Specializations of a Pheromonal Glomerulus in the *Drosophila* Olfactory System

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Abstract

Insect pheromonal glomeruli are thought to track the fine spatiotemporal features of one or a few odorants to aid conspecific localization. However, it is not clear whether they function differently from generalist glomeruli, which respond to many odorants. In this study we test how DA1, a model pheromonal glomerulus in the fruit fly, represents the spatial and temporal properties of its input, in comparison to other glomeruli. We combine calcium imaging and electrical stimulation in an isolated brain preparation for a simultaneous, unbiased comparison of the functional organization of many glomeruli. In contrast to what is found in other glomeruli, we find that ipsilateral and contralateral stimuli elicit distinct spatial patterns of activity within DA1. DA1's output shows a greater preference for ipsilateral stimuli in males than in females. DA1 experiences greater and more rapid inhibition than other glomeruli, allowing it to report slight inter-antennal delays in stimulus onset in a “winner-take-all” manner. DA1's ability to encode spatiotemporal input features distinguishes it from other glomeruli in the fruit fly antennal lobe, but relates it to pheromonal glomeruli in other insect species. We propose that DA1 is specialized to help the fly localize and orient with respect to pheromone sources.
**Keywords**

Pheromone, Olfactory, *Drosophila*, Antennal Lobe, Glomerulus, Specialization

**Introduction**

The antennal lobe is a brain network that guides olfactory behaviors in insects. It is partitioned into globular neuropil called glomeruli, each of which responds to a unique set of odorants. Glomeruli can be classified as generalists or specialists based on the selectivity of their responses. The majority of glomeruli are generalists; they respond to many odorants, and their population activity can combinatorially encode a large variety of odors. Interactions between glomeruli of the generalist subsystem may aid the computation of odor identity, robust to variations in odor concentration and the presence of background odors (Brody and Hopfield 2003; Stopfer et al. 2003). In contrast, some glomeruli are specialists; when activated by specific chemicals, they can trigger innate behaviors (Suh et al. 2004). One well-studied class of specialist glomeruli responds to pheromones, chemicals emitted by individuals that serve as species-specific social cues. A large part of a male insect's olfactory system may be devoted to processing a small number of pheromones, hinting at the unique challenges faced by this system (King et al. 2000). By encoding spatial (Ai and Kanzaki 2004; Heinbockel and Hildebrand 1998) and temporal (Lei et al. 2009) variations in odor concentration, the pheromonal subsystem may aid odor source localization over potentially long distances in turbulent environments (Vickers et al. 2001). Thus, odor processing by pheromonal and generalist glomeruli may exhibit fundamental differences; however, the physiological underpinnings of such differences remain to be identified. Indeed, the structural and functional similarities in the circuitry of the two subsystems bring into question the utility of this dichotomy (Christensen and Hildebrand 2002).

In this study, we explicitly assess the functional specialization of DA1, a model pheromonal glomerulus in *Drosophila melanogaster*. Unlike most glomeruli in the fruit fly AL, DA1 responds selectively to a single odorant, the male pheromone cis-vaccenyl acetate (cVA) (Ha and Smith 2006; Schlief and Wilson 2007). This simple response profile is contrasted by a greater anatomical complexity: while the average glomerulus is innervated by ~2-3 similar projection neurons (PNs) (Stocker et al. 1997), the output cells of the antennal lobe, DA1 is innervated by at least 7 PNs (Datta et al. 2008), many with distinctive features (Marin et al. 2002). The projection pattern of the axons of these PNs differs between males and females, possibly relating to the sex-specific behaviors elicited by
We compare the response properties of DA1 to those of generalist glomeruli, in the context of mechanisms that may aid source localization. Such differences may arise due to peripheral variations in olfactory receptor activation (Hallem et al. 2004; Xu et al. 2005), or central variations in glomerular circuitry (Root et al. 2008). We isolate the latter's contribution by using electrical stimulation to evoke spatially and temporally defined patterns of activity across glomeruli. We find that DA1's heterogeneous input organization and inhibitory circuitry enable it to discriminate exquisitely fine spatiotemporal variations in its input. DA1 recapitulates the defining features of pheromonal glomeruli found in other insects, and these features distinguish DA1 from generalist glomeruli within the fruit fly antennal lobe. Our findings support the view that pheromonal and generalist subsystems process their inputs differently.

Materials and Methods

Fly lines

Flies expressing UAS-G-CaMP 1.6 (Reiff et al. 2005) on the 2nd chromosome were crossed with GH146-Gal4 (for measurements on PNs) or OR83b-Gal4 (for measurements on olfactory receptor neurons (ORNs)). Flies were homozygous for both Gal4 and UAS constructs. For experiments comparing pre- vs. postsynaptic activity (Fig. 3C, Suppl. Fig. 5), flies expressed both GH146 and OR83b-Gal4. For measurement of presynaptic responses to single-pulse stimuli (Suppl. Fig. 10 A-C), flies expressing UAS-G-CAMP3 were used (Tian et al. 2009).

