Inhibitory interactions among olfactory glomeruli do not necessarily reflect spatial proximity

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ABSTRACT

Inhibitory interactions shape the activity of output neurons in primary olfactory centers and promote contrast enhancement of odor representations. Patterns of interglomerular connectivity, however, are largely unknown. To test whether the proximity of glomeruli to one another is correlated with interglomerular inhibitory interactions, we used intracellular recording and staining methods to record the responses of projection (output) neurons (PNs) associated with glomeruli of known olfactory tuning in the primary olfactory center of the moth *Manduca sexta*. We focused on Toroid I, a glomerulus in the male-specific macroglomerular complex (MGC) specialized to one of the two key components of the conspecific females’ sex pheromone, and the adjacent, sexually isomorphic glomerulus 35, which is highly sensitive to Z-3-hexenyl acetate (Z3-6:OAc). We used the two odorants to activate these reference glomeruli and tested the effects of olfactory activation in other glomeruli. We found that Toroid-I PNs were not inhibited by input to G35, whereas G35-PNs were inhibited by input to Toroid-I PNs. We also recorded the responses of PNs arborizing in other sexually isomorphic glomeruli to stimulation with the sex pheromone and Z3-6:OAc. We found that inhibitory responses were not related to proximity to the MGC and G35: both distant and adjacent PNs were inhibited by stimulation with the sex pheromone, some others were affected by only one odorant, and yet others by neither. Similar results were obtained in female PNs recorded in proximity to female-specific glomeruli. Our findings indicate that inhibitory interactions among glomeruli are widespread and independent of their spatial proximity.
INTRODUCTION
In the primary olfactory centers of vertebrates (olfactory bulbs, OBs) and insects (antennal lobes, ALs), each glomerulus contains arborizations of, and synaptic connections among, olfactory receptor cells (ORCs), local interneurons (LNs), and projection (output) neurons (PNs) that send axons to higher brain centers (Boeckh and Tolbert 1993; Jefferis et al. 2002; Shepherd 1972; Tolbert and Hildebrand 1981). Because each type of ORC typically expresses one type of odorant receptor (OR), and the axons of ORCs expressing the same OR converge in the same glomerulus (Buck and Axel 1991; Gao et al. 2000; Mombaerts et al. 1996; Mombaerts 2004), a glomerulus is expected to reflect the odor-response profile of the ORCs converging in it (Root et al. 2007; Vosshall et al. 2000). Furthermore, odorants bind to different ORs with different affinities (e.g. Hallem and Carlson 2006; Malnic et al. 1999) and thus evoke characteristic patterns of glomerular activation (Belluscio and Katz 2001; Friedrich and Korsching 1998; Johnson et al. 1998; Sachse et al. 1999; Wang et al. 2003; Xu et al. 2000). Increasing evidence suggests, however, that glomerular output is refined by interglomerular interactions mediated by GABAergic, inhibitory LNs (Aungst et al. 2003; Friedrich and Laurent 2004; Kashiwadani et al. 1999; Nagayama et al. 2004; Schoppa 2006; Vucinic et al. 2006; Wilson et al. 2004; Silbering and Galizia 2007; Yokoi et al. 1995; Olsen and Wilson 2008). Examination of patterns of inhibitory glomerular interaction in various experimental systems has begun only recently: (a) in the OB, responses of mitral cells are modulated by odorants that activate neighboring glomeruli (Nagayama et al. 2004); (b) modeling studies in honey bees show that interactions between glomeruli are proportional to the similarity of their odor-response profiles regardless of their anatomical proximity (Linster et al. 2005), and optical imaging studies suggest the presence of both a glomerulus-specific and a global inhibitory network (Silbering and Galizia 2007); and (c) in the moth Manduca sexta, the male-specific glomeruli that process sensory information about two key components of the female’s sex pheromone reciprocally inhibit each other (Lei et al. 2002; Christensen and Hildebrand 1997). These findings suggest that interglomerular inhibitory interactions reflect functional relationships between glomeruli. In this study, we tested whether inhibitory interactions among glomeruli also depend on their spatial relationships. We recorded intracellularly the responses of PNs associated
with several neighboring, identified, sexually dimorphic and isomorphic glomeruli with characterized molecular receptive ranges in ALs of male and female *M. sexta*. We measured odor-driven synaptic events and spiking activity on a millisecond time-scale and therefore unambiguously could study odor-driven synaptic inhibition. One of these glomeruli (the Toroid I) belongs to the male-specific macroglomerular complex (MGC) and processes sensory information about one of the two key components of the conspecific females’ sex pheromone (Christensen et al. 1989). Each of the two key sex pheromone components exclusively activates one of two types of male-specific antennal ORCs, and the axons of each of those two types of ORCs project exclusively to one of the two principal MGC glomeruli (Christensen et al. 1995; Kaissling et al. 1989; Christensen and Hildebrand 1987). Therefore, by stimulating antennal inputs with these pheromone components, we could activate one or both MGC glomeruli and test the effect of that activation in other glomeruli. Glomerulus 35 (G35) is sexually isomorphic (i.e., its morphological features and physiological properties are equivalent in males and females), responds preferentially and highly sensitively to the plant-derived volatile compound Z-3-hexenyl-acetate (Z3-6:OAc), and is located adjacent to the MGC in males and to a female-specific glomerulus, the lateral large female glomerulus (latLFG), in females (Reisenman et al. 2005). Therefore, we could stimulate G35 and test the effect of this odorant activation in glomeruli of the MGC. The latLFG is homologous to the male-specific Toroid I (Rospars and Hildebrand 2000) and responds preferentially to stimulation of the antenna with the plant volatile [±]linalool (Roche King et al. 2000), and especially to the [+] enantiomer (Reisenman et al. 2004). We tested if inhibition is related to glomerular proximity by recording: (1) the responses of Toroid-I PNs to the two odorants that activate the adjacent MGC glomerulus (the Cumulus) and G35, (2) the responses of male uniglomerular PNs with arborizations in sexually isomorphic glomeruli (both close and distant to the MGC and G35) to stimulation with the odorants activating these glomeruli, and (3) the responses of female uniglomerular PNs in sexually isomorphic glomeruli to the odorants that preferentially activate the latLFG and G35.
MATERIALS AND METHODS

Preparation. Male and female Manduca sexta (L.) (Lepidoptera: Sphingidae), reared in the laboratory on artificial diet, were used 1–3 days after adult emergence. Animals were dissected and prepared for intracellular recording with established procedures (Roche King et al. 2000). After mechanical removal of the perineural sheath covering the AL, the preparation was continuously superfused with physiological saline solution containing (in mM): 150 NaCl, 3 CaCl₂, 3 KCl, 10 TES buffer (pH 6.9), and 25 sucrose (Christensen and Hildebrand 1987).

