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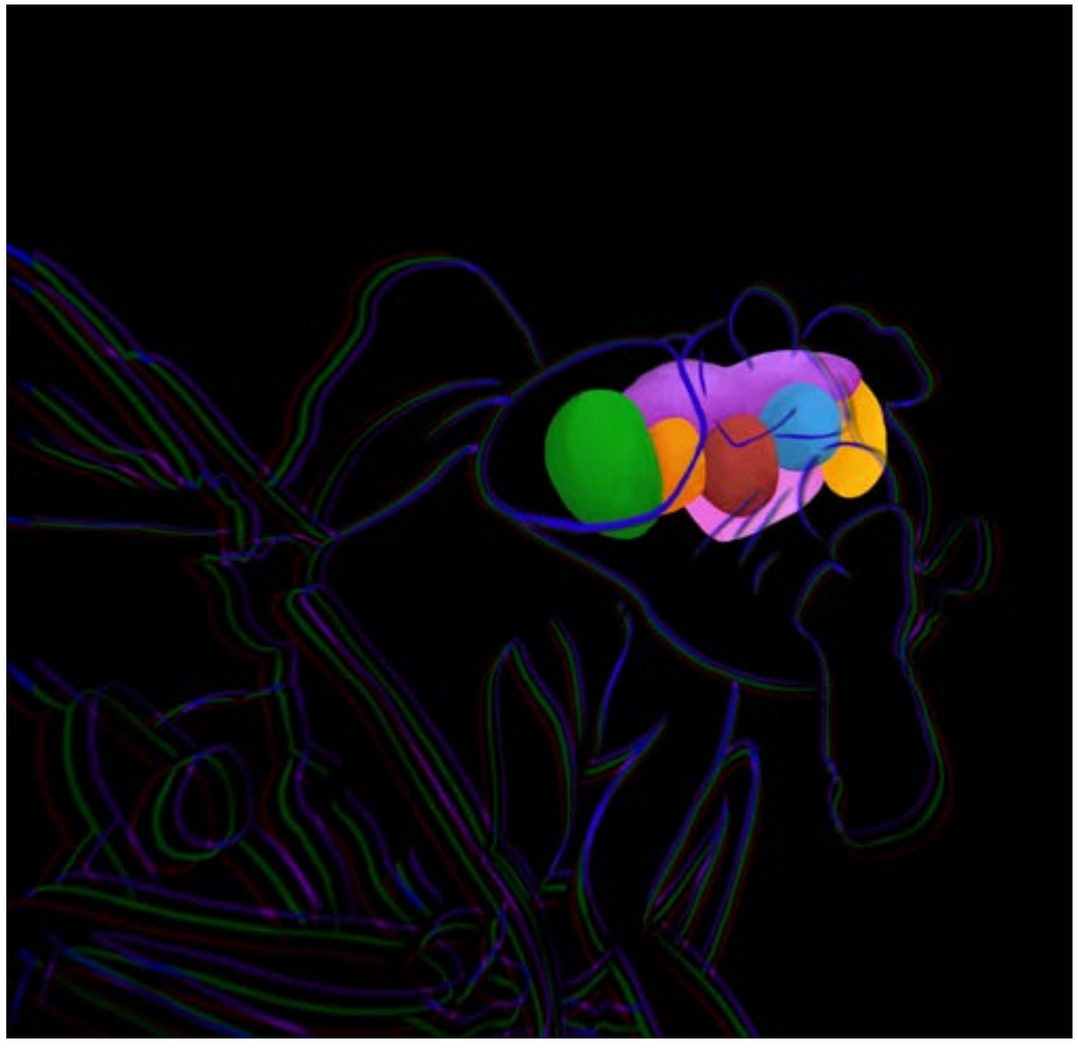

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A Comparative Analysis of Attractive and Repulsive Olfactory Circuits in the Drosophila Connectome

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Duke University
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Abstract

The brain consists of complex sets of neural networks that support sensory integration, computation, and behavior. Olfactory circuits in the *D. melanogaster* brain serve as a model for understanding how the distinct structure and connectivity of these neural circuits mediate diverse behaviors. Currently, the network-level differences between pheromone-detecting olfactory circuits (DA1, VA1v, VA1d) for attraction and odor-detecting circuits (DA2, DC4) for repulsion remain unclear. Conducting a comparative analysis on neural data from the FlyWire connectome of a female adult *D. melanogaster* brain, we found that pheromone-detecting glomeruli are laterally located, while repulsive odor-detecting glomeruli are positioned medially in the antennal lobe (AL) and lateral horn (LH). The DA1 olfactory circuit, characterized by high specificity to its ligand cVA, exhibits few local interneurons (LNs) and minimal lateral inhibition, supporting selective attraction behaviors. In contrast, DC4, associated with acidic compound detection, shows extensive LN connectivity, facilitating broad sensory integration. In higher-order pathways to neuromodulatory neurons, pheromone circuits (DA1, VA1v, VA1d) more directly connect to dopaminergic neurons (DSK, PAM), supporting sexual behavior and associative learning. Furthermore, inputs from olfactory circuits to insulin-producing cells connect through dopaminergic DSK neurons, suggesting a metabolic influence on sexual attraction behaviors. Finally, clock neurons regulating circadian rhythm receive attractive and repulsive olfactory inputs primarily through the lateral horn, which may align innate olfactory-driven behaviors with circadian activity patterns. These findings provide new insights into the structural and functional organization of *D. melanogaster* olfactory circuits, revealing how sensory information is spatially segregated and modulates neural networks for survival, learning, and attraction.

