Hygienic Behavior in the Honey Bee (*Apis mellifera* L.) and the Modulatory Role of Octopamine

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ABSTRACT: **Honey bees, *Apis mellifera*, which perform hygienic behavior, quickly detect, uncap and remove diseased brood from the nest. This behavior, performed by bees 15–20 days old and prior to foraging, is likely mediated by olfactory cues. Because the neuromodulator octopamine (OA) plays a pivotal role in olfactory-based behaviors of honey bees, we examined whether bees bred for hygienic and nonhygienic behavior differed with regard to their OA expression and physiology. We compared the staining intensity of octopamine-immunoreactive (OA-ir) neurons in the deutocerebral region of the brain, medial to the antennal lobes, between hygienic and nonhygienic bees (based on genotype and phenotype). We also tested how the olfactory responses of the two lines, based on electroantennograms (EAGs), were affected by oral administration of OA and of epinastine, a highly specific OA antagonist. Our results revealed that bees expressing hygienic behavior (irrespective of genotype) possessed OA-ir neurons that exhibited more intense labeling than same-aged bees not performing the behavior. In bees bred for nonhygienic behavior, OA significantly increased the EAG response to low concentrations of diseased brood odor. Conversely, in bees bred for hygienic behavior, epinastine significantly reduced the magnitude of the EAG response, a reduction not observed in nonhygienic bees. Our results provide two lines of evidence that OA has the potential to facilitate the detection and response of honey bees to diseased brood. We discuss the contributions of OA for behavioral shaping and its ability to bias the nervous system to express one form of behavior over another.** © 2003 Wiley Periodicals, Inc. J Neurobiol 55: 341–354, 2003

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INTRODUCTION

Honey bees perform multiple and complex behaviors at every age, changing the range of their behaviors with time. The most distinct behavioral change observed is the transition from in-hive tasks such as feeding larvae, performed by younger bees, to outside tasks such as foraging, performed by older bees. Our study focuses on a particular behavior, hygienic behavior (Rothenbuhler, 1964), which is performed by bees between 15–20 days of age (Arathi et al., 2000). These bees are "middle aged"; they are older than typical nurse bees and younger than typical foragers. Bees that perform hygienic behavior have the ability to detect, uncap, and quickly remove brood infected with bacterial (*Paenibacillus larvae*) and fungal diseases (*Ascosphaera apis*) from the nest before the

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pathogens sporulate (Woodrow and Holst, 1942; Rothenbuhler, 1964). They also uncap and remove a portion of brood infested with the parasitic mite *Varroa destructor* (Boecking and Drescher, 1992; Spivak, 1996). This behavior limits the spread of infection, and may slow the reproductive potential of mites; thus, colonies that express this behavior are economically important to beekeepers (Spivak and Reuter, 1998, 2001a, 2001b).

Early work on the genetics of the behavior proposed that the trait was controlled in a Mendelian manner by two recessive loci (Rothenbuhler, 1964). More recent studies, however, using molecular techniques and quantitative trait loci (QTLs) suggest there may be at least seven QTLs associated with hygienic behavior, each controlling 9–15% of the total phenotypic variance (Lapidge et al., 2002). Thus, hygienic behavior is probably inherited in a more quantitative manner than once thought. In addition, the expression of the behavior can be variable both on a colony and individual bee level. For example, the rapidity and efficiency of the removal of abnormal brood can be influenced by resource conditions (Thompson, 1964; Momot and Rothenbuhler, 1971) and by the percentage of bees in the colony capable of performing the task (Arathi and Spivak, 2001).

We use the terms "hygienic" and "nonhygienic" to describe opposite extremes of a quantitative behavioral trait that forms a graded continuum. Typically, in a hygienic colony, individual bees uncap and remove abnormal brood very quickly from the nest, such that the colony rarely if ever shows clinical symptoms of a particular disease (Woodrow and Holst, 1942). In a nonhygienic colony, however, individual bees uncap and remove abnormal brood only very slowly from the nest, and fewer bees engage in the behavior, such that pathogens sporulate, and the colony often has clinical symptoms of a given disease (Woodrow and Holst, 1942). It is estimated that only 10% of honey bee colonies in the U.S. (Spivak and Gilliam, 1993) and Australia (Oldroyd, 1996) are hygienic. This percentage can be increased through selective breeding with no apparent costs to the fitness of the colonies (Spivak and Reuter, 2001b).

We hypothesize that hygienic behavior is mediated by olfactory cues: bees may detect the odor of diseased, parasitized, or dead brood under a wax-capped cell, which stimulates them to uncap and remove the cell's contents. The expression of hygienic behavior may depend on the bees' ability to detect and integrate appropriate olfactory cues. It may also depend on the response threshold of bees to attend to or remove those agents releasing such chemical cues. Response threshold models, also used to explain the division of labor within a social insect colony, suggest that individuals encountering different olfactory cues and those with lower detection thresholds for such cues perform tasks specifically associated with those stimuli (reviewed in Bonabeau and Theraulaz, 1999; Bershers and Fewell, 2001). The genetic predisposition for a heightened detection of abnormal brood odors may, therefore, facilitate the expression of hygienic behavior in a colony.

Support for our hypothesis comes from our previous studies, which used electroantennogram recording (EAG) and proboscis-extension "response" (PER) conditioning to study olfactory thresholds in response to abnormal brood odors. We demonstrated that worker bees collected from a colony bred for rapid hygienic behavior exhibited a greater olfactory sensitivity to low concentrations of the odor of pupae infected with chalkbrood, a fungal disease (Masterman et al., 2001). In addition, hygienic bees were able to discriminate between odors of healthy and diseased brood at a lower stimulus level compared to nonhygienic bees (Masterman et al., 2000, 2001).

What physiological factors might mediate such differences in odor detection thresholds and/or responses among hygienic and nonhygienic bees? Because the neuromodulator octopamine (OA) has been shown to enhance the response of bees to olfactory stimuli (Mercer and Menzel, 1982; Hildebrandt and Muller, 1995) and play pivotal roles in olfactory-based behaviors as a reinforcing signal during olfactory learning (Hammer, 1993; Hammer and Menzel, 1995), we examined whether hygienic and nonhygienic bees differed with regard to their OA expression and physiology. Specifically, we compared the staining intensity of octopamine-immunoreactive (OA-ir) neurons in the brains of hygienic and nonhygienic bees (based on genotype and phenotype). We also tested how the olfactory responses (EAGs) in the two lines were affected by oral administration of OA and of epinastine, a highly specific OA antagonist (Roeder et al., 1998). Our results revealed that the two behavioral types of bees did indeed differ: bees expressing hygienic behavior possessed OA-ir neurons that exhibited more intense labeling. In bees bred for hygienic behavior, epinastine significantly reduced the magnitude of the EAG response to abnormal brood odors, a reduction not observed in nonhygienic bees. Conversely, in bees bred for nonhygienic behavior, OA significantly increased the EAG response. Such observed differences highlight the potential contributions of OA for behavioral shaping and its ability to bias the nervous system to express one form of behavior over another.