Stimulation. The stimulation procedure has been described elsewhere (Reisenman et al. 2004). Briefly, the cut end of one antenna was inserted into a glass capillary tube filled with physiological saline solution, which served both as a holder to position the antenna and as an electrode for monitoring antennal responses (amplified 50-fold with an amplifier, Model M-707, WPI, Sarasota, Florida) to olfactory stimulation. An L-shaped glass tube delivered a constant flow of humidified, charcoal-filtered air to the antenna (1.9 l/min). Plant odorants were injected (2 or 5 ml of odor-bearing air for 200– or 500–ms stimulations, respectively) into the constant air stream by means of a syringe olfactometer (Selchow 1998). Thus, odor stimuli injected (at a velocity of 10 ml/sec) into the air stream (flowing constantly at 32 ml/sec) were diluted approximately 1:4. Sexpheromone components were injected using a solenoid-activated valve (3.8 or 9.6 ml of odor-bearing air for 200– or 500–ms stimulations, respectively). The tip of the syringe containing the odor stimulus was inserted into the air stream through a small hole in the side of the glass tube. In this case, odor stimuli were diluted approximately 1:2.5. A funnel connected to a negative-pressure line was positioned near and behind the preparation to remove stimulus volatiles after delivery to the antenna.

The plant-derived odor compounds used in this study were: [±]linalool ([±]3,7-dimethyl-1,6-octadien-3-ol, catalog no. L2602, 97% pure from Sigma-Aldrich (St. Louis, MO), and Z-3-hexenyl acetate (catalog no. H2137, >97%, hereinafter referred to as Z3-6:OAc (Tokyo Chemical Industries, Tokyo, Japan). These odor compounds are found among the volatiles emitted by hostplants of M. sexta (Fraser et al. 2003; Loughrin et al. 1990; Raguso et al. 2003) and have been shown to evoke responses from antennae (Fraser et al. 2003; C. E. Reisenman, unpublished observations) and/or from antennal
ORCs in type-A trichoid sensilla (Shields and Hildebrand 2001). Plant-associated volatile compounds were diluted in odorless mineral oil (Sigma-Aldrich, St. Louis, MO) and prepared as described elsewhere (Reisenman et al. 2004). Dilutions ranged from $10^{-4}$ to $10^{-2}$ (vol/vol). Fifty $\mu$l of solution were applied to a disk of filter paper inserted into a 20-ml stimulus syringe; control syringes contained 50 $\mu$l of mineral oil alone.

The two main components of the sex-pheromone (E10,Z12-16:Al \textit{[bombykal]}, and E10,E12,Z14-16:Al or its more chemically stable, biologically active mimic, E11,Z13-15:Al \textit{[“C15”]}, Kaissling et al. 1989) were obtained from Dr. Jocelyn Millar, (University of California, Riverside, CA). Single components or a blend (1:1) of the two were diluted in cyclohexane and applied (2–500 ng) to a piece of filter paper (0.5 cm$^2$), which was inserted into a 1-ml glass syringe. In each experiment we first stimulated the antenna with control stimuli, followed by a low stimulus concentration/duration of pheromone components or the blend. If no obvious (mostly inhibitory) responses were observed, the stimulus concentration and/or duration were increased up to a maximum of 500ng/500ms. Therefore, figures present results from neurons stimulated with different concentrations/durations. Hibiscus and ylang-ylang oils (from Select Oils Inc, Prairie Grove, AR), which contain many odor compounds, were used to evoke measurable antennal responses, widely activate the AL network, and test responsiveness of neurons when no obvious responses were observed upon stimulation with the sex pheromone or Z3-6:OAc. Male and female PNs, respectively, were stimulated an average of 4.3 ± 0.18 and 3.61 ± 0.16 (means ± SE) times with each concentration of odorant and odor stimuli.

\textit{Intracellular recording and staining.} Sharp microelectrodes were made from borosilicate glass capillaries with filament (1 mm outer diameter, 0.58 or 0.75 mm internal diameter, Sutter Instruments Co., Novato, CA) on a laser puller (P-2000, Sutter Instruments Co., Novato, CA). The tip of the micropipette was filled with a solution of Lucifer Yellow CH (65 mM, Sigma-Aldrich, St. Louis, MO) in 200 mM LiCl, or with a solution of Alexa Fluor 568 hydrazide (10 mM in 200 mM KCl, Molecular Probes, Eugene, OR), and the shaft, with 2 M LiCl; microelectrodes had resistances in the range 100–350 M$\Omega$. Microelectrodes were manipulated into the glomerular region of the AL above the known location of the MGC and G35, so that MGC-PNs and G35-PNs, or PNs in neighboring glomeruli or distant glomeruli could be impaled. The responses of an
impaled neuron to stimulation of the ipsilateral antenna were amplified 10-50 fold with an amplifier (Axoclamp-2A, Axon Instruments, Molecular Devices, Sunnyvale, CA) coupled to a DC amplifier (LPF 202A, Warner Instruments, Hamden, CT), and digitized at 20 kHz (via a Digidata 1200 series Interface, Axon Instruments, Foster City, CA, or Datapack, Run Technologies, Mission Viejo, CA). Data were analyzed with programs written in Matlab (The Mathworks, Natick, MA).

After physiological characterization, neurons were injected with either Lucifer Yellow or Alexa 568 (see above) by passing hyperpolarizing current (0.2–1 nA) for 6–40 min. Different dyes were used in cases in which recordings were obtained from more than one neuron in the same preparation. The duration of intracellular impalements, including both recording and dye-injection, was variable (15–20 min, 50 min maximum). Upon completion of an experiment, the brain was excised and immersed in 2.5% formaldehyde fixative solution (pH 7.2) for ≥3 hrs, dehydrated through a graded series of aqueous ethanol solutions (from 50% to 100%), and cleared with methyl salicylate (Sigma-Aldrich, St. Louis, MO). Cleared brains were imaged as whole mounts with a laser-scanning confocal microscope (Nikon PCM 2000 or Carl Zeiss 510 Meta, both equipped with a 457-nm Argon laser and a 543-nm Green HeNe laser). When glomerular boundaries could not be visualized in whole mounts, brains were returned to 100% ethanol and embedded in Spurr’s resin (Electron Microscopy Sciences, Ft. Washington, PA) for sectioning at 48 μm and then imaged again.