Introduction

Background –

Modern neuroscience seeks to elucidate how neural circuits orchestrate behavior, attempting to answer questions like: "How do brains produce behavior?", with implications for understanding neurodegenerative diseases, psychiatric disorders, and pain. According to contributors to the *Drosophila melanogaster* connectome, "Without a detailed understanding of how neurons connect to one another, we won't have a basic understanding of what goes right in a healthy brain or what goes wrong in disease" (Fuller-Wright, 2024).

Early efforts at neural circuit mapping, which used light microscopy (1957), electron microscopy (1959), electrophysiology (1997), and targeted lesions (2003), failed to afford scientists this "basic understanding" (Swanson et al., 2022). Indeed, these previous efforts only allowed single-cell and single-region resolution. More recent efforts at neural circuit mapping often used genetic labeling methods to visualize and count specific neuron types and identify upstream and downstream partners of these neurons. However, genetic labeling methods are time-consuming. These methods may also label neurons inconsistently due to varying expression levels, yielding false positives and negatives. Furthermore, recent in vivo studies of neural circuits frequently used brain samples from multiple specimens, which may have introduced confounding variables, therefore making comparisons between neural circuits less feasible (Scheffer et al., 2020).

The FlyWire Brain Dataset FAFB v783 ("FlyWire") is the connectome of a female adult *D. melanogaster* brain. FlyWire represents a groundbreaking advancement in connectomics, enabling unprecedented access to the complete neural circuit of the *D. melanogaster* brain. Flywire maps individual neurons, neurotransmitters, neural circuits, and neuropils within a 3D brain volume (Dorkenwald et al., 2024; Schlegel et al., 2024). This publicly available database consists of 140,000 neurons and over 50 million synapses between them (Dorkenwald et al., 2024). Consequently, in contrast to previous efforts at neural circuit mapping, the *D. melanogaster* connectome enables rapid visual and statistical comparisons across the entire fly brain. These comparisons may enable the use of computational and machine-learning methods to accelerate research on how neural circuits mediate behaviors in flies and other complex organisms.

FlyWire was created in four main steps. First, an adult female *D. melanogaster* brain was fixed and cut into thin sagittal slices. Next, each slice was imaged using focused ion beam scanning electron microscopy (FIB-SEM). Distinct neurons were then identified using automated 3D volume reconstruction and segmentation with flood-filling networks. Finally, synapses and neuropils were identified using automated image analysis and human proofreading.

Despite these advancements, the comparative structure and function of olfactory circuits mediating attraction to pheromones versus repulsion from odors remain poorly understood. Our study focuses on three olfactory circuits (DA1, VA1v, and VA1d) that detect pheromones and mediate mating attraction and two odor-detecting circuits (DA2, DC4) that detect repulsive odors to mediate avoidance. For all five olfactory circuits, ORNs expressing a distinct olfactory receptor detect a specific set of compounds and project to distinct glomeruli in the antennal lobe. From there, projection neurons (PNs) project from that glomerulus to the lateral horn (LH) and mushroom body (MB), from which LH and MB output neurons project to higher-order brain centers. For the DA1 circuit, ORNs expressing the Or67d olfactory receptor detect the cis-vaccenyl acetate (cVA) pheromone produced by male Drosophila flies and project via the DA1 glomerulus to mediate increased copulatory receptivity in females (Kurtovic et al., 2007). For the VA1v circuit, Or47b-expressing ORNs detect methyl laurate (ML) and project via the VA1v glomerulus to mediate copulatory behavior. Similarly, for the VA1d circuit, Or88a-expressing ORNs detect ML, methyl myristate (MM), and methyl palmitate (MP) and project via the VA1d glomerulus to mediate short-range attraction (Dweck et al., 2015). Thus, all three pheromone-detecting olfactory circuits mediate attraction in the female brain. For the DA2 glomerulus, Or56a-expressing ORNs detect geosmin (a bacterial or fungal odorant) and project via the DA2 glomerulus to mediate avoidance (Stensmyr et al., 2012). For the DC4 glomerulus, IR64a-expressing ORNs detect acidic compounds and project via the DC4 glomerulus to mediate avoidance. Thus, both odor-detecting olfactory circuits mediate repulsion (Ai et al., 2010). However, the organization of these neural circuits beyond the lateral horn and mushroom body remains less understood.