Recordings

1-3 day old flies were briefly anesthetized with CO2 and decapitated. Imaging was done on an isolated brain preparation (Wang et al. 2003). Antennae were cut with micro-scissors (FST, Foster City, CA) and cuticle was pulled apart with a pair of forceps. Dissection was done in saline with .1 mM Ca++ (adapted from Silbering and Galizia 2007). Brain was transferred to a glass slide with .5 mL of saline with 2mM Ca++ (Silbering and Galizia 2007). The anterior surface of the brain faced upward and the brain was lightly pressed with forceps so that it adhered to the glass. Glass pipettes were pulled with a micropuller (Sutter, Novato, CA) and fire-polished to match the diameter of the antennal or maxillary
nerve. Pipettes were secured onto electrode holders that were attached to micromanipulators. Electrical stimuli were delivered using a Master-8 stimulator (AMPI, Jerusalem, Israel), triggered by pClamp software (Molecular Devices, Sunnyvale, CA). Acquisition was triggered on a Zeiss LIVE confocal microscope, with a 488 nm diode laser, a CCD camera, a 495 nm long-pass filter, and a 60x objective of .95 NA (Zeiss, Thornwood, NY). Frame rate was 20 Hz.

Two nerves (either 2 antennal or 1 antennal and 1 maxillary nerve) were suctioned into 1 electrode each. Care was taken to have both antennal lobes visible at a similar cross-sectional depth; suction electrodes were slightly adjusted in the z-direction if needed to achieve this. The focal plane was roughly 12 μm beneath the anterior surface of the antennal lobe; the focal depth was roughly 5 μm (half-maximum width of the point-spread function). Stimuli were .1-.3 ms, 10 V electrical pulses, sufficient to elicit EPSPs (Olsen & Wilson 2008) in PNs. Inter-trial interval was 30 s; each trial consisted of a 1.5 second acquisition, stimuli being presented at .5 sec. Pulse trains were 100 Hz for 300 ms. This duration was sufficient to evoke steady-state responses; since the bulk of the response occurred in the first 50 ms (Fig. 7D, Suppl. Fig. 7), our results generalize to other stimulus durations.

For stimulation of ventromedial glomeruli (Fig. 6Cii), ventromedial neuropil was suctioned into electrode and stimulated with a single 10 V, .4 ms pulse. This resulted in selective, bilaterally symmetric activation of ventromedial glomeruli, due to the activation of bilaterally projecting ORNs; results for ipsilateral and contralateral ALs were similar, and were pooled for analysis.

**Pharmacology**

Excitation, mediated by nicotinic acetylcholine receptors, was blocked using 40 μM mecamylamine (Sigma, St. Louis, MO; www.sigmaaldrich.com) (Kazama and Wilson 2008). Stock solution was 10 mM in 10% DMSO. GABA-a was blocked using 20 μM picrotoxin (Sigma, St. Louis, MO); GABA-b was blocked using 50 μM CGP54626 (Tocris, Ellisville, MO; www.tocris.com) (Wilson and Laurent 2005). Drugs were added to preparation with a micropipette, taking care not to move the preparation. There was a 5-minute wait before acquisition was resumed.

To isolate pre- and postsynaptic activity within a single preparation (Fig. 3C, Suppl. Fig. 5), brains from UAS-G-CaMP1.6,GH146-Gal4;OR83b-Gal4 flies were stimulated with a single pulse to evoke a postsynaptic response. Under normal conditions, this protocol minimally evokes a change in presynaptic fluorescence (G. Agarwal & E. Isacoff, unpublished results). This was proceeded by a
blockade of excitation using mecamylamine. This prevented activation of PNs as well as inhibitory circuitry, disinhibiting the presynaptic response. A 30-pulse, 100 Hz stimulus was then used to evoke presynaptic activity.

**Map generation**

All visualization and analysis was performed in Matlab (r2006b, Mathworks, Natick, MA). LSM image stacks were imported into Matlab using DIPimage (www.diplib.org). To make maps of evoked activity, the average of 10 or 20 frames (depending on protocol) post-stimulus was subtracted from the average 10 frames pre-stimulus, and divided by pre-stimulus average (Fig. 1D). To remove the effect of dark-state conversion of G-CaMP1.6 that occurs during illumination, the resultant map was subtracted from a control map generated for a no-stimulus condition. To correct for drift of preparation between trials, each antennal lobe was aligned to the resting fluorescence image from the initial trial using DIPimage. The stability of all proceeding analyses is discussed in Supplementary Fig. 1. All ∆F/F images shown in figures were further processed as such: 1) the region surrounding glomeruli is masked to remove low-fluorescence regions, since these suffer from low signal-to-noise ratio, and 2) image is smoothed with a gaussian filter, σ = .75-1 pixels.

**Analysis**

For ROI (region of interest) analysis, glomeruli were manually selected. They were identifiable in ∆F/F images as contiguous, round regions of relatively uniform intensity. DA1 was identifiable by its distinct morphology and location in the dorsolateral region. To generate average maps of activity across preps, each AL was morphed into a 300-point circle consisting of 10 radial and 30 angular coordinates (Suppl. Fig. 2). Each pixel was binned according to its distance from the boundary of the antennal lobe, and its angle from the centroid of the antennal lobe. Bins were averaged and plotted in polar coordinates and interpolated to generate a smoothed, circular map (using PolarToIm.m, posted on Matlab Central by P. Manandhar). The same procedure was carried out for DA1 average maps, each pixel binned according to its distance from the manually selected boundary of DA1, and its angle from the centroid of DA1. The statistical properties of each average map are described in Supplementary Fig. 3; statistical values for all comparisons are given in Table 1.