METHODS

Breeding

The breeding program for hygienic behavior was initiated in 1993 by selecting colonies of Italian-derived *A. mellifera* bees using a freeze-killed brood assay (Spivak and Downey, 1998). Colonies that uncapped and removed freeze-killed brood within 48 h were considered hygienic; those that took over 6 days to perform the same task were considered nonhygienic. To establish and maintain the lines, queen bees were raised from colonies that displayed the most rapid and least rapid removal rates. For each generation, the daughter hygienic queens were instrumentally inseminated with a mixture of semen from drones from different hygienic colonies (Spivak and Gilliam, 1998). Similarly, daughters from the most nonhygienic queens were inseminated with sperm of drones from the most nonhygienic colonies.

Selection of Experimental Colonies Used for Immunocytochemistry

The OA immunocytochemical experiments were performed from 1994 through 1998. In each year, one hygienic and one nonhygienic colony were chosen to be the source of bees (parental colonies) for the studies that year, and each year different sublines (queen lines) were chosen. The parental colonies were maintained in standard Langstroth beekeeping equipment. The criteria for selection of these colonies were based on their relative rates of removal of freeze-killed brood, but otherwise were similar in populations, and nectar and pollen reserves.

Frames containing eggs from the parental colonies were transferred to and raised by an unselected nursery colony above a queen excluder. Prior to eclosion, pupae were removed from the nursery colony and placed in an incubator (34°C and 50% RH). One-day-old bees were paint marked on the thorax to identify them by age and genetic line, and were introduced into observation hives containing approximately 1000 unmarked bees of various ages, a frame of nectar and pollen, an empty brood comb, and a naturally mated queen. The observation hive colonies were allowed to rear brood from the queen, but the brood was not allowed to eclose. Instead, frames of brood were moved out of the observation hives approximately every 18 days and replaced with a frame of young brood. In this way, the only adult bees in the colony were the original unmarked bees, and the newly added marked bees.

Every 3 days for 4 weeks, 200 marked hygienic and 200 marked nonhygienic bees were added into separate observation hives. A normal age distribution in the colonies was maintained by the continuous addition of marked bees so that older bees did not have to revert to in-hive tasks and nurse bees did not have to become precocious foragers. In each year except 1997, two observation hives composed of bees from either hygienic or nonhygienic parental colonies were used for the source of bees for the experiments. In 1997, the hygienic and nonhygienic bees were housed together within one observation hive, thus forming a mixed genotype colony. Due to this difference in methods, the data from this colony were not comparable to the other years and are not shown here (but see Masterman, 2000). Briefly, the phenotypic expression of the behavior by hygienic bees differed depending on whether they were housed in mixed- versus single-genotype colonies (Arathi et al., 2000; Arathi and Spivak, 2001).

Collection of Bees for Immunocytochemistry

We began collecting bees from the observation hives 4 weeks after the first marked bees were added to the observation hive, when the oldest marked hygienic and nonhygienic bees were 28 days old. In preparation for OA staining**,** bees of known ages, ranging from 1 to 28 days, were removed from colonies through a portal in the glass of the observation hive. This was accomplished by placing a wire screen cage over the bees and allowing them to walk up the side of the cage. This collection technique was used to reduce the effects of stress, which can increase OA levels (Harris and Woodring, 1992).

To collect bees while performing hygienic behavior, a 5 $\times$ 6 cm comb section containing freeze-killed sealed (wax-covered) brood was introduced into the observation hives through the portal, which triggered uncapping and removal behaviors. The freeze-killed brood was presented to the observation hive colony at least 48 h prior to bee collection to expose bees in the colony to cues that elicit hygienic behavior. On the morning of collection for the behavioral study, a new freeze-killed comb section was placed in the colony and bees were collected 2 h later. Bees were collected from the freeze-killed brood section while they were performing hygienic behavior, either uncapping cells or removing pupae. Bees of the same age, but not performing the behavior (control bees), were collected from a random location on the brood comb.

Specimen Preparation and Immunocytochemistry

After collection, bees were brought to the lab and chilled at 4°C until immobile. When the bees were immobilized, the entire brain (fused cerebral and subesophageal ganglion) was dissected out in 4°C bee saline [0.02% KCl, 0.02% CaCl, 0.40% saccharose, 0.90% NaCl (Mercer and Menzel, 1982)]. Brains were fixed for 3 h at 4°C in GPA [15 mL of saturated aqueous picric acid, 5 mL of 25% glutaraldehyde and 0.1 mL glacial acetic acid (Gilchrist et al., 1995)]. Excess fixative was removed by rinsing several times in 70% ethanol until the original tissue color, prior to fixation, was restored. Specimens were further rinsed three times for 30 min each in iso-osmotic Millonig's buffer (13 m*M* NaH_2HPO_4, 86 m*M* Na_2HPO, 75 m*M* NaCl, pH 7.8). Before placement into the primary antibody, the tissue was treated with a buffered 0.05% papainase solution (0.05 *M*

papain, Sigma Chemicals) for 20 min at 36°C to increase tissue permeability. Finally, the tissue was immersed twice for 2 min in a fresh solution of 1% sodium borohydride and 1% sodium metabisulfite to reduce fixative-induced autofluorescence.

After rinsing in Millonig's buffer, brains were placed in 10% normal goat serum (NGS) in Millonig's buffer containing 1% Triton X-100 (Triton-buffer) for 2 h at room temperature. The tissue was then incubated, for 4 days at 4°C, in a 1:200 dilution (in Triton-buffer) of a highly specific OA polyclonal antiserum raised in rabbits against an OA–glutaraldehyde–thyroglobulin complex (Eckert et al., 1992). This highly specific OA antiserum has been shown to stain octopaminergic neurons in a variety of different species including the honey bee *A. mellifera* (Stevenson et al., 1992; Schneider et al., 1993; Kreissl et al., 1994; Gilchrist et al., 1995). After incubation in the OA antiserum, tissue was rinsed in hypo-osmotic Millonig's buffer (13 m*M* NaH_2HPO_4, 86 m*M* Na_2HPO, pH 7.8) for 24 h at 4°C. Brains were then incubated for 2 days in a 1:50 dilution (in Triton-buffer) of cyanine 5.18 (Cy-5)-conjugated donkey antirabbit IgG (see Mesce et al., 1993; Jackson ImmunoResearch, West Grove, PA). After incubation in this secondary antibody, the tissue was rinsed for 48 h at 4°C in hypo-osmotic Millonig's buffer. Brains were dehydrated using a graded series of ethanol solutions (50, 70, and 95%, and twice in absolute), cleared in methyl salicylate for 30 min, and mounted between glass-slide coverslips with pieces of no. 1 cover slips as spacers in DePeX mounting medium (Electron Microscope Services, Ft. Washington, PA).