Data analysis. At the end of the experiment we stimulated the antenna with neat hibiscus or ylang-ylang oil, which unambiguously evoked an antennal response. We thus established the earliest time at which any given odor stimulus could reach the antenna in each preparation. This time, so established for each preparation, was used to calculate the physiological response parameters we describe next. The net number of spikes was calculated by subtracting the number of spikes in a 1-s period before antennal stimulation from the number of spikes counted in a 1-s period post-antennal stimulation. The membrane potential deflection was calculated as the change in potential occurring in a period of up to 2.2 s after the onset of antennal stimulation. Negative and positive deflections indicate hyperpolarization and depolarization, respectively. Although the selection of this time window was arbitrary, our observations indicate that
hyperpolarizations or depolarizations occurring after that time period cannot be attributed to odorant stimulation. Moreover, we found that most odor-evoked changes in membrane potential occurred within 1.5 s after antennal stimulation. In the case of stimulation with control stimuli, we calculated the average membrane potential in a 1-s period after antennal stimulation and subtracted this value from the average resting potential. The net number of spikes and amplitude of the membrane hyperpolarization (or depolarization) were averaged for each combination of PN and stimulus.

To analyze the time course of the spiking response, the spiking activity in a 1-s period after antennal stimulation was divided into 20 50-ms bins. The resting spiking activity was calculated by dividing the 1-s period before odor stimulation into 20 50-ms bins, and the 20 values so obtained were averaged. This single value was subtracted from the activity in each of the 20 50-ms bins of the 1-s period after antennal stimulation. Values were averaged for each combination of PN and stimulus.

Differences between two means were compared with the aid of Wilcoxon-matched pairs tests or Mann Whitney U-tests (Zar 1999). Results were considered statistically significant if p<0.05.

RESULTS

Results were obtained from a total of 40 PNs in 38 males (neurons from the same animal were stained with different dyes), 9 PNs in 9 females, and 4 LNs in 4 females. Some male PNs could not be identified morphologically with certainty (e.g., owing to incomplete staining, in which cases the text refers to “putative” Toroid-I PNs or G35-PNs) but were recognized on the basis of their physiological response properties. Toroid I- PNs were excited by antennal stimulation with E10,Z12-16:Al and were inhibited (or did not respond) to antennal stimulation with E10,E12,Z14-16:Al (the pheromone component that activates MGC-PNs with arborizations in the Cumulus; Heinbockel et al. 1999; Lei et al. 2002) or its more chemically stable, biologically active mimic, E11,Z13-15:Al [“C15”]. G35-PNs were strongly excited by stimulation with Z3-6:OAc, less excited by butyrate and propionate homologs, and not excited by linalool (Reisenman et al. 2005). PNs can be distinguished from LNs in intracellular recordings on the basis of one or more of the following properties: odor-evoked triphasic response (early, rapid
hyperpolarization followed by depolarization with spiking and, thereafter, by long-lasting hyperpolarization), spikes with smaller width at half amplitude than those of LNs, much higher spiking frequency (up to 250 s⁻¹) in response to odor stimulation, and firing irregularity (unpublished observations and Christensen et al. 1993).

Figure 1 shows the morphological features and physiological responses of PNs with dendritic arborizations restricted to three neighboring glomeruli: the Cumulus (Fig. 1A) and Toroid I (Fig 1B) of the MGC, and glomerulus 35 (G35, Fig. 1C). Cumulus-PNs were excited by antennal stimulation with C15 (Fig. 1D, top) and in most cases they were hyperpolarized and their spike activity suppressed in response to stimulation with E10,Z12-16:Al (bombykal; Fig. 1D, middle), the pheromone component that activates PNs in the adjacent Toroid I (Fig. 1D, bottom). Likewise, PNs arborizing in Toroid I were excited and inhibited, respectively, by stimulation with bombykal (Fig. 1E, middle) and C15 (Fig. 1E, top). Although these findings have been reported elsewhere (e.g. Heinbockel et al. 1999; Lei et al. 2002), here we replicated some of the experiments with pheromone components as positive controls; i.e., we confirmed that Toroid-I PNs are inhibited by activation of the Cumulus. This ensured that any lack of inhibitory responses in Toroid-I PNs upon stimulation of G35 or any glomeruli activated by Z3-6:OAc was not due to uncontrolled factors or low concentration of odorants. PNs with arborizations restricted to G35 were excited by stimulation with the plant odorant Z3-6:OAc (Fig. 1F, bottom) and were hyperpolarized by stimulation with either C15 or bombykal (Fig. 1F, top and middle). In contrast, Toroid-I PNs were not affected by stimulation with Z3-6:OAc (typical examples are shown in Figs. 1D and E, bottom), even when this odorant was tested at a concentration (10⁻² vol/vol) at least two orders of magnitude above the sensitivity threshold of G35-PNs (Reisenman et al. 2005).

Figure 2A shows a quantitative population analysis of the odor responses of Toroid-I PNs. The response of these PNs to antennal stimulation with C15 (which activates inputs to the Cumulus) was statistically different from the response to the respective control (Wilcoxon matched pair tests, p<0.05). When the antenna was stimulated with C15 the net number of spikes was lower and the hyperpolarization amplitude was larger than when stimulated with the control cyclohexane stimulus (left parts of Figs. 2A and B). In contrast, the response to Z3-6:OAc was not statistically
different from the response to the mineral-oil control (p>0.05 in both cases, right parts of Figs. 2A and B). The hyperpolarization evoked by the cyclohexane control stimulus was not different from that evoked by the mineral-oil control (Wilcoxon matched-pairs tests, p>0.5), and the response to either control was not statistically different from zero (Student’s t-test for differences between an observed mean and an hypothesized population mean; p>0.5 in both cases). Thus, although Toroid-I PNs receive inhibitory input from the adjacent Cumulus, they were not inhibited by input to the adjacent G35 or any other putative Z3-6:OAc-activated glomeruli. This finding is not due to weak excitatory input to G35, as lower concentrations of Z3-6:OAc elicited hyperpolarization in other glomeruli (Fig. 3B-D). We tested a subset of 6 Toroid-I PNs with antennal stimulation using neat hibiscus oil, which is a mixture of many plant volatiles and thus activates sensory inputs to many glomeruli, and observed obvious suppression of spiking activity and hyperpolarization in 50% of PNs. The net number of spikes and hyperpolarization amplitude (control subtracted, mean ± SEM, n=6) were respectively –6.3 ± 3.7 spikes and –2.2 ± 0.84 mV. This result indicates that Toroid-I PNs receive inhibitory input from at least some sexually isomorphic glomeruli (activated by one or more of the constituents of hibiscus oil).