To calculate the dispersion of activity within a glomerulus, we calculated the mean distance of its pixels from its center of response, weighted by the per-pixel ∆F/F. We used this to calculate “ΔExtent”,...
the log ratio of ipsilateral dispersion to contralateral dispersion. To generate figure 2D, ΔExtent was calculated and assigned to the entire ROI of each glomerulus. The resulting maps were then standardized (Suppl. Fig. 2) and combined to generate an average map.

To determine DA1 boundaries within average maps (e.g. Fig. 2D), DA1 was manually identified in single preparations and its region of interest was assigned a value of 1. The resulting maps were standardized (Suppl. Fig. 2), averaged, and the region whose intensity exceeded 50% of maximum was demarcated as DA1 within average maps. Regions activated by local stimulation (Fig. 6C) were delineated by generating average maps of responses to local stimulation, and thresholding the resultant map at 50% of maximum response. To visualize the medial region activated by maxillary nerve stimulation (Fig. 6Ci), a threshold of 30% was needed.

To enhance color contrast in Fig. 6B and the left panel of Suppl. Fig. 5B, each pixel was assigned a z-score (# standard deviations from the mean) based on the log ratio of intensities in the two channels. For each pixel, this ratio was exaggerated using the hyperbolic tangent function and was used to generate new values for the two channels, keeping the cumulative intensity across its two channels constant (Suppl. Fig. 4). The enhancement was done for visualization purposes alone and did not affect any quantifications.

To calculate the inhibition of the response to the later stimulus in a stimulus pair (Fig. 6E), 1) Stimuli were presented at different inter-pulse intervals; 2) The fluorescence traces of the resulting responses were fit by applying the Matlab function 'nlmfit' on the following formula:

$$f(t) = A_1 g(t) + A_2 g(t - IPI)$$

where

$$g(t) = \begin{cases} 
0 & \text{if } t < 0 \\
\frac{e^{-t/T} - D t}{T} & \text{if } t \geq 0 
\end{cases}$$

and $A_1$ and $A_2$ are the response amplitudes, $g(t)$ is an impulse response function, $T$ is the time constant of decay, $D$ isolates a slight dip of the response below baseline (as seen in Suppl. Fig. 7A), and IPI is the inter-pulse interval for the stimulus; 3) The normalized response to the 2nd pulse was determined as follows:
\[ R_s = \frac{A_1 + A_2 - A_F}{A_S} \]

where \( A_F \) and \( A_S \) are the amplitudes of the responses to the first or second stimulus presented in isolation. This approach was necessary (instead of \( R_S = A_2/A_S \)) because \( A_1 \) and \( A_2 \) could not be separated at small IPI's (\( \leq 50 \) ms).

To plot PN response in time (Fig. 7, Suppl. Fig. 7), the time course of the average fluorescence in a region of interest was bleach-corrected by subtracting the time course of fluorescence in a no-stimulus control movie. The resultant trace was normalized and deconvolved with an exponential with a time constant \( \tau = 363 \) ms, the time constant of fluorescence decay calculated in PNs in response to a single pulse. This corrected for the slow decay intrinsic to the response of G-CaMP 1.6.

To test significance, the two-tailed, two-sample t-test was used; the one-sample (paired) t-test was used when the comparison utilized pairs of measures taken from the same sample (Figs. 4D, 5B, 6D, 7C).

**Results**

**DA1 Encodes Spatial Information**

The bilateral comparison of olfactory stimuli aids source localization in many species (Porter et al. 2007; Rajan et al. 2006), including fruit flies (Borst and Heisenberg 1982; Duistermars et al. 2009). Most glomeruli in the fruit fly antennal lobe are innervated by olfactory receptor neurons (ORNs) originating from both the ipsilateral and contralateral antennae (Stocker et al. 1990). We investigated how these two ORN populations activate DA1 and other glomeruli by visualizing the response to electrical stimulation of either the left or the right antennal nerve (Fig. 2A). We imaged either presynaptic activity in ORN terminal axons (Fig. 2B), or postsynaptic activity in PN dendrites (Fig. 2C). The responses of ventro-lateral glomeruli, in ORNs as well as in PNs, were largely ipsilateral (Fig. 2B,C), matching their innervation by ORNs from the two antennae (Suppl. Fig. 5D). The rest of the glomeruli responded bilaterally: imaging the ORN terminal axons of these glomeruli revealed interlaced regions that responded to either ipsilateral or contralateral stimulation (Fig. 2B).

Unlike the segregation of ipsi and contralateral activity in ORN inputs, the PNs of each bilaterally responsive glomerulus exhibited virtually identical spatial patterns of activity in response to ipsilateral
and contralateral stimulation (Fig 2C, white regions; Suppl. Fig. 6). However, this was not the case for the PN population innervating DA1. Here, ipsilateral stimulation resulted in the focal activation of a subregion within DA1, whereas contralateral stimulation triggered a more spatially distributed response (Fig. 2C-F). The appearance of distinct spatial patterns within DA1 is not simply a byproduct of DA1's large size, as this property is also apparent in DL3 (arrowheads in Fig. 2C, D), a smaller, cVA-responsive pheromonal glomerulus (van der Goes van Naters and Carlson 2007) dorsal to DA1.