Specificity of the OA antiserum was verified in the bee brain by preadsorption of the primary antiserum with an OA–thyroglobulin conjugate or a combination of a tyramine–thyroglobulin and an OA–thyroglobulin conjugate using methods described elsewhere (Eckert et al., 1992; Gilchrist et al., 1995). Omission of the primary antiserum with the addition of the secondary antiserum was also conducted as a control.

Confocal Microscopy and Image Analysis

Immunostained whole-mounted brains were examined with a Bio-Rad Lasersharp 1024 (or 1000 model) Confocal Imaging System (Bio-Rad Laboratories, Hercules, CA) mounted on an Olympus AX70 microscope equipped for epifluorescence (Lake Success, NY). The Cy5 fluorphore was imaged using 647 nm excitation and a red bandpass emission filter (i.e., 664–696 nm). The distribution of OA-ir cells was visualized by calculating a projection from a series of optical sections acquired through the entire brain using the Confocal Assistant Program, written by T.C. Brelje (University of Minnesota, Minneapolis, MN). Because OA-ir somata in cluster 3 (Kreissl et al., 1994) routinely showed some level of OA immunoreactivity, this particular group of cells was examined as described below.

Brains from four different groups of bees were studied: (1) bees performing hygienic behavior collected from a hygienic colony; (2) same-aged bees not performing the behavior, obtained from a hygienic colony; (3) bees performing hygienic behavior collected from a nonhygienic colony; and (4) same-aged bees, not performing the behavior, from a nonhygienic colony. All groups were collected, dissected, and processed together and the OA-ir cells in cluster 3 were scored under identical imaging parameters, such as gain and laser intensity. Each projection of the OA-ir cells in cluster 3 consisted of 11 optical sections acquired at 5-μm intervals.

The staining intensity of the OA-ir cells in cluster 3, within each brain sample, was initially rated qualitatively as "intense/bright" or "weak/low." All ratings were conducted blindly with no knowledge of which group of bees a given sample belonged. The brightest cell observed among cluster 3, determined by all or part of the cell showing solid white pixels, was used to score the sample. Standards for the levels of brightness were chosen and pictures of the OA staining were used for reference. Because the specimens were labeled with Cy5, which exhibits minimal photobleaching, standard samples were rechecked on the confocal microscope periodically to determine whether the laser maintained its intensity. To ensure that our subjective ratings truly warranted a division into an intense or weak category, 25 randomly selected samples of varying intensities were measured quantitatively. Each projection was imported into Adobe Photoshop 6 (Adobe Systems, Mountain View, CA) and the staining intensity of the brightest OA-ir cell in cluster 3 was quantified using the Elliptical Marquee Tool and Histogram feature of the image function. For the brightest cell in each full projection of cluster 3 (all projections contained 11 sections), the median intensity value of the cell, based on the frequency distribution of pixels at each intensity level, was calculated over a standard area of 224 pixels. This number of pixels matched the average area of each cell without exceeding each cell's perimeter. The units of the intensity level ranged between 0–255. Specimens rated as having weak staining never exceeded a median value of 185, whereas samples rated as intense had median values of 228 or higher.

OA Staining Statistical Analysis

The data were analyzed by fitting a generalized linear mixed model (Wolfinger and O'Connell, 1993; McCulloch and Searle, 2001) using the routine glmmPQL (R Statistical Software, 2002). Year of study was modeled as a random effect, and behavior (whether a bee was collected while performing hygienic behavior) and line (whether the bee was from a hygienic or nonhygienic colony) were modeled as fixed effects. The analysis is based on the odds of having bright staining in the set of cells medial to the antennal lobes in the deutocerebrum relative to the behavior and line of the bees.

Collection of Bees for Electroantennogram Recordings

The EAGs of bees treated with OA were obtained during late summer (August–September 2000). Studies of the effects of epinastine on EAG activity were conducted during the same time of year, but in year 2001. For these experiments, three hygienic and three nonhygienic colonies were chosen from the seventh (2000) or eighth (2001) generation of selected colonies, all derived from different sublines. These colonies were maintained in small "nucleus" boxes, each containing three to four frames of brood and one to two frames of stored nectar and pollen. All bees developed in their respective colonies; they were not transferred to a nursery colony. Cohorts of 1-day-old bees were marked each day for 1 week with a unique color of paint and were replaced into their respective colonies. After 2 weeks, marked bees between the ages of 15–18 days (mean age 16.8 days) were selected randomly from their colonies for EAG studies. No stimuli, such as dead or diseased brood that could have elicited hygienic behavior, were experimentally introduced into the colony before collection of the bees. These methods allowed us to establish the baseline olfactory responses of the bees, regardless of colony genotype composition or behavioral status at the time of collection.

Electroantennogram Recordings

Electrophysiological methods used for EAG recordings are described in detail in Masterman et al. (2001). Briefly, recording electrodes consisted of glass microcapillary tubes containing chlorided silver wire and physiological saline [210 m*M*/L NaCl, 3.1 m*M*/L KCl, 10 m*M*/L $CaCl_2$, 2.1 m*M*/L $NaHCO_3$, 0.1 m*M*/L NaH_2PO_4 (Patte et al., 1989)]. Bees were chilled until they became inactive and then placed into 1-mL tapered pipette tips so that only the head protruded from the tapered end. The ground electrode was inserted into the hemolymph of the posterior region of the head capsule, just above the occipital foramen. Insertion of the ground electrode into the head capsule limited movement of the head, which resulted in more stable recordings. A small portion of the distal tip of the bee antenna was removed and the recording electrode was advanced slightly into the tip opening until electrical continuity with ground was achieved. EAG responses were amplified and recorded using a Cornerstone IX2-700 intracellular DC pre-amplifier (Dagan, Minneapolis, MN.) and a Maclab digital acquisition system using the Chart program.

A constant flow (125 mL/min) of charcoal-filtered humidified air was passed continuously over the bee during recording sessions. Application of a constant, unvarying air stream resulted in adaptation of antennal mechanoreceptors, which resulted in more reliable and repeatable measures of the summed antennal olfactory responses (Bhagavan and Smith, 1997).

Experiments were performed at approximately 23°C. Hexane (saturated vapor pressure (SVP) of 0.68) was used as the control odorant at 1/23 of SVP (370 ppm) (Patte et al., 1989). Odorant preparations consisted of diseased-brood extract dissolved in hexane. The disease used was the fungal disease, chalkbrood, caused by *Ascosphaera apis*. One chalkbrood equivalent (CBE) was defined as one diseased pupa dissolved in 2 mL of control strength hexane. Subsequent dilutions were made by addition of hexane. A 5-μL aliquot of odorant was placed onto a filter strip inserted into glass tubes. Tubes were sealed until use and discarded after each trial presentation. Stimuli were presented to bees by shunting odorant into the constant air stream. For all presentations, the duration of stimulus delivery was 2 s, and interstimulus intervals equaled 2 min. Order of presentation was as follows: control, 0.1 CBE, control, 0.5 CBE, control, 1 CBE. Peak amplitude of the response was measured using Maclab software. Subtracting the response to hexane (control) from the response to each CBE allowed for standardized data.

