between odor pairs as a function of increasing task demands (sic decreasing odor concentration). Consistent with our previous findings, we observe that the ability to differentially respond to the CS+ and CS− systematically increased with increasing concentration.

By comparison with saline-injected controls, we could not detect differences in the effect of GABA$_A$ blockade on either

differential concentration-response functions or discrimination threshold measures between the closely related and unrelated odor pairs. That is, the effect of GABA$_A$ blockade was independent of the similarity of the odor pairs. This was evidenced by a 2 log step increase in the concentration necessary to elicit a significant differential CR to the CS+ and CS− for both similar and dissimilar odor pairs. This 2 log step increase in concentration required to discriminate between odors that was independent of odor similarity implies that the ability to detect differences in odor identity has been generally affected. The finding that discrimination of dissimilar odor pairs was disrupted is in contrast to those observed using the generalization paradigm where

discrimination of unrelated odors persists. Again, it is worth highlighting that this finding was consistent across 2 GABA$_A$ antagonists.

How can these differing results be reconciled? The difference in results, in our opinion, can be attributed to differences in the effectiveness of the 2 experimental methods at resolving changes in discrimination between molecularly different odors. As mentioned, generalization paradigms test whether a novel stimulus "X" is perceived to be similar to another stimulus "Y," which has been previously associated with reward. Assumed in this case is that there is a theoretical dimension that we can term "relatedness" that any 2 stimuli can be measured on. A differential conditioning task, on the other hand, specifically establishes a differential reward structure whereby X is rewarded and Y is not. In this case, prior conditioning experiences with both X and Y results in a differential CR. Thus, even closely related odors, so long as they can be discriminated, will come to elicit a differential CR. This is true in cases where a generalization paradigm may show a high degree of generalization or no generalization. Thus differential conditioning more explicitly assesses the ability to discriminate stimuli. In the case of the discrimination threshold paradigm, the animal must differentially respond to X and Y under the increased task demand of lowered odor concentration. The discrimination threshold method therefore represents the most sensitive measure of discrimination relative to standard differential conditioning or generalization methods.

In the current study, we used undiluted stimuli in the generalization experiment; this is consistent with previous studies in honeybee. In this case, we propose that the task demands are sufficiently low that even if discrimination of molecularly different odors were affected by GABA$_A$ disruption, as we show in Experiment 2, it would not necessarily yield a measurable effect between CS and D in a generalization paradigm. This is simply because D is sufficiently different that it is still perceived as not like the CS. For example, Figure 11 is a theoretical generalization gradient that describes the relatedness of the CS, S, and D odors under normal and GABA$_A$-impaired conditions. This figure illustrates how D can be sufficiently different from the CS, that when GABA$_A$ is disrupted D will not have moved up the slope of the gradient. In this particular case, the effect of GABA$_A$ blockade on S and D are equal. Yet as we postulate in Figure 11, the only observable effect of GABA$_A$ blockade will be on the S odor specifically in the generalization paradigm. Again, this model assumes only that there is a perceptual dimension, relatedness, and as S moves toward the CS along this dimension, it moves up the CS generalization gradient but as D moves toward the CS it does not. It is also worth mention that, in principle, the CS could also be shifted from the peak of the gradient under the experimental conditions we used.

Interestingly, comparative psychophysical studies in rats suggest that the effectiveness of the generalization paradigm
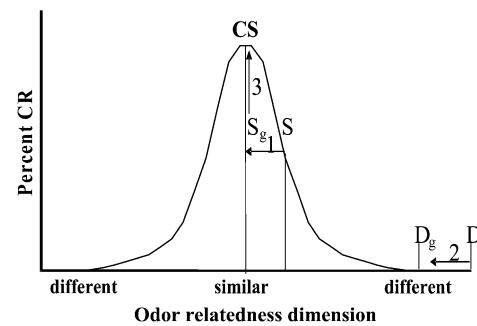

Figure 11 A theoretical generalization gradient showing the probability of a CR for the conditioning odor (CS), a molecularly similar odor (S), and a dissimilar odor (D). Note that the x axis represents an odor relatedness dimension where the CS and S are more related to each other than to D. S$_g$ and D$_g$ represent theoretical change in positions on the x axis of S and D, respectively, under conditions of GABA impairment. Numerals 1 and 2 indicate the magnitude of the theoretical effect of drug treatment for S (1) and D (2) odors relative to the CS, and in this case, they are equal. Numeral 3 indicates the theoretical increase in the percentage of moths eliciting a CR in response to S as a result of moving toward the CS on the x axis. This movement on the y axis represents an increase in generalization. Note that the prediction of this model is that although the perceptual similarity of CS and D has been affected equally to CS and S, there is no increase in generalization.