We observed that the inhibitory responses of individual Toroid-I PNs were variable in temporal profile, some having more prolonged inhibitory epochs or longer delays to the onset of the inhibition than others, and some not showing obvious suppression of spiking. To address the possibility that we underestimated the degree of inhibition in our population analysis by calculating spiking activity in a relatively long time period (1 s, Fig. 2A), we analyzed the spiking activity of each PN in 20 50-ms bins. The response to stimulation with C15 was statistically different from the control in 11 time bins (asterisks, Fig. 2C, top), started about 300 ms after the odor reached the antenna, and lasted about 500–600 ms. By contrast, stimulation with Z3-6:OAc did not cause reduction in spiking activity (the response was statistically different in only one out of 20 bins, which is the false-positive rate expected under a p=0.05 criterion; Fig. 2C, bottom). Although Z3-6:OAc might activate other glomeruli besides G35 (Reisenman et al. 2005), this negative result allowed us to conclude unambiguously that Toroid-I PNs do not receive inhibitory input from G35 or from any other glomerulus activated by Z3-
6:OAc. We recorded the activity of a small subset of male G35-PNs (n=5) to Z3-6:OAc and to the two key sex-pheromone components and/or a blend of the two. All G35-PNs, as expected (Reisenman et al. 2005), were excited by antennal stimulation with Z3-6:OAc, but were hyperpolarized by stimulation with either of the two key components of the sex-pheromone blend (an example is shown in Fig. 1F; results from responses of three G35-PNs to stimulation with the pheromone blend are included in the analysis presented in Fig. 4A-B). Because pheromone-responsive ORCs project exclusively to the MGC glomeruli (Christensen et al. 1995; Kaissling et al. 1989; Christensen and Hildebrand 1987), these results indicate that G35-PNs are inhibited by input to either one or both of the main MGC glomeruli (Cumulus and Toroid I). Because we have shown that the opposite is not true (activation of G35 did not inhibit Toroid-I PNs; Fig. 2), these results suggest that inhibitory interactions between the MGC and G35 are not reciprocal.

We next asked if inhibitory interactions among the MGC and sexually isomorphic glomeruli, or between sexually isomorphic glomeruli, are general phenomena. We recorded the activity of 11 and 14 uniglomerular PNs with arborizations in glomeruli adjacent (or 1-2 glomeruli away) and more distant to the MGC and G35, respectively, in response to antennal stimulation with the sex-pheromone blend and Z3-6:OAc. Two examples of each of these PNs are shown in Fig. 3G-J. All 25 PNs were stained intracellularly, so that in each case the position of the glomerulus containing the arborizations of the recorded PN with respect to the MGC and G35 could be precisely established. We found that some PNs were inhibited by stimulation with both the sex-pheromone blend and Z3-6:OAc regardless of their proximity to the MGC and G35 (e.g., Fig. 3A and 3D show examples of two PNs with arborizations respectively in glomeruli adjacent and distant to the MGC). Other PNs were inhibited by stimulation with Z3-6:OAc but not by stimulation with the sex pheromone (e.g. Fig. 3B and 3F), and vice-versa (e.g. Fig. 3C). Again, this did not depend on the position of the glomerulus from which the recording was obtained. Other PNs were not inhibited by either odor stimulus (e.g. Fig. 3E), even when odorants were tested at high concentrations. In particular, the finding that a lower concentration of Z3-6:OAc (10⁻³ vol/vol) than that used to stimulate Toroid-I PNs (10⁻² vol/vol) was sufficient to hyperpolarize some PNs in sexually isomorphic glomeruli (Fig. 3A and 3D), confirms our conclusion about the lack of
inhibitory input from G35 (or any Z3-6:OAc) on Toroid-I PNs. At the end of the experiment all PNs were stimulated with hibiscus or ylang-ylang oil to test that they were responsive to odor stimulation even if no response was observed to stimulation with the sex-pheromone blend or Z3-6:OAc.

Figure 4 shows a quantitative population analysis of the responses of PNs with arborizations restricted to sexually isomorphic glomeruli to stimulation with the pheromone blend and Z3-6:OAc, respectively. In this figure, each symbol represents the response (control-subtracted for clarity) of one PN; the closed symbol represents the average across PNs. The scattering of data points in this figure illustrates the variability of responses to odor stimulation in PNs with arborizations in glomeruli both adjacent to and distant from the MGC and G35. Despite this variability, the net number of spikes and hyperpolarization amplitude evoked by stimulation with the sex-pheromone blend was statistically different from the respective control in PNs in both neighboring (n=11 PNs, including three G35 PNs, Wilcoxon matched pairs tests, p<0.05) and distant (n=14 PNs; p<0.05) glomeruli. The net number of spikes and hyperpolarization amplitude evoked by pheromone stimulation were not different between neighboring and distant PNs (Mann Whitney-U tests, p<0.05). These results indicate that pheromonal stimulation inhibits PNs in sexually isomorphic glomeruli regardless of their distance to the MGC (Fig. 4A-B). The response of PNs to stimulation with Z3-6:OAc was also variable, as indicated by the scattering of data points in Figure 4C-D (the responses of G35-PNs are not included in these panels because stimulation with Z3-6:OAc caused a strong excitatory response in these PNs). The net number of spikes and hyperpolarization amplitude of PNs close to the MGC and G35 was not statistically different from the response to the respective control (Wilcoxon matched pairs tests, p>0.05, Fig. 4C-D). In contrast, stimulation with Z3-6:OAc caused suppression of spikes and hyperpolarization in many PNs with arborizations in glomeruli distant from the MGC and G35 (Fig. 4C-D, right). The net number of spikes and hyperpolarization amplitude were statistically different from the respective control (Wilcoxon matched pairs tests, p<0.05). Because Z3-6:OAc might also activate glomeruli other than G35 (Reisenman et al. 2005), we cannot conclude that the inhibition observed by Z3-6:OAc stimulation in these PNs necessarily reflects connectivity between G35 and the recorded glomerulus. Specific connectivity patterns
can be established with certainty only in cases in which we did not observe hyperpolarization and/or suppression of spiking (e.g. Fig. 3C, E, F; PNs with values $\approx 0$ in Fig. 4, and Toroid-I PNs, Fig. 2).

