CHAPTER II

Histamine acts as an inhibitory transmitter in the honeybee antennal lobe

ABSTRACT

Odors are represented by specific spatio-temporal activity patterns in the olfactory bulb of vertebrates, or its insect analogue, the antennal lobe (AL). In honeybees inhibitory circuits in the AL are involved in the processing of odors to shape afferent odor responses. GABA has been identified as a constituent of the inhibitory network, but not all interneurons are GABAergic. Therefore we sought to analyze the functional role of the inhibitory transmitter histamine for the processing of odors in the honeybee AL. We optically recorded the representation of odors during histamine application at the input level, estimated by a compound signal, and at the output level, by selectively measuring the projection neurons (PNs). Histamine led to a strong and reversible reduction of the odor-evoked responses of both the input and the output neurons. The PN responses were more sensitive to histamine than the compound responses, suggesting different histaminergic inputs to receptor neurons (RNs) and PNs, respectively. We conclude that histamine, in addition to GABA, acts as an inhibitory transmitter in the honeybee AL and plays an important role in odor processing.

INTRODUCTION

The antennal lobe (AL) of insects is the functional analogue of the olfactory bulb (OB) in mammals, which is the first central neuropil where information from the olfactory receptor neurons (RNs) is processed. Both, the AL and OB, consist of different neuron types which modulate and optimize the afferent input in a complex network. It has been shown that intrinsic inhibitory circuits within the olfactory bulb and the AL shape temporal and spatial aspects of the odor-evoked patterns to improve odor detection and discrimination (Christensen et al. 1993; Yokoi et al. 1995; Sachse and Galizia 2002). However, little is known about the synaptic interactions among the olfactory neurons involved in odor processing in insects.

In the honeybee, 95% of the sensory cells located on the antenna are olfactory neurons (Esslen and Kaissling 1976) which convey the olfactory information to two categories of AL neurons, namely 4000 local interneurons (LNs) (Witthöft 1967) and 800 projection neurons (PNs) (Bicker et al. 1993). LNs branch exclusively within the AL, whereas PNs relay the olfactory information to higher order brain centers. Almost all synaptic contacts between the sensory neurons, LNs and PNs are strictly located in areas of high synaptic density, the olfactory glomeruli (Gascuel and Masson 1991). Each of the 156 glomeruli represents an identifiable morphological and functional subunit, arranged in a single layer around the honeybee AL (Flanagan and Mercer 1989; Galizia et al. 1999a). Similar to the olfactory system of lobsters and moths (Christensen et al. 1993; Schmidt and Ache 1996), honeybees have anatomically and functionally distinct classes of olfactory LNs (Fonta et al. 1993). The majority of them, heterogeneous LNs (hetero LNs), have a high density of dendrite branches in one particular glomerulus and sparser branches distributed across other glomeruli. Homogeneous LNs (homo LNs) distribute their branches more homogeneously over the whole AL. It is conceivable that these different LN types are involved in functionally distinct inhibitory networks to shape the odor responses of olfactory PNs. Indeed, in vertebrates (reviewed by Mori and Yoshihara 1995) and lobsters (Wachowiak and Ache 1997; Wachowiak and Ache 1998) dual inhibitory pathways at the first synaptic level have been well characterized. In lobsters the existence of both GABA- and histaminergic inhibitory pathways has been reported (Wachowiak and Ache 1997), whereas in vertebrates both pathways are mediated by the inhibitory transmitter GABA (reviewed by Shipley and Ennis 1996).

Two observations suggest that such a processing mechanism based on two distinct inhibitory pathways may also exist in the honeybee AL: First, the majority of honeybee LNs appears to be inhibitory, unlike the LNs of moths and cockroaches, in which most of the LNs are GABA-immunoreactive (Hoskins et al. 1986; Distler 1989); only 20% of them have been shown to be GABAergic in the bee (Witthöft 1967; Schäfer and Bicker 1986). Yet the transmitter of the remaining 80% of LNs is still unclear. Interestingly, a strong population of histamine-immunoreactive LNs has been found in the honeybee AL (Bornhauser and Meyer 1997), suggesting histamine as a possible candidate similar to the lobsters olfactory system. The existence of histaminergic AL neurons is not ubiquitous to insects; apart from honeybees, only cockroaches and crickets possess histaminergic olfactory cells, though these form a very minor cell population in both species (reviewed by Nässel 1999). The ALs of moths, locusts and dipteran flies totally lack histamine-immunoreactivity (reviewed by Nässel 1999). Second, in our previous study we have shown the existence of two separate inhibitory networks in the honeybee AL, which both shape the odor-induced PN responses (Sachse and Galizia 2002). One is picrotoxin (PTX)-sensitive and thus likely GABAergic and constitutes a global gain control mechanism. The second is a glomerulus-