The relatively focal contralateral activation found within DA1 PNs mirrors the distribution of ORN inputs (Suppl. Fig. 5). This difference in the spatial 'peakedness' of the glomerular response to ipsilateral and contralateral stimuli is quantified by the \( \Delta \text{Extent} \) measure (see Methods). For other glomeruli, the \( \Delta \text{Extent} \) was much smaller in ORNs and roughly zero in PNs (Suppl. Fig. 5E). We aligned 16 DA1's mapped to polar coordinates and plotted the average relative response of each subregion (30 angular x 10 radial bins; see Image Analysis section in Methods) to ipsilateral and contralateral stimuli. The ventro-medial region had a relatively large response to ipsilateral stimulation, while a more contralaterally-responsive region was centered on a dorso-lateral locus (Fig. 2F).

Thus, the activation of ORNs originating from the left and right antennae elicits unique spatial patterns of activity in DA1 PNs, a feature not seen in the PNs of generalist glomeruli.

**DA1's Spatial Response is Sexually Dimorphic**

Since DA1 activation triggers sex-specific behaviors in *Drosophila* (Kurtovic et al. 2007), we looked for differences in the side-specific responses of male and female DA1s. Unlike male DA1's, which responded more strongly to ipsilateral stimulation (Fig. 3A), female DA1's showed similar magnitudes of response to ipsilateral and contralateral stimulation (Fig. 3B), and in some cases an even stronger response to contralateral stimulation (e.g. female AL in Fig. 2C). This difference could not be explained by the relative density of presynaptic innervation, since both male and female DA1 ORN responses were largely ipsilateral (Fig. 3C).

The difference between males and females in ipsilateral preference of DA1 PNs was seen across the range of stimulus durations tested. Longer trains resulted in a greater ipsilateral preference in both males and females, in DA1 as well as in other glomeruli (Fig 3D). This was the result of the ipsilateral response having a sustained component to prolonged stimuli that was absent in contralateral responses.
The sexual dimorphism in ipsilateral bias seen in DA1 was small or undetectable in other glomeruli (Fig. 3D, Suppl. Fig. 8).

**DA1's Responsivity Rapidly Decreases Upon Activation**

The ability of pheromonal PNs to report rapid fluctuations in odor concentration is critical for localizing odor sources (Lei et al. 2009). We therefore investigated the specialization in the response dynamics of DA1 PNs relative to the PNs of other glomeruli. The activation of ORNs triggers excitatory and inhibitory interactions within and among glomeruli that together transform the output of PNs (Olsen et al. 2007; Olsen and Wilson 2008). We developed a protocol to measure the net impact of circuit interactions on PN excitability in the moments following initial activation. We stimulated one antennal nerve to activate excitatory and inhibitory neurons across the antennal lobe, and then stimulated the contralateral nerve with varying delays to map time-sensitive variations in the ability of the ORNs to excite the PNs in wake of the prior stimulation (Suppl. Fig. 9, Movie S1). Introducing a delay in the 2nd pulse attenuated its contribution to the total evoked response, due to the predominance of inhibition evoked by the 1st pulse (See below). We visualized this attenuation within different glomeruli by superimposing the response to simultaneous inputs and 25-ms-staggered inputs (Fig. 4B, C). Compared to other glomeruli, DA1 showed a decrease in its response to stimuli delayed by as little as 5 ms (Fig. 4D); this decrease was more than 4x larger for a delay of 25 ms. In contrast, simultaneous inputs more linearly summed in DA1 than in other glomeruli (Fig. 4E). Plotting the per-pixel response to simultaneous vs. staggered inputs showed linear distributions of pixels belonging to DA1 or neighboring glomeruli (Fig. 4F). The linear and bimodal clustering of these pixels indicates that the decrease is uniform and confined within DA1.

**GABA-a Mediates Rapid Inhibition in DA1**

In moths, the ability to track pheromone dynamics relies on GABAergic inhibition (Lei et al. 2009), an interaction that is pronounced in the fruit fly antennal lobe (Olsen and Wilson 2008; Root et al. 2008). We pharmacologically assessed how GABA shapes the spatiotemporal response properties of DA1 PNs. Block of either GABA-a or GABA-b receptors increased the response of DA1 PNs (Fig. 5D). Normalizing these responses allowed us to assess how each pathway shaped the temporal dynamics of input integration in DA1. Block of GABA-a receptors eliminated the relatively rapid decrease in DA1's responsivity (Fig. 5A, B), unmasking an excitatory interaction that peaked at the 10 ms inter-pulse
interval. Interestingly, removing fast inhibition induced a complementary increase in late-phase inhibition (Fig. 5A, Suppl. Fig. 10), suggesting a greater activation of inhibitory local neurons. Block of GABA-b receptors decreased the late phase of the delay-dependent response attenuation. Only fast GABA-a inhibition shaped the spatial extent of DA1 activation, as was apparent in the distribution of activity along the proximal-distal axis (relative to PN output, see Fig. 5C) of DA1 PN dendrites. Whereas the activity profile scaled linearly upon blocking GABA-b receptors, GABA-a receptor block preferentially disinhibited the proximal dendrites of DA1 PNs (Fig. 5D). The results show that fast inhibition is evoked strongly and rapidly enough to restrict the spread of activity, as well as the influence of slow inhibition, within the PNs of DA1.