We also recorded the activity of nine female PNs with arborizations in glomeruli near the sexually dimorphic latLFG and the sexually isomorphic G35 to stimulation with the odorants that preferentially activate those glomeruli. Figures 5A-B show an example of such a PN that was hyperpolarized by both $[\pm]$linalool (which stimulates latLFG-PNs, Roche King et al. 2000) and Z3-6:OAc (which stimulates G35-PNs, Reisenman et al. 2004). This example illustrates that at least certain PNs in sexually isomorphic glomeruli in the female AL might receive inhibitory input from the sexually dimorphic glomeruli and G35 (or by glomeruli activated by $[\pm]$linalool and Z3-6:OAc, respectively). Overall, more PNs were inhibited by stimulation with $[\pm]$linalool than with Z3-6:OAc: 66% and 33% of PNs, respectively, were obviously hyperpolarized by these odorants. The hyperpolarization evoked by $[\pm]$linalool (but not by Z3-6:OAc) was statistically different from the control (p<0.05, asterisk, Fig. 4C). Thus, it appears that activation of the latLFG and/or other glomeruli with linalool (this odorant can activate PNs in other glomeruli besides the latLFG, Reisenman et al. 2004), has a stronger inhibitory effect in neighboring, sexually isomorphic glomeruli than activation of G35 or any other glomeruli activated by Z3-6:OAc.

**DISCUSSION**

In this study we tested the whether spatial relationships between olfactory glomeruli determine interglomerular inhibitory interactions. To this end, we used specific odorants (sex-pheromone components and Z3-6:OAc) to activate PNs in morphologically and functionally identified reference glomeruli (the MGC glomeruli and G35, respectively) and tested the effect of this olfactory activation in neighboring and distant glomeruli. We found that interglomerular inhibitory interactions occur throughout the glomerular array in the AL and are not simply a reflection of the spatial relationships among glomeruli. While PNs in Toroid I of the MGC were inhibited by activation of the neighboring MGC Cumulus, these MGC-PNs were not inhibited by antennal stimulation with Z3-6:OAc (Figs. 1-2), i.e., by activation of input to the neighboring G35 glomerulus.
We recorded the responses of PNs arborizing in sexually isomorphic glomeruli (other than G35) to stimulation with the sex pheromone and Z3-6:OAc and found that inhibitory responses were not related to proximity to the MGC and G35: both distant and adjacent PNs were inhibited by stimulation with the sex pheromone, some others were affected by only one odorant, and yet others by neither (Figs 3-4). Similar results were obtained in female PNs recorded in the proximity of female-specific glomeruli. These results indicate that inhibitory interglomerular interactions are not determined by proximity of the glomeruli involved in these interactions.

It is thought that a major function of inhibition in the vertebrate OB is sharpening glomerular output through two OB neuronal circuits. One circuit involves reciprocal synapses between the lateral dendrites of output neurons (mitral cells) and GABAergic local interneurons (granule cells), and the other circuit involves inhibitory interactions between glomeruli (Vucinic et al. 2006; Halabisky and Strowbridge 2003; Egger et al. 2003; Schoppa and Urban 2003). Thus, in the OB, activation of short-axon cells mediates “center-surround” inhibition of distal mitral cells (Aungst et al. 2003). These in vitro studies, using electrical stimulation in OB brain slices, revealed synaptic circuits underlying inhibition, but they did not use behaviorally relevant stimuli and did not investigate functional interglomerular interactions. Testing patterns of interglomerular connectivity requires knowledge of the natural odor stimuli that activate at least some identifiable glomeruli, a requisite met by the *M. sexta* olfactory system. In our experiments, we activated a reference glomerulus (e.g. Toroid I) and recorded the activity of PNs in distant and neighboring glomeruli. We could study such interactions unambiguously because the two key sex-pheromone components exclusively activate male-specific ORCs that project exclusively to and activate only the two principal MGC glomeruli (Christensen et al. 1995; Kaissling et al. 1989; Christensen and Hildebrand 1987). The techniques used in this study allowed us to measure synaptic events (hyperpolarization) and spiking activity in single neurons with millisecond resolution and to determine the glomerular associations of the recorded PNs.

As expected, Toroid-I PNs and G35-PNs were strongly excited by antennal stimulation with their respective, preferred odor compounds (bombykal and Z3-6:OAc, respectively). Stimulation with E10,E12,Z14-16:Al or its chemical mimic C15 elicited
hyperpolarization and suppression of spiking in Toroid-I PNs (Figs. 1-2). Although this finding has been reported previously (Heinbockel et al. 1999, 2004), we repeated some of the earlier experiments to validate the findings discussed below. We found that Toroid-I PNs were not inhibited by stimulation with Z3-6:OAc (Figs. 1E, 2), an odorant that preferentially activates PNs in the adjacent G35 glomerulus. This negative result unambiguously demonstrated that MGC-PNs do not receive inhibitory input from G35 or from other glomeruli that might be activated to some extent by Z3-6:OAc. The fact that Toroid-I PNs were inhibited by stimulation with a blend of odorants (hibiscus oil), however, demonstrates that these PNs can receive inhibitory input from glomeruli other than G35 or upon stimulation with a high concentration of odorant mixtures. In contrast, G35-PNs were hyperpolarized by antennal stimulation with sex-pheromone components (Fig. 1F). This demonstrates that interactions between glomeruli do not have to be reciprocal in nature. The generality of this finding must be validated by testing the interactions among other glomeruli of characterized odor input.