Octopamine and Epinastine Treatment

Bees were fed 10 μL of water containing a 10 m*M* (1.896 mg/mL) concentration of OA (Sigma, St. Louis, MO). Control bees were fed 10 μL of water alone. This dosage of OA was used based on previous experiments in which oral administration of an equivalent dose (2.0 mg/mL OA) to a colony of honey bees affected the onset of foraging of individual bees (Schulz and Robinson, 2001). To administer either solution, the antenna of each bee was touched with a drop of 2 *M* sucrose solution, which stimulated the bee to extend its proboscis (the proboscis-extension response). Bees that would not imbibe the OA–water treatment or the water alone were discarded (less than 10%). In all, 30 bees from each line (10 per colony) were successfully given OA treatments, and 30 bees from each line were fed water alone.

For epinastine treatment, 36 bees from each line (12 per colony) were fed 10 μL of water containing 100 m*M* epinastine and 36 bees were fed water alone. Epinastine (3-amino-9, 13 *b*-dihydro-1 *H*-dibenz(c,f)imidazo(1,5a)-azepine hydrochloride) was generously provided by Dr. Thomas Roeder (Universität Hamburg, Germany). This dose was the same as the lowest concentration at which a precipitous change in firing frequency was seen in the DCMD visual interneuron in the locust (Roeder et al. 1998). As above, proboscis extension was elicited by touching a drop of 2 *M* sucrose solution to the antenna of each bee, and then feeding the bee either the epinastine solution, or water alone. Bees that would not consume the solutions were discarded (less than 10%). Approximately 10 min elapsed between both OA and epinastine treatment and electrophysiological measurements.

EAG Statistical Analysis

The main question was whether there was a treatment effect on the bees' ability to perceive odors at each odor concentration. As the data were distributed normally, one-way

ANOVAs (PROC ANOVA; SAS Institute, 1989) were used to compare the responses between the treated hygienic bees, untreated hygienic bees, treated nonhygienic bees, and untreated nonhygienic bees at each odor concentration level (0.1, 0.5, and 1 CBE). Post hoc comparisons of mean EAG responses at each concentration were made using Tukey's HSD test. The responses of bees were pooled among the three hygienic colonies and among the three nonhygienic colonies within each treatment group and concentration level because no significant colony-level differences were found between them. Our previous results showed a significant effect of odor intensity (CBE concentration) on the EAG response of untreated hygienic and nonhygienic bees (Masterman et al., 2001); thus, it was not necessary to repeat that level of analysis here.

RESULTS

Neuronal Distributions of OA-ir Neurons in Wholemounted Honey Bee Brains

In this first report of OA staining in whole-mounted bee brains, we observed seven clusters of OA-ir somata in the brain and subesophageal ganglion, as well as OA-ir processes within particular brain regions, such as the protocerebrum and antennal lobes. All of the described OA-ir cell clusters were found in all age groups ranging from 1- to 28-day-old bees. The overall distribution of OA-ir clusters and stained pathways matched the OA-ir profiles described previously in sectioned bee brain material (Kreissl et al., 1994). In all preadsorption control samples ($n = 6$), no staining was observed, and no secondary-alone staining was observed. Figure 1(A) shows a low magnification (3.6×) frontal view (anterior plane) of the cerebral and subesophagael ganglion showing the antennal lobes, OA-ir cell cluster 3 (arrow; see Kreissl et al., 1994 for nomenclature) and the OA-ir midline somata in the subesophageal ganglion (cluster 7). Figure 1(B) shows a higher magnification of the anteriorventral midline region of the protocerebrum and the two antennal lobes of the deutocerebrum. The arrow points to the relatively tight cluster of OA-ir somata medial to each antennal lobe (cluster 3); above this group is cluster 2 as defined by Kreissl et al. (1994). We observed that cluster 3 stained the most reliably among all the somata. Kreissl et al. (1994) reported that their strongest OA staining was restricted to this specific cell cluster as well. Thus, cluster 3 was chosen as the group of OA-ir cells to study regarding differences in staining intensity among the hygienic and nonhygienic bees. Figure 1(C) and (D) shows a posterior frontal view of the brain and subesophageal ganglion showing the OA-ir central body of the protocerebrum (upper arrow) and cluster 5 (middle arrow). In the subesophageal ganglion, one of the two lateral cells in cluster 6 (at right, no arrow) can be seen as well as cluster 7 (lower arrow), which contains the ventral median cells (Hammer, 1993; Bicker, 1999). Octopamine staining of the antennal lobes is shown in Figure 1(E)–(G). Figure 1(E) shows a full projection of the antennal lobe from a young bee (9 days old) and its associated low levels of OA staining. In contrast, Figure 1(F) shows the antennal lobe of a much older bee at 26 days of age. Typically, such older bees had much more robust OA staining than did young bees. Figure 1(G) [a single optical section from the projection shown in Figure 1(F)] reveals the OA-ir fibers associated with the olfactory glomeruli.

Figure 2(A)–(D) depicts the OA-ir somata of cluster 3, in which we routinely observed between eight to nine stained cells in each side of the brain. Kreissl et al. (1994) reported seeing six to seven OA-ir cells in each cluster in sectioned material. Because the cells in cluster 3 are often tightly packed together, our whole-mount method, coupled with confocal microscopy, resolved more cells. The different samples presented in Figure 2(A)–(D) show the typical staining intensities of OA-ir cells observed among the two bee lines, and among bees performing hygienic behavior or not. Examples that are typical of low OA immunoreactivity (low/weak, median scores of 102 and 172, respectively) are shown in Figure 2(A) and (B). Figure 2(C) and (D) contain representative samples of cells with higher levels of OA immunoreactivity (intense/bright, median scores of 234 and 245, respectively).

Behaviorally Based Differences in OA-IR Staining Intensity

Over the 4 years of study (Table 1), the odds of a bee having bright OA-ir staining in the cluster-3 cells were significantly higher when the bee was observed performing hygienic behavior than when it was not performing this behavior (likelihood ratio = 9.80; $df = 1$; $p = .0017$). The odds were not significantly different for the two genetic lines of the bee (hygienic or nonhygienic, likelihood ratio = 1.88; $df = 1$; $p = .1694$), and there was no significant interaction between behavior and line (likelihood ratio = 0.15; $df = 1$; $p = .6945$). The odds of having bright staining were 13 times greater in bees performing the behavior than in bees from either genetic line.