**Weak Inter-gomerular Inhibition in DA1**

The greater inhibition that we find in DA1 could arise from intra-glomerular or inter-glomerular interactions, both of which would be triggered by our antennal nerve stimulation protocol (Fig. 4A). To determine whether inter-gomerular inhibition is sufficient to elicit DA1's relatively strong and rapid inhibition, we delivered the initial stimulus to the maxillary nerve instead of the antennal nerve (Fig. 6A). Since the two nerves activate different sets of glomeruli (Rajashekar and Shamprasad 2004), any decrease in DA1's response should result exclusively from inter-gomerular inhibition. Comparing the response to simultaneous stimulation of the two nerves to the response when the maxillary nerve was stimulated first revealed that a dorso-ventral strip of glomeruli down the middle of the AL experiences greater inhibition (magenta region, Fig. 6B) than the medial and lateral glomeruli, including DA1 (Fig. 6B, Ci). Blockade of GABA-a receptors further increased the lateral inhibition triggered by maxillary nerve stimulation (consistent with the effect seen in Fig. 5A), but once again, DA1 was among the least inhibited (Suppl. Fig. 10D).

To make sure that this pattern of lateral inhibition does not result specifically from the activation of maxillary glomeruli, we measured the inhibition of the response to antennal nerve stimulation resulting from 100 ms prior direct stimulation of ventromedial glomeruli. Maxillary nerve stimulation (Fig. 6Ci) and ventromedial neuropil stimulation (Fig. 6Cii) evoked similar distributions of lateral inhibition: in both cases the greatest inhibition was in dorsal glomeruli, with weaker inhibition in DA1. Furthermore, the onset of inhibition following maxillary nerve stimulation (Fig. 6D) was slower than that observed following antennal nerve stimulation (Fig. 4D). Thus inter-gomerular inhibition failed to account for the rapid-onset inhibition that differentiates DA1 from other glomeruli (Fig. 4E). This suggests that
intra-glomerular interactions contribute to DA1's distinctive inhibitory response following antennal nerve stimulation.

DA1 Discriminates Direction of Stimulus Onset

One cue that enables odor localization in several species is the sequence in which an odor plume comes into contact with spatially separated sensors (von Bekesy 1964; Gardiner 2010; Rajan et al. 2006). Responding to this cue requires that an animal make a persistent decision that far outlasts a potentially transient difference in sensor activation. We tested the ability of DA1 to report such brief temporal asymmetries in the activation of inputs originating from the left and right antenna. We stimulated each nerve with a 100 Hz train for .3 s (Fig. 7A), a frequency and duration within the range of ORN responses to naturally occurring odor plume filaments (van der Goes van Naters and Carlson 2007; Justus et al. 2002), and introduced varying delays in the onset of right nerve stimulation. With a relative delay of only 25 ms, less than one tenth of the total stimulus duration, the pattern of activity in left and right DA1's resembled the pattern evoked by stimulating the left nerve alone (Fig. 7B). We quantified this with the spatial correlation between the response to paired stimulation and that of stimulating either nerve alone (Fig. 7C). For delays of 25 ms or more, the later stimulus contributed minimally to the final spatial pattern of activity. This can be seen in the time course of activity following dual-nerve stimulation; activation of DA1 by the left nerve strongly and rapidly inhibits the transient evoked by right nerve stimulation (Fig. 7D), matching the onset of GABA-a mediated inhibition (Fig. 5A). DA1's persistent inhibition enables it to capture relative delays in stimulus onset between the two antennae (Movie S2).

Discussion

The anatomy and ethology of pheromonal and generalist subsystems suggests that they process inputs uniquely. We characterize functional differences among glomeruli of the fruit fly antennal lobe, focusing on the specialization of the pheromonal glomerulus DA1. We find that the organization of inputs and inhibitory interactions in DA1 are distinct from those in other glomeruli. DA1's properties enable it to discriminate the direction of odor onset, suggesting that DA1 is specialized to localize odor sources.

Our methodology provides a useful complement to electrophysiology in understanding the Drosophila
olfactory circuit. First, we gain simultaneous access to a large cross-section of the antennal lobe, allowing us to understand it at a population level. This provides a view of the topographic organization of the AL in which nearby glomeruli tend to have more similar response properties. For example, we find that medial glomeruli are bilaterally activated (Fig. 2B, C; Suppl. Fig. 5D), and that dorsal glomeruli receive relatively strong inter-glomerular inhibition (Fig. 5B, C). This topography is contrasted by the apparent lack of chemotopy, as ORNs with similar chemical sensitivities innervate widely distributed glomeruli (Hallem & Carlson 2004). An intriguing implication of this duality is that a generalist odor may simultaneously activate multiple glomeruli that have unique response properties. Second, we gain simultaneous, high-resolution access to many points within the projection arbors of olfactory neurons. This reveals that the inhibition of delayed input is roughly uniform within glomeruli (Fig. 4E); and, that there can exist different functional zones within even a ~10 µm-wide neuropil (DL3, Fig. 2).

Conversely, one must keep in mind the limitations of our methodology. We elicit activity via the simultaneous activation of all ORNs in a nerve bundle; how our observations extend to more naturalistic stimulus conditions remains unknown. Unlike our stimuli, an odorant could activate only a subset of responsive ORNs originating from one antenna. Would more selective stimulation reveal additional spatial patterns of activity within DA1 and other glomeruli? In addition, we uncover DA1's enhanced inhibition, relative to other glomeruli, using antennal nerve stimulation, which triggers both intra- and inter-glomerular sources of inhibition. Relative to other glomeruli, DA1 receives little inter-glomerular inhibition (Fig. 6); is selective activation of DA1 sufficient to invoke its distinctively strong and rapid inhibition seen during antennal nerve stimulation? An optogenetic approach (Root et al. 2008; Suh et al. 2007) will provide key insights into these and other unresolved methodological questions.