What might be the functional consequences of such non-reciprocal interaction? In the MGC, reciprocal inhibition between the Cumulus and Toroid-I synchronizes the outputs of the glomeruli processing information about the key components of the sex-pheromone blend (Lei et al. 2002) and thus might “bind” the features of the blend in higher brain centers downstream from the AL. Our findings suggest that the naturally occurring, simultaneous presence of sex pheromone and plant odors such as Z3-6:OAc would not lead to inhibition of the responses of MGC-PNs to sex-pheromone stimulation. Because we found that antennal stimulation with sex-pheromone components inhibits PNs in sexually isomorphic glomeruli (Figs. 1F, 3-4), this hyperpolarization could enhance the responses of these PNs to their preferred odor input when the two odorants concurrently stimulate the antenna. We have observed that in hyperpolarized PNs the responses to their preferred odor input is stronger than in PNs at their resting membrane potential (unpublished observations). Another possibility is that activation of the sexually dimorphic glomeruli “shuts down” responses in glomeruli involved in detection and discrimination of plant odors, which in the presence of a conspecific female may become behaviorally less significant to a male moth. These ideas are supported by our finding that stimulation of the sexually dimorphic glomeruli has a strong inhibitory effect in PNs
in sexually dimorphic glomeruli (Figs. 3-4). Odor input to the brain might be processed in a hierarchical manner such that processing of odorants involved in reproductive behaviors takes priority over processing of food-related odorants (plant volatiles). These speculations remain to be tested both physiologically and behaviorally, e.g., by concurrent stimulation with sex pheromone and plant odorants. Moreover, the finding that MGC-PNs were inhibited by sexually isomorphic glomeruli upon antennal stimulation with high concentrations of complex odor blends (hibiscus oil), merits further study of the relationship between the sexually dimorphic and isomorphic olfactory subsystems.

Our recordings from PNs with arborizations in glomeruli adjacent to and distant from the MGC and G35 show that inhibitory interactions are not related to glomerular proximity. Overall, we found that most PNs, irrespective of their position, were inhibited by antennal stimulation with the sex-pheromone blend (Fig. 4A-B). Some PNs, mostly those in glomeruli distant from the MGC and G35, also were inhibited by stimulation with Z3-6:OAc (Figs. 3-4). Other PNs were inhibited by stimulation with Z3-6:OAc but not by stimulation with the sex pheromone (e.g., Fig. 3B and 3F). Again, this did not depend on the positions of the glomeruli containing the arborizations of the recorded PNs. Because pheromone-responsive ORCs project exclusively to the MGC, we could unequivocally establish whether or not PNs in the recorded glomeruli received inhibition from the MGC. In contrast, Z3-6:OAc activates other glomeruli in addition to G35, albeit to a lesser extent (Reisenman et al. 2005). Therefore, we could establish conclusive connectivity patterns between G35 and the recorded glomerulus only in those cases in which we did not observe inhibition. For instance, we found that some PNs were inhibited by stimulation with sex pheromone but not by Z3-6:OAc (e.g., Fig. 3C), and other PNs were not inhibited by either odorant (e.g., Fig. 3E-J) even when odorants were tested at high concentrations. Therefore, we can conclude unambiguously that these PNs do not receive inhibitory input from G35 or any other Z3-6:OAc-sensitive glomerulus.

In female *M. sexta*, we found that antennal stimulation with [±]linalool (which activates PNs in the female-specific latLFG, Roche King et al. 2000; Reisenman et al. 2004) produced hyperpolarization in most recorded PNs associated with neighboring glomeruli (Fig. 5). Z3-6:OAc stimulation (which activates the adjacent G35, Reisenman
et al. 2005) produced hyperpolarization in some PNs (Fig. 5B), but the response across PNs was not statistically different from the control (Fig. 5C). Furthermore, we have previously shown that G35-PNs are hyperpolarized by antennal stimulation with [±]linalool (Reisenman et al. 2005). Caution should be taken, however, in interpreting these results in terms of glomerular connectivity, because these odorants also activate – to a lesser extent – PNs in other glomeruli. Nevertheless, these results parallel our findings in males, showing that activation of the sexually dimorphic subsystem appears to have a stronger inhibitory effect in sexually isomorphic glomeruli than does stimulation with Z3-6:OAc.

The finding that activation of Toroid-I PNs (Figs. 3-4) inhibited PNs in sexually isomorphic glomeruli shows that interglomerular inhibition extends beyond a cluster of functionally related glomeruli (in this case, the MGC). We observed that Toroid-I PNs received stronger inhibitory input (i.e., larger hyperpolarization) from the Cumulus than did sexually isomorphic PNs from the MGC (compare Figs. 2 and 4). Thus it is possible that sex-pheromonal inhibition has dual roles: one to promote synchronous firing of PNs within a glomerular cluster that processes components of the sex-pheromone blend (Lei et al. 2002), and the other to promote overall inhibition through a mechanism resembling contrast enhancement. Recent studies in D. melanogaster similarly have proposed the parallel existence of glomerulus-specific and global inhibitory networks (Silbering and Galizia 2007).

In both D. melanogaster and M. sexta, odor stimulation can cause suppression of spiking in ORCs (Hallem et al. 2004; Shields and Hildebrand 2001). Because in M. sexta ORCs projecting to sexually isomorphic glomeruli are insensitive to sex-pheromone components (Kalinová et al. 2001; Shields and Hildebrand 2001; B. Kalinová, personal communication), the inhibitory responses we observed in sexually isomorphic glomeruli probably are mediated by inhibitory LNs. In any case, inhibition of ORCs would produce a decrease in the amount of neurotransmitter released by the presynaptic terminal. Since ORCs are excitatory (e.g. Berkowicz et al., 1994; Squire et al., 2003; Nickell et al., 1996), this would at most cause a reduction of spiking activity in postsynaptic AL neurons (but not hyperpolarization). Reduction of spiking activity in PNs also could be caused by other central mechanisms such as presynaptic inhibition of ORC terminals.
mediated by GABAergic interneurons (Schoppa and Urban 2003; Olsen and Wilson 2008).