EAG Results: Octopamine Treatment

In Figure 3, are the pooled mean EAG responses (with 95% confidence intervals) to three concentrations of

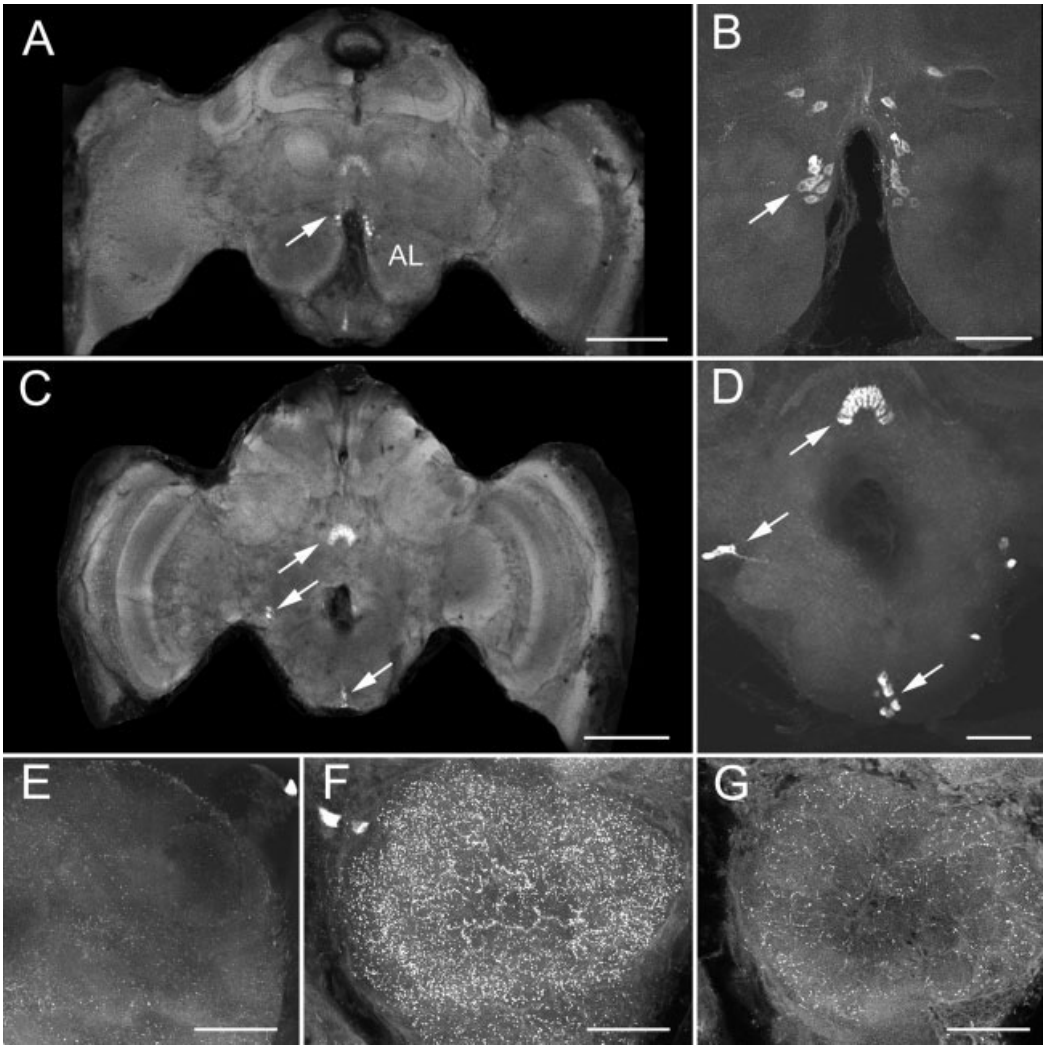

Figure 1 Laser scanning confocal images of octopamine-immunoreactive (OA-ir) neurons (Cy5 fluorescence) in the brain (fused cerebral and subesophageal ganglia) of the honey bee *Apis mellifera*. (A) Low magnification (3.6×) anterior view of a whole-mounted brain, showing major brain regions and prominent optic and antennal lobes (AL). Arrow points to a group of OA-ir neurons residing in the deutocerebrum medial to the antennal lobes. This group of OA-ir cells is referred to as cluster 3, according to the nomenclature of Kreissl et al. (1994). (B) Different preparation from (A) showing higher magnification (18× objective) image of OA-ir neurons in cluster 3 (arrow), and other OA-ir cells located mediodorsal to the antennal lobes (cells of cluster 2). (C) Posterior view of entire brain showing OA immunoreactivity in the lower division of the central body (top arrow), the OA-ir lateral cells of cluster 5 (middle arrow), and the ventral median cells of the subesophageal ganglion comprising cluster 7 (bottom arrow). (D) Higher magnification image of similar OA-ir cells in (C), but different preparation, showing the inclusion of one of the lateral OA-ir cells in cluster 6 (lower cell not marked by arrow). (E) Antennal lobe of a young bee at age 9 days showing low OA immunoreactivity (full projection of 34 optical sections at 2-μ intervals). (F) Octopamine-ir fibers within the antennal lobe of an older bee at 26 days of age (similar projection of 34 optical sections at 2-μ intervals). Punctate OA-ir fibers appear to outline the individual glomeruli within each antennal lobe. (G) Single confocal optical section from preparation in (F), depicting the association of OA-stained fibers with the glomeruli. Scale bars = 500 μm (A,C); 100 μm (B and D–G).

CBE (see Methods) among the four treatment groups of bees. At the lowest odor concentration, 0.1 CBE (ANOVA: $F = 26.35$; $df = 3, 119$; $p < .001$), the untreated nonhygienic bees had significantly lower mean EAG responses than OA-fed nonhygienic bees, OA-fed hygienic bees, and untreated hygienic bees.