Most glomeruli in the *Drosophila* AL are generalists that are coupled via a profuse lateral network of excitatory (Olsen et al. 2007; Shang et al. 2007) and inhibitory connections (Olsen and Wilson 2008). In response to an odor, these glomeruli modify each others' outputs in a manner that may enable downstream circuits to classify their population activity (Luo et al. 2010; Olsen et al. 2010). According to this model, the activation level of a generalist glomerulus conveys information that allows the fly to identify an odor from among a potentially vast number of choices. In contrast to generalists, DA1 responds to a single identified odorant, cVA (Schlief and Wilson 2007). Notwithstanding its seemingly
simple chemical response profile, we propose that DA1's complex anatomical (Marin et al. 2002) and the functional organization we find here support surprisingly subtle discriminations.

We find different zones of activity within DA1 corresponding to ipsilateral and contralateral inputs. Can different spatial patterns of activity within DA1 be discriminated by downstream circuits and ultimately inform behavior? Glomeruli are commonly treated as a monolithic unit of olfactory input (Wachowiak et al. 2004). Indeed, the organization of several Drosophila glomeruli may prevent them from discriminating the spatial identity of activated ORNs (Gouwens and Wilson 2009; Kazama and Wilson 2008). Nonetheless, for several insect species, pheromonal ORNs (Christensen et al. 1995) and PNs (Hösl 1990) restrict their processes to subregions of the glomerulus in a manner that corresponds to their spatial receptive fields along the antenna. Unlike other Drosophila glomeruli, DA1 is non-uniformly innervated by several types of PNs with unique arborizations (Marin et al. 2002). In light of this, our findings suggest that the activation of ipsilateral and contralateral ORNs induces unique patterns of activity across the PN population innervating DA1.

The magnitude and spatial spread of DA1's response within its PN dendrites were strongly suppressed within 25 ms of initial activation due to fast GABA-a mediated inhibition. Additionally, DA1 is known to strongly express GABA-b receptors (Root et al. 2008). However, lateral interactions are insufficient for accounting for the strong inhibition we find within DA1 relative to other glomeruli. This is consistent with the observation that several types of inhibitory local neurons that interconnect the AL avoid pheromonal glomeruli (Chou et al. 2010; Seki et al. 2010; Wilson and Laurent 2005). While we did not comprehensively assess DA1's interactions with all other glomeruli, lateral inhibition appears to largely reflect the cumulative activation of ORNs, independent of their glomerular identity (Olsen et al. 2010). Given the significance of pheromonal blends in Drosophila (Griffith and Ejima 2009) and other species (Christensen and Hildebrand 1997; Anton and Hansson 1996), it will be important in future work to probe interactions between DA1 and its neighboring pheromonal glomeruli.

Fruit flies can navigate towards odors using inter-antennal differences in activation (Borst and Heisenberg 1982; Duistermars et al. 2009), a behavior known as osmotropotaxis. This ability is surprising, since ORN's have been shown to evoke EPSP's of similar magnitude and latency within ipsilateral and contralateral PNs (Kazama & Wilson 2009). Our findings provide two mechanisms that distinguish ipsilateral from contralateral responses: 1) unlike medial glomeruli, dorsolateral glomeruli
respond more strongly to ipsilateral stimuli (Fig. 2). This behavior was not previously reported, as all glomeruli tested were medial (Kazama & Wilson 2009). The preference of lateral glomeruli for ipsilateral stimuli is consistent with their relative innervation by ORNs from the two antennae (e.g. glomerulus VA1d in Berdnik et al. 2006; Suppl. Fig. 5); 2) for generalists as well as specialists, ipsilateral glomeruli show a larger steady-state response to prolonged stimuli (Suppl. Fig. 7).

We present a third mechanism whereby each DA1 glomerulus is able to use input timing as a cue to constrain pheromone location. The strong mutual inhibition of ipsi- and contralateral DA1 ORNs allows the discrimination of slight differences in onset of activity on the two sides. This is even true for prolonged inputs: an activated input simultaneously establishes a spatial pattern of activity and minimizes the impact of future inputs on DA1, preventing the modification of the initial pattern. This behavior is characteristic of “winner-take-all” networks (Coultrip et al. 1992), which settle on one of several possible activity patterns based on an initial asymmetry. Thus, a transient difference in activity between the two antennae could serve as a persistent orientation cue (Movie S2). The mutual inhibition of two neural populations with identical chemical tuning, but distinct spatial receptive fields, is found in diverse circuits, from pairs of glomeruli in the olfactory bulb (Lodovichi et al. 2003; Yan et al. 2008), to antiphasic 'flip-flops' in the left and right lateral accessory lobes of moths (Mishima and Kanzaki 1999). The circuit we describe in Drosophila may thus represent a variant of a widespread design principle.