specific inhibitory network, which contrast-enhances overlapping glomerular response profiles, and is picrotoxin-insensitive. Since PTX blocks chloride channels, the second network is either constituted by metabotropic GABA receptors or a different transmitter system altogether. A likely transmitter could be histamine.

In this study we analyzed the effect of histamine application to the honeybee AL by optically recording two different processing levels. During histamine application we measured odor-evoked responses of a compound signal mainly reflecting the afferent input to the AL (Galizia et al. 1998), and of PNs which represents the AL output. The results provide first evidence that histamine acts as a second functional inhibitory transmitter in the honeybee AL, besides the already established GABAergic one.

MATERIALS AND METHODS

Animal preparation and staining

Adult worker honeybees (*Apis mellifera*) were caught from different hives, quickly anesthetized by cooling and placed in a Plexiglas stage using dental wax. The antennae were fixed with two silicone components (Kwik-Sil TM, WPI) at their scapus and covered with a coverslip while leaving the head accessible for preparation. The head capsule was opened and glands and tracheae were carefully removed. The animals were then either stained with calcium green to estimate RN responses (Galizia et al. 1998), or selectively labeled with fura to record PN responses. In the case of calcium green, the brain was floated with a solution of calcium green 2 AM (Molecular Probes, Eugene, OR; 50 µg dye was first dissolved in 50 µl Pluronic in DMSO and then diluted in 950 µl Ringer solution: 130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich, Deisenhofen). After staining for 1 h, the brain was rinsed with fresh Ringer and the recording stage placed under the microscope. PNs were selectively stained as previously reported (Sachse and Galizia 2002). A glass electrode, which was coated with crystals of fura-dextran (potassium salt, 10,000 MW, Molecular probes, Eugene, OR), dissolved in 2% bovine serum albumin solution, was inserted into the deutocerebrum, aiming for the projection neurons (PNs) of the lateral antenno-cerebralis tract (l-ACT). The brain was then rinsed with Ringer solution to remove extracellular dye. After 3 h of staining, successful PN loading was visible by a strong staining of the l-ACT somata at the AL under the fluorescence microscope. Only bees whose PNs were stained were used for calcium measurements.

Optical recording

Imaging was done using a T.I.L.L. Photonics imaging system (Martinsried, Germany). In the case of calcium green, monochromatic excitation light was 475 nm, dicroic: 510 nm, emission: BP 515-565 nm, for fura the excitation light alternated between 340 nm and 380 nm, dicroic: 410 nm, emission: LP 440 nm. Measurements were made with an upright microscope (Olympus BX 50WI), using 20 x water immersion objectives (NA 0.95 for calcium green, NA 0.5 for fura). Pixel image size was 2.4 x 2.4 µm. For each fura recording, a series of 60 frames was taken with a frequency of 6 Hz. Since odor-evoked compound signals measured with calcium green lasted longer than PN responses, we took recordings of 20 s (i.e. 100 frames with 5 Hz). Light was turned off between frames. Interstimulus interval was at least 1 min.

Odors were delivered to the antennae using a custom-made and computer-controlled olfactometer by switching from a constant air stream to an odor stream in order to eliminate mechanical stimulation (Galizia et al. 1997). Stimulus duration was 1 s for calcium green and 2 s for fura measurements. Odors used differed between experiments, and were 1-hexanol, 2-octanol, 1-nonanol and linalool (Sigma-Aldrich, Deisenhofen). For each odor, 4 µl of the odorant dissolved in mineral oil were applied to a filter paper (1 cm^2) in a plastic syringe. Dilutions were adjusted to equalize effective vapor pressure for the different odorants. The control stimulus was a syringe plus filter paper with mineral oil.

Solutions of histamine (Sigma-Aldrich, Deisenhofen) were first dissolved in Ringer for final concentrations of 10 mM and 50 mM and then bath-applied to the brain. In control experiments lower histamine concentrations (0.01, 0.1 and 1 mM) were also applied to the brain (data not shown).