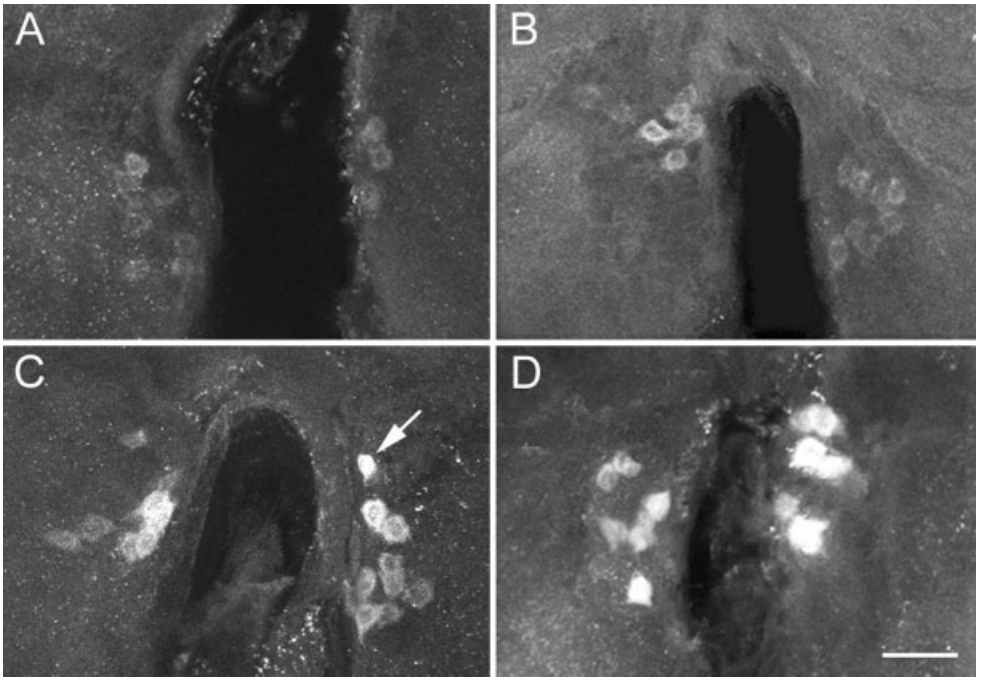

Figure 2 Representative samples of confocal images containing deutocerebral OA-ir neurons belonging to cluster 3. All brain samples were segregated into categories of bright or low staining levels with no knowledge of the genotype or behavioral history of the bee associated with each sample. (A,B) Two different brain samples exemplifying what was considered to be weak/low OA staining. Samples in which the brain contained no evidence of OA immunoreactivity were not included in our analysis. In addition, the outlines of the OA-ir cells had to be discerned. (C,D) Examples of brain samples in which the OA-ir neuronal somata of cluster 3 expressed intense/bright staining. Each bilaterally paired cluster contained between 8–9 somata. Although the sample in (D) was rated as having brighter staining than (C), the two samples were grouped together as being "bright" because of the one cell in sample (C) that had solid pixels/intense staining (arrow). Quantitative image analysis (see methods) revealed that weak and bright staining levels were matched by nonoverlapping numerical intensity values. Image projections of 11 optical sections at 5-μm intervals. Scale bar = 50 μm.

There were no significant differences between the OA-fed and untreated hygienic bees, and the OA-fed nonhygienic bees. The same result was found at the middle odor concentration, 0.5 CBE ($F = 34.43$; $df = 3, 119$; $p < .001$). Again, untreated nonhygienic bees had significantly lower mean EAG responses than the other groups. At the highest concentration, 1.0 CBE ($F = 8.79$; $df = 3, 119$; $p < .001$), the untreated nonhygienic bees had a significantly lower response than the OA-fed and untreated hygienic bees. However, there was no difference between the OA-fed nonhygienic bees and the other three groups.

EAG Results: Epinastine Treatment

The opposite effect (above) was observed when bees were fed epinastine, an OA blocker (Figure 4). At the lowest odor concentration, 0.1 CBE ($F = 19.91$; $df = 3, 143$; $p < .001$), the untreated hygienic bees had significantly higher mean EAG responses than epinastine-fed hygienic bees, epinastine-fed nonhygienic bees, and untreated nonhygienic bees. There were no significant differences between the epinastine-fed and untreated nonhygienic bees. However, the response of the epinastine-fed hygienic bees was intermediate; it was significantly lower than the untreated hygienic bees, and significantly higher than the treated and untreated nonhygienic bees. At the middle odor concentration, 0.5CBE ($F = 11.724$; $df = 3, 143$; $p < 0.001$), the untreated hygienic bees had significantly higher mean EAG responses than the other groups. There were no significant differences between treated and untreated nonhygienic bees and treated hygienic bees. At the highest concentration, 1.0 CBE ($F = 2.39$; $df = 3, 143$; $p = .0714$), there were no significant differences between the four groups.

Table 1 Intensity Rating of OA-ir Neurons Medial to the Antennal Lobes (Cluster 3) in the Honey Bee Brain

	Hygienic Colony				Nonhygienic Colony			
	Bees Not Performing Behavior		Bees Performing Hygienic Behavior		Bees Not Performing Behavior		Bees Performing Hygienic Behavior	
Year	Number of Bees Analyzed	Number of Bees with Bright Cells	Number of Bees Analyzed	Number of Bees with Bright Cells	Number of bees analyzed	Number of bees with bright cells	Number of Bees Analyzed	Number of Bees with Bright Cells
1994	5	1	4	4	5	0	2	2
1995	4	1	7	3	6	1	1	0
1996	8	3	23	16	17	6	-	-
1998	5	2	7	5	8	2	3	2
Total	22	7	41	28	36	9	6	4
		36%		68%		25%		67%

The number of bees analyzed represents a subsest of all bees that were collected from a colony in a particular year. Samples in which the brain contained no evidence of OA immunoreactivity were not included in our analysis. All samples were rated using identical imaging parameters (see Methods).

DISCUSSION

General Discussion

Our results revealed that the probability of having brightly stained OA-ir neurons, in the deutocerebrum medial to the antennal lobes (cluster 3), was significantly greater in 15–20-day-old bees collected while performing hygienic behavior than in same-aged bees not performing the behavior. Although slight differences in staining intensity were observed between the hygienic and nonhygienic genotypes, statistically significant differences were observed only between bees expressing the behavior, independent of their genotype. For example, an average of 68% (hygienic line) and 67% (nonhygienic line) of bees possessed brightly stained cells, compared to the 36% (hygienic line) and 25% (nonhygienic line) of bees that did not perform hygienic behavior. In Table 1, it is important to note that the sample sizes reflect the probability that a bee could be observed performing the behavior. Nonhygienic bees do perform hygienic behavior although at a very low frequency (Arathi and Spivak, 2001); hence, we were able to collect and obtain reliable staining of only six bees over the 4 years. In contrast, it was relatively easy to collect bees performing the behavior within a hygienic colony, and we obtained reliable staining in 41 of those collected. Thus, although the genotype of an individual bee influences its predisposition to express all or some components of hygienic behavior (i.e., facilitates the detection, uncapping, and/or removal of dead brood), clearly other factors must play a role in regulating the underlying neural networks responsible for hygienic behavior. These networks and regulatory mechanisms are probably shared because all honey bees, regardless of their genotype, have the ability to detect, uncap, and remove dead brood.