Sensing variations in the onset of activity of spatially separated ORNs may help insects to localize pheromone sources. However, it is unclear how the structure and salience of the fruit fly pheromonal plume compare to those of much larger pheromonal models, such as moth. Further, the small separation of their antennae (~2 mm) may not accommodate the long-range navigational cues available to more widespread sensors (Webster et al. 2001). For DA1 to discriminate the directionality of an odor plume, the plume's lateral velocity relative to the fly's head would need to be less than ~1 cm/s (.2 mm inter-antennal spacing ÷ 25 ms inter-antennal delay detectable by DA1), more than an order of magnitude slower than the velocities encountered in anemotactic orientation during Drosophila free flight (Budick and Dickinson 2006). One possibility is that spatiotemporal variations in cVA concentration are useful in orienting the fly to short-range mating cues. DA1 activation stimulates mating in females but suppresses it in males (Kurtovic et al. 2007). The sexual dimorphism we see in DA1's response to contralateral stimuli may support differences in how males and females orient to cVA-emitting sources.
In conclusion, we propose a dual relationship between DA1 and other glomeruli. DA1 exhibits specializations in spatial and temporal processing of inputs that distinguish it from other glomeruli in the *Drosophila* AL. These specializations indicate that DA1 shares organizing principles with pheromonal glomeruli in other insect species. It remains to be seen how these specializations are adapted to meet the challenges specific to the fly's pheromonal environment. An understanding of DA1's computations will benefit from exploiting the analytic power of the fruit fly genetic model in the context of the rich methodologies developed to explore the structures, functions, and behaviors in classical models of pheromone perception.

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References


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Figure Legends

Figure 1. Recording maps of activity in the antennal lobe. A) Isolated Brain Preparation, dorsal view. Antennal nerve is sucked into a glass pipette and electrically stimulated. B) The antennal lobe receives input from two pairs of nerves innervating bilateral pairs of glomeruli. Antennal nerves are oriented ventrally for clarity. C) Expression of Ca-sensor G-CaMP1.6 in projection neurons, the outputs of the antennal lobe. Resting fluorescence is visible in cell bodies and dendrites. Viewed from the anterior side. D) Normalized change in fluorescence (ΔF/F) upon stimulation of antennal nerves reveals an increase of calcium levels in projection neuron dendrites. In panels C and D, dashed line demarcates DA1 border; orientation is analogous to panel B. All scale bars = 10 µm.

Figure 2. Spatial distribution of responses to left or right antennal nerve stimulation. A) Schematic of color-coded responses to left or right nerve stimulation, depicted in magenta or green, respectively, for panels B, C, and E. B) Olfactory receptor neuron (ORN) responses to left (magenta) or right (green) nerve stimulation. Cholinergic transmission is blocked. C) Projection neuron (PN) responses. Regions that respond to both left and right nerve stimulation are white. D) Difference in spatial extent of PN responses to ipsilateral vs. contralateral stimulation in the antennal lobe (AL). Warm regions indicate glomeruli with a more widespread response to ipsilateral stimulation. Average map of 16 antennal lobes. E) Average map of DA1 PNs showing regions activated by ipsilateral or contralateral stimuli. F) Regional variation within DA1 of relative response to ipsilateral or contralateral stimulation. Average map of 16 DA1's. In panels B through F, dashed line demarcates border of DA1. In panels C and D, arrowhead points to DL3. Panels B, C, and E are from females. All scale bars = 10 µm.

Figure 3. Sexual dimorphism in DA1 PN response. A-B) Close-ups of left and right DA1. Responses to left and right nerve stimulation are colored purple and green, respectively. A) Male, B) Female. C) Preference for ipsilateral over contralateral stimuli in DA1 for males and females. Sexual dimorphism in preference is only seen postsynaptically. Ipsilateral preference in female PNs differs from that of ORNs and males (n = 8 DA1's for each column). ** p < .01. D) Dependence of preference of PNs on number of pulses, for DA1 and other glomeruli in males and females. The DA1 bias in females is significantly lower than that of males for all pulse numbers, p < .001. Data pooled from 8 male and 8 female antennal lobes. Ipsilateral preference = log(∆Fipsi /∆Fcontra). A value greater than 0 indicates that glomerulus responds more to stimulation of the ipsilateral nerve than to stimulation of the contralateral nerve. All responses are integrated over the 0.5 s period following start of stimulation.
**Figure 4. Time dependence of response to bilateral inputs.** A) Response to stimulation of each nerve with a single pulse, either simultaneously or with a 25 ms inter-pulse interval (IPI), represented as purple or green, respectively. B) Map of relative response to simultaneous vs. staggered inputs. In this example, the right nerve, whose input was delayed by 0 or 25 ms, was stimulated with 2 pulses to increase image quality. Scale, 10 µm. C) Averaged map of response attenuation, ratio of ΔF/F at 0 vs. 25 ms (n = 26 antennal lobes). Cold regions show greater attenuation. In panels B and C, dashed line demarcates DA1 border. D) Average responses in DA1 and other glomeruli to IPI's ranging from 0 to 500ms. * p < .05, ** p < .01, *** p < .001. E) Linearity of responses for DA1 and other glomeruli at 0 ms IPI. Values less than 1 indicate a sublinear response (i.e. dual nerve stimulation evokes a smaller response than that predicted from single nerve stimulation). For panels D and E, data were pooled from 15 brains. F) Representative example of response attenuation of points in a contiguous region. Each point represents a pixel's ΔF/F at 0 ms vs. 25 ms IPI. Pixels within DA1 are purple. Dashed black line represents 0 attenuation.