Data processing

All analyses were done using custom software written in IDL (Research Systems, CO). The raw data were median-filtered for shot noise reduction (filter size 3 pixels in two spatial and one temporal dimension) and were corrected for scattered light by calculating an unsharp image with a radius of 50 µm and subtracting this from each frame. The calcium green signals (i.e. 475 nm measurements) were calculated as $\Delta F/F$ [%], where the mean of 19 frames measured before stimulus was used as F. These measurements were corrected for bleaching by fitting a logarithmic function computed for each measurement. In the case of fura-recordings, we calculated the ratio 340 nm / 380 nm and multiplied it with 100; these values are labeled as $\Delta(F340/F380)$ [%] in the figures. Since each glomerulus had an individual background fluorescence ratio, all time traces were shifted to zero shortly before the stimulus onset by subtracting the background using frames 5-16 (i.e. before stimulus onset at frame 18). This allows a comparison between the traces of different glomeruli. We identi-

fied the strongest glomeruli on the basis of their response activities, using their published glomerular response profiles (Galizia et al. 1999b; Sachse and Galizia 2002).

For the false-color coded images (Fig. 1A, 2A), we averaged the fluorescence changes between frames 25-45 for the compound responses (i.e. from stimulus onset until 3 s after stimulus offset) and frames 18-30 for the PNs (i.e. from stimulus onset until stimulus offset). For time courses of identified glomeruli (Figs. 1B, 2B) squares of 11 x 11 pixels (corresponding to 26.4 µm side length) were placed on the center of a glomerulus, their values were averaged and plotted against time.

We averaged the responses of the most activated glomeruli during Ringer, histamine and in the wash over all animals measured (n = 7 for calcium green, n = 5 for fura; Figs. 1C, 2C). Beforehand, for each animal the glomerular response was calculated as the maximum during stimulus onset until 3 s after stimulus offset and repeated stimulations were averaged. In order to compare animals with different background fluorescences and thus different maximal activities, we normalized by defining the glomerular response within each animal to each odor before the pharmacological treatment as 1 and scaled the other responses accordingly. Significant differences were determined using a two-tailed paired t-test, performed on the original data.

RESULTS

We investigated the influence of histamine on the odor-evoked glomerular responses in the honeybee AL. We visualized different glomerular processing levels, using two different staining protocols in different animals. We measured the compound signals with calcium green 2-AM, which emphasizes the afferent input to the AL (i.e. RNs) (Galizia et al. 1998). In further experiments we selectively stained PNs using fura-dextran, thus measuring the AL output. In the compound signal stimulation with odors led to strong, long-lasting and odor-specific calcium signals in several glomeruli before the histamine treatment (Fig. 1A). The time courses of two identified glomeruli during stimulation with nonanol are shown in Figure 1B. After application of 10 mM histamine to the brain, the calcium activity patterns remained unchanged, whereas 50 mM histamine totally abolished the odor-induced responses. In the wash the odor-specific calcium signals recovered completely, but appeared slightly reduced. The histamine effect observed in this animal was typical for all animals measured (n = 7; Fig. 1C). The reduction of the odor-evoked compound responses at 50 mM histamine was highly significant.