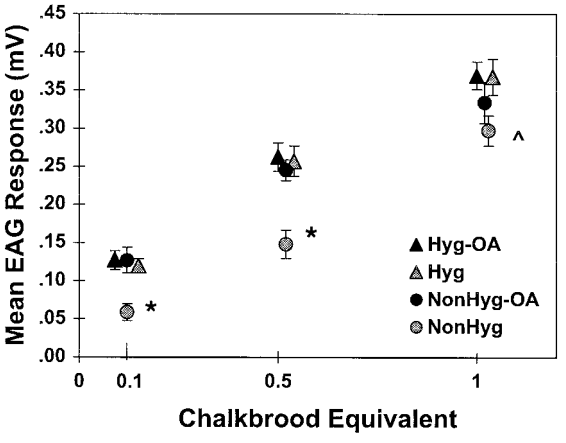

Figure 3 For four groups of bees, the mean EAG responses (±95% confidence intervals) to the three concentrations of the odor of diseased brood (chalkbrood equivalent, CBE). Each symbol represents the mean response of 30 bees. Hygienic bees treated with OA (Hyg-OA), untreated hygienic bees (Hyg), and nonhygienic bees treated with OA (NonHyg-OA) had statistically similar olfactory sensitivities (mean EAG response) to 0.1, 0.5, and 1.0 CBE. Untreated nonhygienic bees (NonHyg), however, had significantly lower olfactory sensitivity (lower mean EAG response) than the other three groups at 0.1 and 0.5 CBE (denoted by askterisks). At 1.0 CBE, the untreated nonhygienic bees had a significantly lower response than treated and untreated hygienic bees, but had a similar response as the OA-treated nonhygienic bees (denoted by caret).

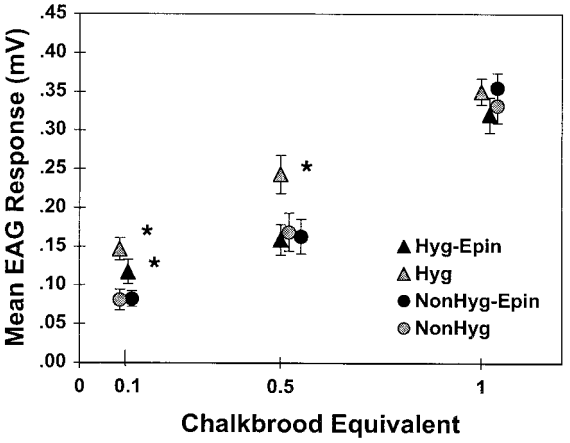

Figure 4 Mean EAG responses (with 95% confidence intervals) of the four groups of bees to the three odor concentrations (CBE). Each mean symbol represents the mean response of 36 bees. Untreated hygienic bees (Hyg) had significantly higher mean EAG responses than epinastine treated hygienic bees (Hyg-Epin), treated nonhygienic bees (NonHyg-Epin) and untreated nonhygienic bees (NonHyg) at 0.1 and 0.5 CBE (denoted by askterisks), but at 1.0 CBE, the responses of all four groups was similar. Epinastine-treated hygienic bees had a significantly higher EAG response than the treated and untreated nonhygienic bees at 0.1 CBE (denoted by askterisk), but at the higher odor concentrations, the responses of the treated hygienic, treated nonhygienic, and untreated nonhygienic bees were not different.

Assuming that the increased staining of OA-ir neurons reflects an upregulation of OA production in bees performing hygienic behavior, further studies are needed to determine the mechanisms by which this potential upregulation controls or modulates the expression of hygienic behavior. Presently, it remains unclear whether such possible changes in OA are causal in nature or are a result of feedback mechanisms stemming from behavioral performance.

The OA-ir neurons and other OA-ir profiles we examined in the two honey bee lines were representative of cells that express OA and not some other biogenic amine. The OA antiserum that we used was highly specific to OA, and it has been used previously to identify octopaminergic neurons across a number of different animal preparations (Eckert et al., 1992; Stevenson et al., 1992; Schneider et al., 1993; Gilchrist et al., 1995; Lehman et al., 2000; Crisp at al., 2002). Most importantly, the locations and distributions of OA-ir neurons we observed in whole-mounted bee brains were similar to those reported previously in sections of the honey bee brain by Kreissl et al. (1994).

Although the actual performance of hygienic behavior, and not the hygienic genonotype per se, was most correlated with the staining intensity of OA-ir neurons in the brain, baseline genetic differences between the hygienic and nonhygienic genotypes were evidenced by EAG differences in this study and in Masterman et al. (2001). Bees between 15–20 days old, collected randomly from a colony bred for hygienic behavior (i.e., not collected while performing the behavior), were more sensitive to lower concentrations of the odor of diseased brood than were same-age bees collected from a nonhygienic colony (Masterman et al., 2001). In the present study, the response of EAGs to OA treatment differed between the two gentoypes. Oral administration of OA increased olfactory sensitivity in bees selected for nonhygienic behavior, whereas the same treatment had no effect on the olfactory sensitivity of bees selected for hygienic behavior. In turn, oral administration of the OA receptor antagonist, epinastine, had no effect on the olfactory sensitivity of bees selected for nonhygienic behavior, but reduced the sensitivity of bees selected for hygienic behavior. For example, at the two lowest concentrations (0.1 and 0.5) of CBE tested, the magnitudes of the EAG responses in untreated hygienic bees were greater compared with epinastine-treated hygienic bees or untreated or treated nonhygienic bees. These results show that oral administration of OA to an individual nonhygienic bee increases its sensitivity to a behaviorally relevant odor. The ability to perceive this odor at a low stimulus level could facilitate the detection of diseased brood and trigger the expression of hygienic behavior by an individual bee.

If the brighter OA-ir cells we observed in bees displaying hygienic behavior were correlated with higher levels of OA in the nervous system, then such levels could modulate the olfactory sensitivity of the bee, as we have shown with OA oral administration. This enhancement could contribute to a bee's detection of olfactory cues emitted from diseased or abnormal pupae, which would elicit hygienic behavior. Because of the well-documented sensitizing effects of OA on olfactory systems of the honey bee, and its actions on "motivational" state (see Menzel, 2001, for review), OA has the potential to facilitate both the detection and response of the honey bee to diseased brood. This modulation could effectively increase the probability that honey bees would initiate uncapping or removal behaviors.

Possible Mechanistic Roles of OA in the Expression of Hygienic Behavior

Results from both the OA and epinastine studies imply that the response thresholds were lower in the CBE-sensitive chemosensory neurons contributing to the summed potentials comprising the EAG recordings, and that such neurons are targets of OA modulation. Because the magnitudes of the EAGs in the epinastine trials were similar among the different genetic lines at higher concentrations of odorant, this supports the idea that overall the complement of antennal chemosensory neurons is similar between the two lines. However, such results also suggest that olfactory excitation thresholds are differentially modulated, especially at lower stimulus values. Perhaps, differences in OA action or receptor distributions play an important role in regulating neuronal sensitivity, but when odorant concentrations cause a suprathreshold excitation of the chemosensory neurons, such modulatory effects are minimized. This may help to explain why epinastine lost its ability to reduce the sensitivity of olfactory neurons in the hygienic bees when antennae were exposed to higher concentrations of chalkbrood. Previous studies by Masterman et al. (2001) indicate that a defining feature of hygienic bees is that they can detect particular odors at lower concentrations; at higher concentrations, olfactory responses are similar between the two genotypes. Thus, the differential actions of OA may well only function within a relatively narrow range of odorant concentrations, the boundaries of which we have yet to determine.