The hypothesis that glomeruli are organized in synaptically interacting clusters is supported by the fact that in *M. sexta* many LNs, the main inhibitory elements in the AL (Hoskins et al. 1986; Christensen et al. 1993), have primary neurites that arborize in only a few glomeruli (Matsumoto and Hildebrand 1981; Fig. 6C-D). These LNs can serve as neural substrates for the selective inhibitory interglomerular interactions described above. Although further studies of the synaptic architecture of LNs (e.g., location of input and output synapses) are needed to test this hypothesis, the arborization pattern of such LNs provides at least a neural connection among the glomeruli that process the individual components of an innately significant odor blend. Moreover, each glomerulus receives arborizations from LNs arborizing in different but overlapping sets of glomeruli, and this pattern could provide a combinatorial scheme for coding of odor blends. In *M. sexta* (Fig. 6A-B) and other insects, however, there are LNs that arborize in all of the glomeruli (Matsumoto and Hildebrand 1981; Abel et al. 2001; Wilson and Laurent 2005). In *D. melanogaster*, Wilson and Laurent (2005) found that each LN releases GABA widely throughout the AL, but apparently it does so non-uniformly. In *M. sexta* this type of wide-field LN (e.g. Fig. 6A, B) might have a different function (e.g. overall gain control) than more restricted types such as the one shown in Fig. 6C. These different morphological types of LNs could provide an anatomical substrate for the two parallel inhibitory networks, one that acts globally and one that is glomerulus-specific as proposed for the *D. melanogaster* AL (Silbering and Galizia 2007). Future studies of the odor-response properties and specificity of such different kinds of LNs, and of the patterns of synaptic connectivity among LNs and PNs, should clarify the issue. Both vertebrate-like GABA_A (ionotropic) and GABA_B (metabotropic) receptors shape PN odor responses (Root et al. 2007; Wilson and Laurent 2005; Christensen et al. 1998; Silbering and Galizia 2007; Olsen and Wilson 2008). The kinetics of the odor-evoked interglomerular inhibition we observed in *M. sexta* PNs (slow, long-lasting) suggest that this inhibition is likely mediated by GABA_B receptors, but this remains to be investigated. A recent study in *D. melanogaster* described a population of excitatory (cholinergic) LNs, and thus added a new neural element to the AL circuitry in this insect
species. The authors suggested that such excitatory LNs could augment PN output through lateral excitation (Shang et al. 2007). In this regard, Olsen et al. (2007) showed that PNs receive indirect excitatory input from other glomeruli, and found that the spatial relationships among glomeruli do not predict the strength of these excitatory interactions. These results contrast with those from a recent study in the species, which found that ORCs are the main source of excitatory input to PNs, and that lateral inhibition is prevalent in the AL (Root et al. 2007).

Overall, our results indicate that inhibitory interglomerular interactions are globally distributed in the AL and cannot be explained simply by spatial proximity of the interacting glomeruli. The rules that govern these interactions (e.g., response properties, chemical relatedness, or other functional relationships) merit further study. We expect that patterns of glomerular interaction will be found to reflect a functional and biologically relevant organization of glomeruli since integration of signals among glomeruli is an important mechanism for processing of olfactory information about behaviorally significant, naturally occurring odor blends.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1:** Morphological features and typical odor responses of PNs arborizing in three neighboring glomeruli in the antennal lobe (AL) of male *M. sexta*. The Cumulus (A) and Toroid I (B) are the two main glomeruli of the male-specific macrogglomerular complex (MGC); the adjacent glomerulus 35 (G35, C) is a sexually isomorphic glomerulus. MGC-PNs and G35-PNs have their cell bodies respectively in the medial group of AL neuronal cell bodies (MC in A, B) and in the anterior group (AC in C). Electrophysiological traces obtained from a Cumulus-PN (D), a Toroid-I PN (E), and a G35-PN. All PN classes gave excitatory responses to stimulation with their respective key odor inputs, the sexpheromone components E10,E12,Z14-16:Al (here substituted by the stable chemical mimic, E11,Z13-15:Al or “C15”) and E10,Z12-16:Al (bombykal), and the plant volatile Z-3-hexenyl acetate (Z3-6:OAc). Cumulus-PNs and Toroid-I PNs respectively were inhibited by stimulation with bombykal and C15 (D-E, deflections below the resting membrane potential indicated by the horizontal dashed lines), i.e. each by excitatory input to the other MGC glomerulus. These PNs were not inhibited by Z3-6:OAc (D, E, third row), the compound that activates PNs in the adjacent G35. Stimulation with C15 or bombykal inhibited G35-PNs (F, first and second rows). The solvents used to dilute the pheromone components and Z3-6:OAc (cyclohexane and mineral oil, respectively – solvent controls) elicited no response (not shown in this figure). The solid line below each record shows the activation of the device controlling the stimulus delivery system and the duration (200 ms) of the stimulus. Concentrations or amount of compounds are indicated between parentheses below each record. Calibration: 10 mV, 500ms. D: dorsal, L: lateral. Scale bars: 150 μm.
**Figure 2:** Quantification of the responses of Toroid-I PNs (left, mean ± SEM, n=11 PNs) to stimulation with bombykal (bomb), C15, Z3-6:OAc, and the two respective solvent controls (cyclohexane in the case of pheromone components, mineral oil in the case of Z3-6:OAc). **A.** The net number of spikes in a 1-s period after the onset of antennal stimulation. **B.** The amplitude of the odor-evoked deflection (negative values indicate hyperpolarization). PNs were stimulated with 20 ng of bombykal (except 1 PN was stimulated with 2 ng), and with 20, 200, or 500 ng of C15 loaded on filter paper. Stimulus duration was 200 ms (except 500 ms in 3 PNs). Asterisks indicate significant differences between C15 and the respective control (Wilcoxon matched pairs tests; *: p<0.05, ***: p<0.005). In neither case was the response to Z3-6:OAc statistically different from the response evoked by mineral oil (Wilcoxon matched pairs tests: p>0.05). **C.** Time course of the responses of Toroid-I PNs. The net number of spikes (mean ± SEM, n=8 PNs) during a 1-s period after the odor had reached the antenna was broken down into 20 50-ms bins. First row: net response to stimulation with the C15 (black symbols) and the cyclohexane control; second row: net response to stimulation with Z3-6:OAc 10⁻² vol/vol (black symbols) and the mineral oil control. This panel includes only those PNs that were stimulated with 200-ms pulses. Asterisks indicate statistical differences between the response to the odorant and the respective control for each time bin (Wilcoxon matched pair tests, p<0.05). Note that the response to C15 was statistically different from that to the control in 11 consecutive time bins. The response to Z3-6:OAc was different from the response to the control in only one of the 20 time bins, which is the false positive rate expected under a p=0.05 criterion. These results show that Toroid-I PNs do not receive inhibitory input from the adjacent G35 glomerulus or any other Z3-6:OAc-activated glomerulus.