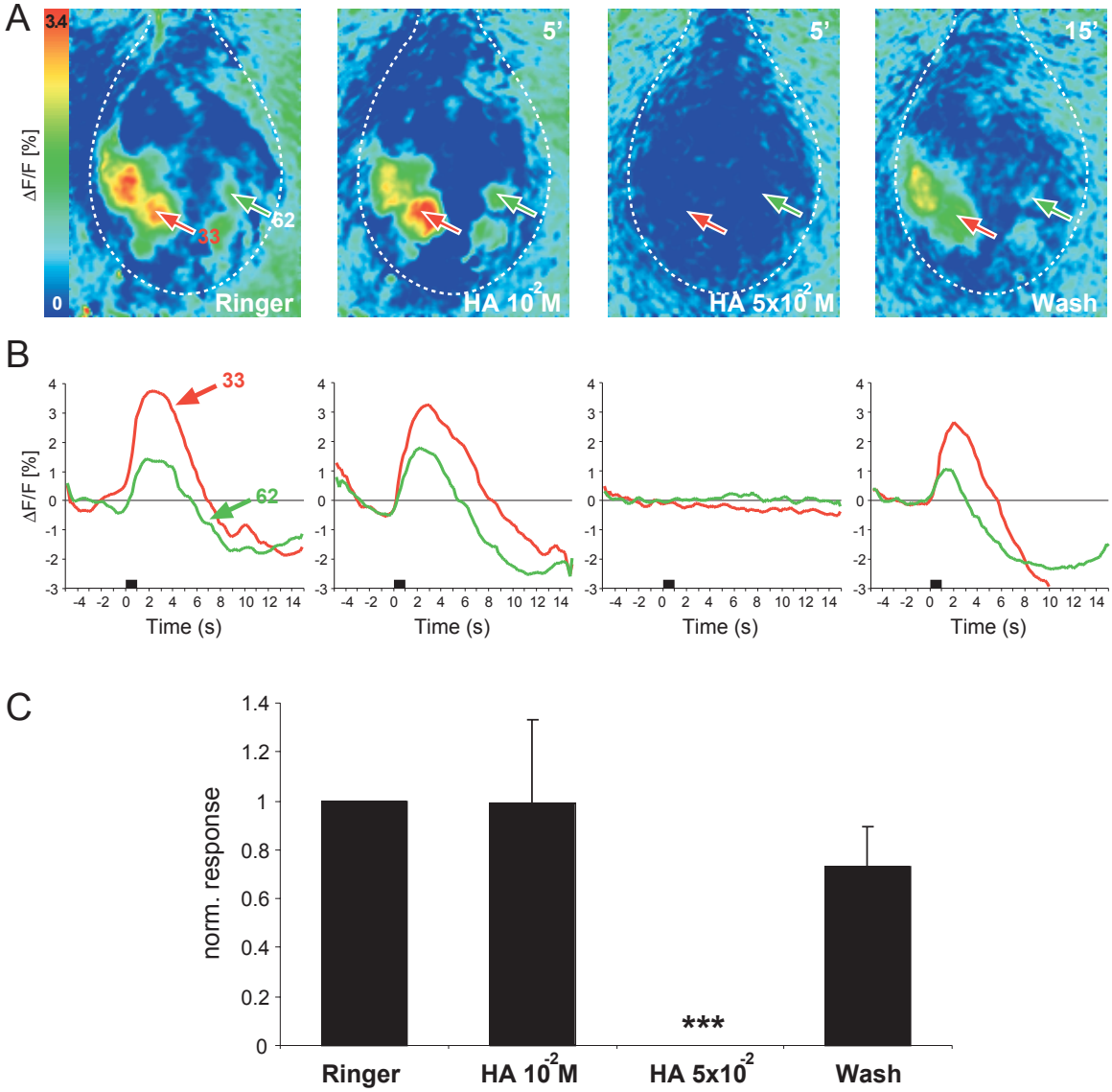

Figure 1 Influence of histamine on the odor-induced compound responses of the honeybee AL. **A**, False-color coded spatial activity patterns to the odor 1-nonanol before, during and after histamine application. Histamine was successively applied with increasing concentrations. The AL border is marked with a dotted line, antennal nerve is at the top. The positions of two identified glomeruli are indicated in each frame. The numbers at the top right in each image indicate the time elapsed from the latest treatment change. **B**, Time traces of the two identified glomeruli, whose positions are marked in A. Odor application is shown by a black bar. A histamine concentration of 50 mM completely abolished the spatial and temporal calcium responses, which were reversible after wash-out. **C**, Bar chart of the odor-evoked responses averaged over all animals (mean and SEM, n = 7). Only the most-responsive glomeruli were included in the plot. The arrangement of the different bars from left to right reflects the temporal sequence of the experiment. Asterisks give significant differences to the Ringer measurement (***P < 0.001, two-tailed paired t-test, performed on the original data). The histamine effect observed for the animal in A, B was confirmed in each of the 7 animals tested.

Similar to the compound signals, PNs revealed a strong calcium increase following odor application (Fig. 2A, B). However, due to interglomerular processing these responses were temporally more complex compared to the afferent input to the AL (Müller 1999; Abel et al. 2001; Sachse and Galizia 2002). PNs were spontaneously active (not shown) and showed odor responses consisting of excitatory and inhibitory phases. For example, the odor octanol elicited a weak on- and off-response (i.e. calcium increase after stimulus offset) in glomerulus 24; the latter is due to the release from inhibitory input. In contrast to the compound responses, application of histamine at a concentration of 10 mM strongly affected the PN signals (Fig. 2A, B). The spontaneous activity as well as the excitatory and inhibitory odor-induced responses were almost abolished in all glomeruli. In the wash a complete recovery of both spontaneous activity and odor responses could be observed, which were in a few cases even increased. The histamine effect could be observed in all animals measured (n = 5; Fig. 2C). Concentrations lower than 10 mM did not influence the calcium signals in any animal measured (data not shown).