Unknown/Question –

Despite extensive research on the DC4, DA2, VA1d, VA1v, and DA1 glomeruli, it remains unclear how their respective circuits compare in terms of morphology, connectivity, and elicited behaviors. More specifically, the circuit-level differences between pheromone-detecting circuits (DA1, VA1v, VA1d) for attraction and odor-detecting circuits (DA2, DC4) for repulsion remain unclear. Structural differences between attractive and repulsive circuits may inform hypotheses that explain functional differences between these olfactory circuits. Consequently, our analysis revolves around the following research questions: How does the number of each neuron type differ between the DC4, DA2, VA1d, VA1v, and DA1 circuits? How do the morphologies of and connections between the neuron types vary amongst the five circuits? What does neuron count, structure, and connectivity of each circuit reveal about the divergent behaviors elicited by the five circuits?

Experimental approach –

Using FlyWire's Codex Connectome Data Explorer, we determined the numbers of olfactory receptor neurons (ORNs), projection neurons (PNs), local interneurons (LNs), lateral horn neurons (LHNs), and mushroom body neurons (MBONs) that connect to each of the 5 glomeruli of interest. We also identified pathways between PNs and target cells and visualized these pathways on 3D reconstructions of the *D. melanogaster* hemibrain.

Materials and Methods

Within FlyWire, the Codex Connectome Data Explorer was used to explore the five glomeruli of interest and their associated neural circuits. Individual neurons were identified within the "Search" tab, summary statistics were identified within the "Stats" tab, 3D views of neural circuits in the fruit fly brain were visualized within the "3D Viewer" tab, and neural circuit pathways were identified within the "Connectivity" tab to "Pathways" sub-tab.

Stats –

The "Stats" tab of FlyWire was primarily used to find the number of ORNs, PNs, LHNs, MBONs, and LNs and their NT type. For each type of olfactory neuron within a glomerulus, the upstream/downstream partners, numbers of synapses, types of connected cells, and input and output regions were also identified this way. The following search terms were used:

ORNs: "[Glomerulus] && type == ORN_[Glomerulus] && side == right"

PNs: "[Glomerulus] && class == ALPN && side == right"

LNs: "[Glomerulus] && side == right"

LHNs: "[Glomerulus] && class == ALPN && side == right"

MBONs: "[Glomerulus] && class == ALPN && side == right"

LN counts were determined through the PNs that connect to each glomerulus, following the " □[#] upstream" link in the "Related Cells" column for each individual PN and identifying all cells that had "AL local neuron, ALLN" listed under the "Community Labels" column. Many of the same LNs connected to different PNs within the same glomerulus, so these were identified by hand to ensure no LNs were double-counted in the total. LHN counts were determined by looking at downstream partners of PNs labeled with "LH" within the "Top Output Types (Synapses / Partners)" section. Similarly, MBON counts were determined by looking at downstream partners of PNs labeled with "KC" (Kenyon cells) within the "Top Output Types (Synapses / Partners)" section.

Pathways –

The following search terms were used to establish connections between PNs and target cells:

Input search: "[Glomerulus] && class == ALPN && side == right".

Output searches: "NT == DA", "NT == SER", "Insulin-Producing", and "Clock"

Individual neurons were selected, and the "pathways" tab was navigated using the "connectivity" drop-down menu. The simplest pathway was visualized by clicking "view pathways chart" and selecting the option with the fewest number of hops. For context, it was important to identify the pathway with the minimum number of synapses between points A and B while simultaneously prioritizing synapse strength. Unique cell IDs were revealed by clicking on each "connected cell, and these IDs were then recorded for 3D visualization on the Drosophila brain.

Imaging –

A chosen pathway was visualized in 3D by separating the relevant "cell IDs" with commas and inputting them into the FlyWire search box. "Color By" was changed to "Color By Type," and "layer list panel" and "layer side panel" were selected to determine the colors of particular cell types. If the color labels of two or more cells were too similar to one another (e.g. aqua and blue), new color labels were generated. The "render" option under the "layer list panel" was selected, and the wheel next to "color seed" was clicked. This process was repeated until all cells were colored distinctly. A screenshot of the 3D viewer was taken to preserve our work and visually present neuron morphology and pathways.