to quantify the perceptual relatedness between odors is far narrower than other behavioral methods such as differential conditioning (Cleland et al. 2002). The implication is that stimulus generalization paradigms are less able to quantify the degree of perceptual relatedness of unrelated odors; this is consistent with our findings. On the other hand, other studies have looked more carefully at subtle differences between 2-DG representations in the rat olfactory bulb (OB) for closely related odor pairs (Linster et al. 2002). This study correlated 2-DG measures of OB activation to behavioral measures of discrimination. Their results also suggest that differential conditioning, not stimulus generalization, provided a greater correspondence to 2-DG measures. Importantly, these studies as well as our own clearly point to the conclusion that the behavioral measure implemented in an experiment can impact the experimental results and, hence, affects the conclusions that are inevitably drawn.

GABA$_A$ blockade increases detection thresholds

Finally, in a previous report, we established that odor detection thresholds can be characterized psychophysically and that these detection thresholds correlate well with matched physiological measures (Daly et al. 2007). By implementing detection threshold methods in drug-treated versus saline control experiments, we show that GABA$_A$ disruption, in turn, disrupts the detection of odor. Obviously, a prerequisite to discrimination of any 2 stimuli is that they are detectable. Typically, moths detect the odors used herein at concentrations one or more orders of magnitude below what is necessary for discrimination (Daly et al. forthcoming). We therefore suggest that if detection thresholds generally

increased, it would follow that discrimination thresholds would also have to increase, though any causal relationship between detection and discrimination remains unknown.

Subsequent assessment of moths' ability to elicit an unconditioned response to the sucrose solution strongly supports the conclusion that the increased detection thresholds cannot be attributed to an inability to respond with a behavioral response. That is, the sensory-motor circuitry responsible for driving the activation and maintenance of feeding behavior, at least in response to gustatory input, remains intact. Furthermore, the fact that higher concentrations of odor stimuli were able to elicit a significant CR suggests that the sensory-motor circuitry involved in driving the olfactory-mediated CR's was at least partially functional.

In conclusion, physiological studies from the sphinx moth, the honeybee, and the fruit fly, indicate that when $GABA_A$ is blocked, the resulting output from primary olfactory networks, among other effects, is increased (Christensen et al. 1998; Sachse and Galizia 2002; Wilson et al. 2004). In spite of the overall increase in output, detection and discrimination thresholds are increased. This, in our opinion, suggests that $GABA_A$ blockade results in the disruption of indirect output pathways from the AL (Christensen et al. 1998), which results in a decrease in the information available to AL's projection fields. At the same time, a loss of inhibitory control within the AL increases noise in the output response; this is manifest by an increase in overall odor-driven PN spike rate. Thus, the salient odor signal, which we presume forms the basis of both detection and discrimination, is effectively lost.

Funding

Acknowledgements

References

Bhagavan S, Smith BH. 1997. Olfactory conditioning in the honey bee, Apis mellifera: effects of odor intensity. Physiol Behav. 61:107–117.

Buck L, Axel R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 65:175–187.

Christensen TA, Waldrop BR, Harrow ID, Hildebrand JG. 1993. Local interneurons and information processing in the olfactory glomeruli of the moth Manduca sexta. J Comp Physiol [A]. 173:385–399.

Christensen TA, Waldrop BR, Hildebrand JG. 1998. Multitasking in the olfactory system: context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. J Neurosci. 18:5999–6008.

Cleland TA, Morse A, Yue EL, Linster C. 2002. Behavioral models of odor similarity. Behav Neurosci. 116:222–231.

Cleland TA, Narla VA. 2003. Intensity modulation of olfactory acuity. Behav Neurosci. 117:1434–1440.

Couto A, Alenius M, Dickson BJ. 2005. Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr Biol. 15: 1535–1547.

Daly KC, Carrell LA, Mwilaria E. 2007. Detection versus perception: physiological and behavioral analysis of olfactory sensitivity in the moth (Manduca sexta). Behav Neurosci. 121:794–807.

Daly KC, Chandra S, Durtschi ML, Smith BH. 2001. The generalization of an olfactory-based conditioned response reveals unique but overlapping odour representations in the moth Manduca sexta. J Exp Biol. 204:3085–3095.

Daly KC, Christensen TA, Lei H, Smith BH, Hildebrand JG. 2004. Learning modulates the ensemble representations for odors in primary olfactory networks. Proc Natl Acad Sci USA. 101:10476–10481.

Daly KC, Durtschi ML, Smith BH. 2001. Olfactory-based discrimination learning in the moth, Manduca sexta. J Insect Physiol. 47:375–384.