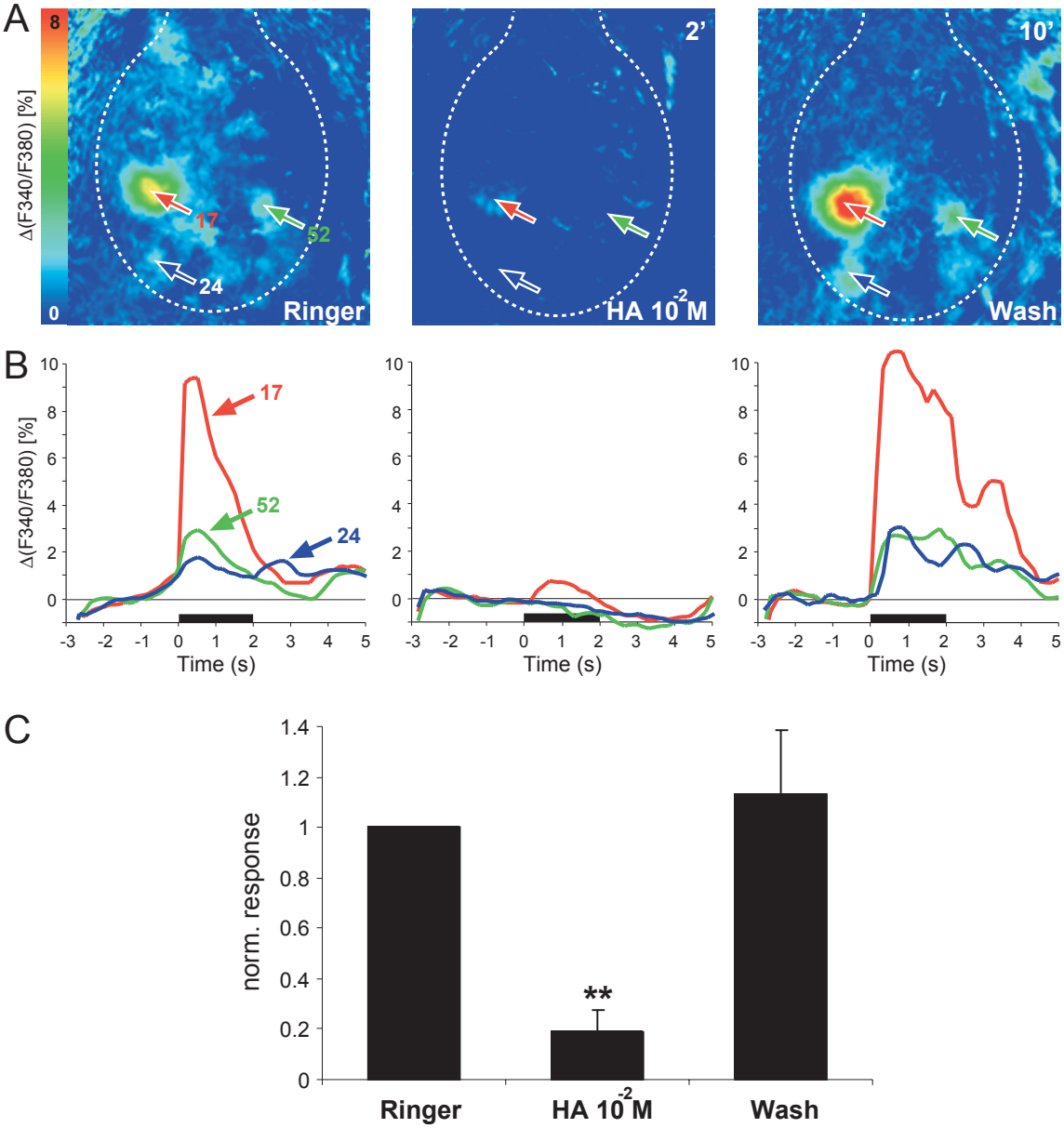

Figure 2 Influence of histamine on the odor-induced PN responses of the honeybee AL.
A, False-color coded spatial activity patterns to the odor 2-octanol before, during and after histamine application. Only one histamine concentration was tested in this animal. The AL border is marked with a dotted line, antennal nerve is at the top. The positions of three identified glomeruli are indicated in each frame. The numbers at the top right in each image indicate the time elapsed from the latest treatment. **B**, Time traces of the three identified glomeruli, whose positions are marked in A. Odor application is shown by a black bar. Contrary to the compound signals, PN responses were temporally complex and were strongly sensitive to a histamine concentration of 10 mM. The responses reappeared in the wash and were even stronger. **C**, Odor-induced PN responses averaged over all animals (mean and SEM, n = 5) of the most responsive glomeruli. Asterisks give significant differences to the Ringer measurement (**P < 0.01, two-tailed paired t-test, performed on the original data). Histamine significantly reduced the odor-evoked signals.

DISCUSSION

In this study we investigated the role of the transmitter histamine in the olfactory system of the honeybee. To this end, we optically recorded odor-evoked responses during histamine application either of the afferent input to the AL, estimated by a compound response, or of the AL output neurons (i.e. PNs). The results show that applying histamine to the bee brain led to a strong and reversible reduction of both the compound and the PN odor responses (Figs. 1, 2). Additionally, the spontaneous activity of PNs was totally abolished due to histamine. These findings are in line with electrophysiological recordings of lobster olfactory neurons, which reported that histamine application suppressed both spontaneous and odor-evoked activity in RNs (Bayer et al. 1989; Wachowiak and Cohen 1999) as well as electrically-induced responses in olfactory PNs (Wachowiak and Ache 1997). Our applied histamine concentrations were at least 10- to 50-fold higher than described elsewhere (Wachowiak and Ache 1997). However, those studies were carried out in isolated brain preparations in lobsters. In contrast we used an *in-vivo* preparation of the whole animal, so the actually effective histamine concentration at the cellular level is hard to estimate but is likely to be lower than the concentration applied to the whole brain.