Studies using the OA antagonist, epinastine, indicate that oral administration of OA and its effects on EAG magnitude are a direct result of OA action and not a consequence of a different neuroactive substance or other biogenic amine. Epinastine has both a high affinity and a high specificity for insect neuronal octopamine receptors, including the honey bee OA receptor (Roeder et al., 1998; Roeder, 1999; Degen et al., 2000). Furthermore, in the silkmoth *Antheraea polyphemus,* Pophof (2000) showed that epinastine caused a decrease in the firing rate of antennal olfactory neurons in response to pheromone concentrations, whereas OA caused an increase in nerve impulse frequency. These and other studies (von Nickisch-Rosenegk et al., 1996) brought Pophof (2000) to the conclusion that OA was likely acting via a type-3 OA receptor located on the olfactory neurons and not by way of secondary alterations in OA metabolism.

In a number of invertebrate preparations, it has been well documented that OA can modulate the sensitivity of various types of sensory systems (Ramirez and Orchard, 1990; Zhang et al., 1992; Pophof, 2000, 2002; Dolzer et al., 2001; Grosmaitre et al., 2001; Lim and Wasserman, 2001). For example, OA has been shown to modulate neuronal firing activity and to increase the amplitude of receptor potentials in the pheromone-sensitive antennal neurons of the male moth, *Bombyx mori*, but not the receptor potentials of olfactory neurons in other moth species (Pophof, 2000; Grosmaitre et al., 2001). In some cases, these actions appear to be mediated by the direct effects of OA on the receptor neurons and not the receptor-associated supporting cells (Pophof, 2000). Thus, these and other studies are consistent with our findings that the honey bee olfactory neurons are targets of octopaminergic neuromodulation, and that such modulation can be selective and even species specific. In addition, a recent study by Pophof (2002) demonstrated that female general-odorant-sensitive antennal neurons were not responsive to OA, whereas the male pheromone-sensitive neurons were. Such findings indicate that OA can have differential actions on the sensitivities of antennal olfactory neurons of individuals within a species. Similarly, we observed that the actions of OA were different among the hygienic and non-hygienic genotypes within a single species *Apis mellifera*. Thus, such aminergic modulation appears to facilitate a broadening of the behavioral repertoires expressed by individuals of the same species.

As putative targets of OA modulation, the honey bee olfactory neurons we examined may possess OA receptor subtypes and/or receptor distributions that are differentially expressed on the sensory neurons and supporting cells of hygienic and nonhygienic bees. It has been shown that amine receptor subtypes are quite variable and can become differentially altered between animals of the same species in response to particular internal or environmental influences (Yeh et al., 1996; Edwards et al., 2002). Thus, a slight difference in the physiological "state" of the honey bee, genetically based or not, can have a great influence on the excitability thresholds of olfactory neurons that are targets of OA.

The increased occurrence of bright OA-ir somata in cluster 3, which we observed in bees performing hygienic behavior, suggests that OA is related to the performance of this behavior, but as mentioned previously, we do not know whether this increase is causal in any way. Unfortunately, the specific functions of the OA-ir cells in question are not yet known and it is unclear where they project. Future studies are warranted to determine whether the cluster-3 cells

mediate olfactory modulation at the level of the olfactory glomeruli.

Age and Colony-Specific Factors Regulating Behavior

As part of our control studies, OA-ir cells were also examined as bees aged and became adult workers. We found that each group of OA-ir neurons in the bee brain was present at all ages examined (1, 7, 14, 21, and 28 days old, $n \geq 6$ at each age). In addition to the presence of all OA-ir neuronal groups originally described by Kreissl et al. (1994), variations in staining intensity were found in every age group, but we did not systematically rate this staining intensity among cell groups other than the ones reported. Age-related changes in the levels of OA using HPLC analysis, however, have revealed that older foraging bees possess greater amounts of OA (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). These findings are consistent with our preliminary observations that the terminals of unidentified OA-ir cells within the antennal lobes become brighter with age. Aside from this change in glomerular staining, no other OA-ir cells in the brain or OA-ir processes were obviously brighter as the bee aged. Further studies are needed to determine which OA-ir neurons contribute to the HPLC-detected increases of OA in older bees.

Octopamine levels have also been shown to change with the acquisition of specific hive-related behavioral tasks in honey bees. In particular, HPLC analysis has revealed that increased levels of OA are observed in foraging bees, regardless of age (Wagener-Hulme et al., 1999). Furthermore, Schulz and Robinson (1999) have shown that bees forced to forage precociously show forager-like levels of OA in their antennal lobes; such levels decrease when foragers revert to nursing. Importantly, bees treated (by feeding the colony) with OA are more likely to become precocious foragers (Schulz and Robinson, 2001). Additional studies by these authors revealed that OA levels in the antennal lobes and the remaining protocerebral and deutocerebral regions varied with worker behavior, but not with age. In contrast, mushroom body analysis revealed differences in OA levels based on age, but not behavior. Such studies underscore the important behavioral and developmental roles of OA, but also emphasize that the influences of OA are quite complex and likely depend on the region-specific actions of this amine.

Although oral administration of OA to nonhygienic bees increased their sensitivity to the odor of diseased pupae, we did not test if these treated bees, and if hygienic bees in general, had increased sensitivity to other odors not associated with hygienic behavior, such as floral odors or brood pheromone. Similarly, we do not know whether feeding OA to a nonhygienic bee colony (using methods of Schulz and Robinson, 1999; 2001) would elicit more rapid uncapping and removal of diseased or dead brood. Our preliminary data (M. Spivak, unpublished data) suggest that hygienic bees may be precocious foragers. Although we did not measure region-specific levels of OA in the bee brain, such regional differences could modulate both the expression of hygienic behavior in middle-aged bees and influence the tendency of older bees to forage. It is likely that the expression of these behaviors is determined by complex interactions among the genetic predisposition of the individual bee, its physiological state, social interactions, and environmental constraints on the colony.

Our study supports the idea that the OA modulation of olfactory-related systems may enable honey bees to respond to chemical cues that facilitate the performance of various behavioral tasks. Thus, the stimulus threshold for performance of a particular task can change with time and resource conditions (Masterman et al., 2001; reviewed in Beshers and Fewell, 2001). The aminergic modulation of such tasks, whether genetically biased or not, could afford the bee a high degree of behavioral flexibility and enable an individual bee or an entire bee colony to respond adaptively to its dynamic environment. How precisely OA contributes to a sculpting of hygienic behavior is a compelling problem that awaits future investigations.

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