**Figure 5. Complementary effects of fast and slow inhibition** A) Dependence of DA1 PN responses on IPI shifts with blockade of fast or slow inhibition. Normalized to 0 ms IPI. Green and red asterisks mark time points where GABA-a and GABA-b block, respectively, significantly differ from saline condition (n = 29 (saline), 8 (GABA-a), 11 (GABA-b) DA1’s). B) Larger attenuation in DA1’s response at 25 ms IPI is eliminated by blocking fast inhibition. Blue, saline; green, GABA-a blocked; red, GABA-b blocked; data pooled from 15 (saline), 4 (GABA-a), and 6 (GABA-b) brains. C) Normalized close-ups of DA1 response at 0ms IPI, before vs. after GABA-a or -b block. Warmer regions represent larger responses. Dashed line demarcates DA1 border. In top right panel, 'P' and 'D' indicate proximal and distal regions of PN dendrites, relative to PN output. D) Average response in DA1 as a function of position along the proximal-distal (relative to PN output) axis, in response to stimulation of one antennal nerve with a single pulse. Blocking fast inhibition preferentially increases activity in the proximal part of PN dendrites, while blocking slow inhibition scales activity up approximately linearly. For group data, profiles for individual DA1’s were normalized to their maximal response (n = 19 (saline), 8 (GABA-a), 11 (GABA-b) DA1’s, average of ipsilateral and contralateral responses). Individual responses were normalized with respect to position with maximal response. Pink inset: Unscaled plots show larger responses upon blockade of either GABA-a or -b. For A, B, and D, * p < .05, ** p < .01, *** p < .001.
**Figure 6. Mapping of inter-glomerular inhibition.** A) Schematic of color-coded responses to stimulation of the maxillary and antennal nerves, each with a single pulse, either simultaneously (magenta) or with the maxillary nerve stimulus preceding the antennal nerve stimulus by an inter-pulse interval (IPI) of 100ms (green). Bi) Responses to 0 (magenta) or 100 ms (green) IPI. Bii) Color contrast is enhanced to reveal inter-glomerular differences (Suppl. Fig. 2). C) Averaged ratio of the two delays, Ci) with maxillary nerve stimulation, 28 antennal lobes; Cii) with ventro-medial neuropil stimulation, 16 antennal lobes. Cold regions show greater decrease at 100ms. D) Responses to paired stimulation of antennal and maxillary nerves, at IPI’s ranging from 0 to 500 ms. E) % inhibition of the response to a delayed stimulus, following stimulation of either antennal, maxillary, or ventromedial glomeruli. Compared to dorsal glomeruli, DA1 experiences greater overall inhibition due to intra-glomerular plus inter-glomerular inputs (antennal nerve stimulation), but weaker purely inter-glomerular inhibition (maxillary nerve or ventromedial stimulation). * p < .05, ** p < .001; data pooled from 13 (antennal), 14 (maxillary), and 8 (ventromedial) brains. In panels B and C, DA1 is demarcated with a dashed line and ‘+’ outlines indicate regions activated by maxillary nerve stimulation (B, Ci) or ventromedial neuropil stimulation (Cii) in order to trigger inhibition.

**Figure 7. DA1 reports inter-antennal difference in stimulus onset.** A) Close-up of left and right DA1 from a female. Purple and green depict regions activated by ipsilateral or contralateral stimuli. B) Response to sustained stimuli (30 pulses at 100Hz) i, simultaneous, ii, offset by 25 ms. C) For onset delays of 25ms or longer, the spatial pattern of response correlates much more strongly to the response to left nerve stimulation alone than the response to right nerve stimulation alone. ** p < .01, *** p < .001, n = 14 DA1’s. D) Temporal profile of average (n = 14 DA1’s) DA1 response at different onset delays, indicated in legend. Traces are corrected for slow decay of G-CaMP response. Solid black bar indicates time when left nerve is stimulated. Dashed line indicates timecourse of delay-dependent inhibition of transient response to right nerve stimulation. Normalized to peak response to right-nerve only stimulation.
Table 1 – Quantification of relationships presented in different images.

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Corresponding quantification</th>
<th>Description of quantification</th>
<th>Map</th>
<th>Plot</th>
<th># preps</th>
<th>mean ± sem</th>
<th>p</th>
<th>Ratio (%)</th>
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</table>
| 2B   | ΔExtent                      | Methods, Results             | --  | Supp 5E | 8       | DA1 = .098 ± .010 (n = 16) **
Other = .025 ± .003 (n = 211) ** | 5x10⁻¹¹ | 392     |
| 2C   | ΔExtent                      | Methods, Results             | 2D  | Supp 5E | 8       | DA1 = .058 ± .011 (n = 16) **
Other = -.004 ± .002 (n = 211) ** | 6x10⁻¹⁵ | -1450   |
| 2E   | ΔExtent                      | Methods, Results             | 2F  | Supp 5E | 8       | .058 ± .011 (n = 16) ** | --    | --        |
| 4B   | % response                   | Results                      | 4C  | 4D    | 13      | DA1 = 73.7 ± 3.4
Other = 96.6 ± 3.1 | 9x10⁻⁵ | 76      |
| 6B   | % inhibition of 2nd response | Methods, Results             | 6C  | 6E    | 14      | DA1 = 15.8 ± 2.0
Dorsal = 30.6 ± 3.0 | 1x10⁻⁶ | 52      |
| 7A/B | Correlation                  | Results                      | --  | 7C    | 7       | 1st = .68 ± .08
2nd = .25 ± .10 | .01   | 272     |

1Location in original manuscript
2Since average ΔExtent depends on circumscribing non-DA1 glomeruli individually, sample size reflects total number of glomeruli in all preparations.
3Using average values for each group: DA1/Other, DA1/Dorsal, 1st train/2nd train.