**Figure 3:** Responses and morphological features of PNs in sexually isomorphic glomeruli. **A-C, G-H.** PNs in glomeruli adjacent to or 1-2 glomeruli away from the MGC and G35; **D-F, I-J.** PNs in glomeruli distant to the MGC. Records shown in **A, B, D,** and **E** were obtained, respectively, from the neurons shown in **G, H, I,** and **J.** PNs in both adjacent (**A, G**) and distant (**D, I**) glomeruli were inhibited by stimulation with either odorant. PNs in both adjacent (**B, H**) and distant (**F, J**) glomeruli were inhibited only by
Z3-6:OAc. Panel C shows an example of a PN that was inhibited only by the sex pheromone, and panel E, J shows an example of a PN that was not inhibited by either key component of the sex pheromone. PNs were stimulated with the odor compounds and concentrations indicated. The respective control stimuli (cyclohexane and mineral oil) elicited no response (not shown in this figure). The resting potential is indicated by the horizontal dotted lines. These results show that not all PNs, regardless of their position, receive inhibitory input from the MGC or G35 (or any other glomeruli activated by Z3-6:OAc). Calibration bars: 10 mV in all records. D: dorsal, L: lateral; scale bars = 200 μm. Dotted lines indicate the outline of the MGC glomeruli. The cell body of the PN shown in I (not visible in this section) was in the anterior group of neuronal cell bodies.

**Figure 4:** Quantification of the responses of PNs in glomeruli adjacent to or 1-2 glomeruli away from the MGC or distant to the MGC and G35 to stimulation with the sex-pheromone blend (A-B) and Z3-6:OAc (C-D). A, C. The net number of spikes in a 1-s period post-antennal stimulation. B-D. The amplitude of the odor-evoked change in membrane potential. The open symbols represent the average response (control-subtracted for clarity) of each PN to illustrate the response variability. The closed symbol represents the average response across PNs. The net number of spikes and the change in membrane potential evoked by the pheromone blend were statistically different from those evoked by the cyclohexane solvent-control stimulus in PNs in glomeruli both nearby and distant to the MGC (A-B, asterisks, Wilcoxon matched pairs tests, n=11 and 14, p<0.05). The responses evoked by Z3-6:OAc were statistically different from that to the mineral-oil control stimulus only in PNs in glomeruli distant from the MGC and G35 (C-D, asterisks; Wilcoxon matched pairs tests; G35-PNs were not included in this analysis because Z3-6:OAc evokes a strong excitatory response in these PNs). PNs were stimulated with pheromone blend (200 ng or 500 ng; duration: 200 ms or 500 ms), the cyclohexane control (200 or 500 ms), Z3-6:OAc (1:100 vol/vol in most cases; G35-PNs were stimulated with 1:1,000 or 1:10,000 vol/vol; duration=200ms), and the mineral oil control (200 ms; see Methods for an explanation of the different concentration/stimulus duration used). These results indicate that many PNs in sexually isomorphic glomeruli receive inhibitory input from the MGC regardless of their position, and that PNs in
distant glomeruli receive inhibitory input from G35 and/or other Z3-6:OAc activated glomeruli.

**Figure 5:** Responses of PNs in sexually isomorphic glomeruli of female ALs near the female-specific lateral large female glomerulus (latLFG) and G35 to stimulation with linalool and Z3-6:OAc. These odorants respectively strongly stimulated PNs in the latLFG and G35 and to a less extent, PNs in other glomeruli (Roche King et al. 2000; Reisenman et al. 2004; Reisenman et al. 2005). **A.** Morphology of a PN with arborizations restricted to a glomerulus near the latLFG (dotted lines) and G35 (not visible in this section; image obtained after sectioning). This PN had its cell body in the lateral group of neuronal cell bodies (LC). Scale bar: 200 µm. **B.** Electrophysiological responses of this PN to stimulation with the mineral oil control, Z3-6:OAc, and [±]linalool.. Note that both Z3-6:OAc and [±]linalool elicited hyperpolarization in this PN (deflections below the resting potential, dotted lines). Calibration bars: 5 mV in all panels. **C.** Deflection evoked by stimulation with [±]linalool and Z3-6:OAc in PNs (n=9) with dendritic arborizations in the neighborhood of the latLFG and G35. The open symbols represent the average response (control-subtracted for clarity) of each PN to illustrate the response variability across neurons (only 6 PNs could be tested with Z3-6:OAc). The closed symbols represent the average response across PNs. Concentration of odorants was $10^{-3}$ or $10^{-2}$ vol/vol (duration = 200 ms). The response to [±]linalool, but not to Z3-6:OAc, was statistically different from the response to the control (asterisk, Wilcoxon matched pairs test, $p<0.05$). These results show that the latLFG and/or any other linalool activated glomeruli cause inhibition in PNs in nearby glomeruli.

**Figure 6:** Examples of morphological types of local interneurons (LNs) in the ALs of female *M. sexta*. LNs are confined to the ALs. **A.** This type of LN exhibits a symmetrical arborization pattern in which the dendrites branch radially from the major neurite in the central, coarse neuropil and ramify widely in the glomeruli. **B.** This type of LN is distinguished by the marked asymmetry in the branching pattern of their neurites into the glomeruli and also ramifies widely in the glomeruli. **C, D.** This type of LN shows an asymmetric pattern, but the arborizations are limited to a smaller number of glomeruli. **D.**
Confocal microscopic image obtained from the neuron shown in C after embedding in plastic and sectioning. This neuron had two main neurites, one connecting a large number of glomeruli (arrows in C, D), which includes the latLFG (dotted lines in D), and the other connecting fewer glomeruli (arrowhead in C). Scale bars: 100 μm. This figure illustrates the different types of LNs that mediate interglomerular interactions. The different morphologies provide a neuronal substrate for proposed global and glomerulus-specific inhibitory networks (Silbering and Galizia 2007).
A  pheromone stimulation

B  

C  Z3-6:OAc stimulation

D  

Net number of spikes

Deflection (mV)

nearby PNs  distant PNs
**Figure A** shows a microscopic view of a brain section, highlighting the lateral frontal gyrus (latLFG) and the locus coeruleus (LC).

**Figure B** displays neural activity in response to different conditions:
- **Control**
- **Z3-6:OAc**
- **Linalool**

**Figure C** illustrates the net number of spikes and deflection (mV) under different conditions:
- **Net number of spikes**
- **Deflection (mV)**

*Significant differences are indicated by asterisks (*) in the graph.*