The receptors on the lobsters RNs are histamine-gated chloride channels similar to those found in the fly visual system (McClintock and Ache 1989). In the honeybee the existence of histamine-sensitive chloride channels is also conceivable, but has not yet been shown. Interestingly, PNs were more sensitive to histamine than the compound signals. Several explanations could account for this difference. First, since the two preparations differ, diffusion barriers may differ too. Second, it is conceivable that along the pathway from RNs to PNs there are multiple occurrences of histaminergic channels. While the PN signal will be affected by all of them, the compound signal may be affected only by some. This may reduce the effect because of the resulting small numbers of synapses or also, because different populations of histaminergic receptors may have different affinities. In *Drosophila* visual system at least two histaminergic channels have been described (Gisselmann et al. 2002; Zheng et al. 2002). The fact that histamine totally abolishes responses in the compound signal indicates that there is direct histaminergic input onto RN synaptic terminals, such as is found in lobsters (Orona et al. 1990).

Taken together, our results indicate for the first time that histamine acts as an inhibitory transmitter in the honeybee's olfactory system. Thus, it is conceivable that GABA and histamine may be constituents of a dual system of inhibitory transmitters in the AL, similar to findings in lobsters (Wachowiak and Ache 1997; Wachowiak and Ache 1998). However, it is still unclear whether histaminergic and GABAergic LNs correspond to morphologically distinct LN types. Two types of LNs have been described in the honeybee: homo LNs and hetero LNs. In a previous study we have proposed that the homo LNs are GABAergic. This

would leave the hetero LNs as likely histaminergic neurons. These neurons have a very specific role in the olfactory code, since they specifically inhibit a limited number of glomeruli only when they are excited themselves (Sachse and Galizia 2002). There are insect species that apparently lack the histaminergic network (Nässel 1999), such as the locusts. While it may be possible that locusts use another transmitter for the same purpose, the difference in total count of LNs (~4000 LNs in honeybees vs. ~300 in locusts; Witthöft 1967; Laurent et al. 2001) make it more likely that olfactory processing and coding may follow different rules in different insect species, such as locusts and bees. Further immunocytochemical experiments using antibodies to GABA and histamine are needed to simultaneously identify and localize both GABA- and histaminergic inputs in the AL of a single animal. Moreover, pharmacological experiments with respective antagonists to histamine receptors will help to elucidate histamine's role in odor processing in the honeybee's olfactory system.

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