Daly KC, Carrell LA, Mwilaria E. Forthcoming. Characterizing Psychophysical Measures of Discrimination Thresholds and the Effects of Concentration on Discrimination Learning in the Moth Manduca sexta Chem Senses. doi:10.1093/chemse/bjm068.

Daly KC, Smith BH. 2000. Associative olfactory learning in the moth Manduca sexta. J Exp Biol. 203:2025–2038.

Daly KC, Wright GA, Smith BH. 2004. Molecular features of odorants systematically influence slow temporal responses across clusters of coordinated antennal lobe units in the moth Manduca sexta. J Neurophysiol. 92: 236–254.

Eaton JL. 1971. Morphology of the head and thorax of the adult tobacco hornworm Manduca sexta (Lepidoptera: Sphingidae). 1. Skeleton muscles. Ann Entomol Soc Am. 64:437–445.

Fan RJ, Hansson BS. 2001. Olfactory discrimination conditioning in the moth Spodoptera littoralis. Physiol Behav. 72:159–165.

Fishilevich E, Domingos AI, Asahina K, Naef F, Vosshall LB, Louis M. 2005. Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila. Curr Biol. 15:2086–2096.

Hosler JS, Buxton KL, Smith BH. 2000. Impairment of olfactory discrimination by blockade of GABA and nitric oxide activity in the honey bee antennal lobes. Behav Neurosci. 114:514–525.

Hosler JS, Smith BH. 2000. Blocking and the detection of odor components in blends. J Exp Biol. 203:2797–2806.

King TL, Horine FM, Daly KC, Smith BH. 2004. Explosives detection with hardwired moths. IEEE Trans Instrum Meas. 53:1113–1118.

Laska M, Galizia CG, Giurfa M, Menzel R. 1999. Olfactory discrimination ability and odor structure-activity relationships in honeybees. Chem Senses. 24:429–438.

Laurent G, Davidowitz H. 1994. Encoding of olfactory information with oscillating neural assemblies. Science. 265:1872–1875.

Laurent G, Stopfer M, Friedrich RW, Rabinovich MI, Volkovskii A, Abarbanel HDI. 2001. Odor encoding as an active, dynamical process: experiments, computation, and theory. Annu Rev Neurosci. 24:263–297.

Leitch B, Laurent G. 1996. GABAergic synapses in the antennal lobe and mushroom body of the locust olfactory system. J Comp Neurol. 372: 487–514.

Linster C, Johnson BA, Morse A, Yue E, Leon M. 2002. Spontaneous versus reinforced olfactory discriminations. J Neurosci. 22:6842–6845.

Matsumoto SG, Hildebrand JG. 1981. Olfactory mechanisms in the moth manduca sexta: response characteristics and morphology of central neurons in the antennal lobe. Proc R Soc Lond B Biol Sci. 213:249–277.

Olsen SR, Bhandawat V, Wilson RI. 2007. Excitatory interactions between olfactory processing channels in the Drosophila antennal lobe. Neuron. 54:89–103.

Sachse S, Galizia CG. 2002. Role of inhibition for temporal and spatial odor representation in olfactory output neurons: a calcium imaging study. J Neurophysiol. 87:1106–1117.

Shang Y, Claridge-Chang A, Sjulson L, Pypaert M, Miesenbock G. 2007. Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. Cell. 128:601–612.

Shepherd GM, Firestein S. 1991. Toward a pharmacology of odor receptors and the processing of odor images. J Steroid Biochem Mol Biol. 39:583–592.

Shipley MT, Ennis M. 1996. Functional organization of olfactory system. J Neurobiol. 30:123–176.

Skiri HT, Stranden M, Sandoz JC, Menzel R, Mustaparta H. 2005. Associative learning of plant odorants activating the same or different receptor neurones in the moth Heliothis virescens. J Exp Biol. 208:787–796.

Stopfer M, Bhagavan S, Smith BH, Laurent G. 1997. Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. Nature. 390:70–74.

Vosshall LB, Wong AM, Axel R. 2000. An olfactory sensory map in the fly brain. Cell. 102:147–159.

Waldrop B, Christensen TA, Hildebrand JG. 1987. GABA-mediated synaptic inhibition of projection neurons in the antennal lobes of the sphinx moth, Manduca sexta. J Comp Physiol [A]. 161:23–32.

Wilson RI, Turner GC, Laurent G. 2004. Transformation of olfactory representations in the Drosophila antennal lobe. Science. 303:366–370.

Wright GA, Skinner BD, Smith BH. 2002. Ability of honeybee, Apis mellifera, to detect and discriminate odors of varieties of canola (Brassica rapa and Brassica napus) and snapdragon flowers (Antirrhinum majus). J Chem Ecol. 28:721–740.