Olfactory Receptor Neurons (ORNs) –

The analysis of olfactory receptor neurons (ORNs) across the five examined glomeruli revealed several interesting patterns. DA1 stood out with the highest number of ORNs, totaling 66 (Table 1). Acetylcholine (ACh) was the predominant neurotransmitter used by ORNs across most glomeruli. However, some exceptions were noted: a small subset of DA1 and DA2 ORNs utilized serotonin, while the majority of DC4 ORNs with identified neurotransmitters employed GABA. Interestingly, for VA1v and VA1d glomeruli, the neurotransmitter type remained unidentified for most ORNs (Table 1). A consistent feature observed across all five glomeruli was the bilateral projection of ORNs to both antennal lobes, highlighting the symmetrical nature of these olfactory circuits.

Antennal Lobe Local Neurons (ALLNs) –

In the antennal lobe, the DA1 and DA2 glomeruli connected to a relatively low number of local neurons (LNs), whereas the DC4 glomerulus had the highest number of connecting LNs. Specifically, the DA1 and DA2 glomeruli connected to 38 and 18 LNs, respectively, while the DC4 glomerulus connected to 63 LNs (Table 1). In addition, pheromone-detecting glomeruli were localized to the lateral antennal lobe, whereas repulsive odor-detecting glomeruli were localized more medially.

Figure 1: Localization of pheromone and repulsive food odor-detecting glomeruli in the antennal lobe. Pheromone-detecting glomeruli (DA1, VA1v, VA1d) are located in the lateral AL, whereas repulsive food odor-detecting glomeruli (DA2, DC4) are located in the medial AL.

Projection Neurons (PNs) –

The findings for projection neurons (PNs) in olfactory circuits revealed several key characteristics. We observed convergence from olfactory receptor neurons (ORNs) onto PNs in each glomerulus, as evidenced by the lower number of PNs compared to ORNs. Among the glomeruli studied, DA1 had the highest number of PNs at 11. Most PNs in each glomerulus utilized acetylcholine (ACh) as their neurotransmitter, except for DC4 PNs, which predominantly used GABA.

In the antennal lobe, PNs can be categorized as either ventral (vPNs) or lateral (lPNs) based on their cell body location and projection patterns. The vPNs have cell bodies situated ventral to the antennal lobe and project via the medial antennocerebral tract (mACT) pathway exclusively to the LH (Marin et al., 2002). In contrast, lPNs have cell bodies located lateral to the antennal lobe and project via the inner antennocerebral tract (iACT) pathway to both the MB and LH (Figure 2).

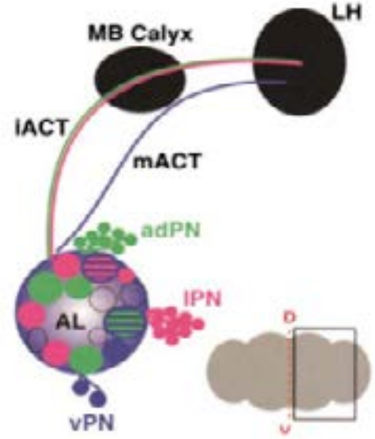


Figure 2: Diagram of the projection pathways of PNs derived from three neuroblast types. Anterodorsal PNs (adPNs) and lateral PNs (lPNs) project via the medial antennocerebral tract (mACT) pathway to both the MB and LH. Ventral PNs (vPNs) project via the inner antennocerebral tract (iACT) pathway to the LH only. Figure adapted from Marin et al., 2002.

The DA1 glomerulus has both lateral PNs (lPNs) and lateral ventral PNs (lvPNs) with cell bodies positioned lateral to the antennal lobe, projecting to both the MB and LH via the iACT pathway. DA1 vPNs, however, project solely to the LH via the mACT pathway (Figure 3). Similar projection patterns were observed in DC4, VA1v, and VA1d glomeruli, with lPNs projecting to both MB and LH via iACT, and vPNs projecting only to LH via mACT (Table 1).

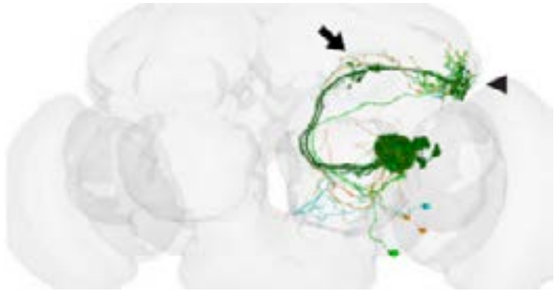


Figure 3: 3D visualization of projections from DA1 glomerular PNs to lateral horn (LH) and mushroom body (MB) neuropils. DA1 lateral PNs (lPNs) are dark green, and ventral PNs (vPNs) are light green. Multi-glomerular lateral ventral PNs (lvPNs) are orange and aqua. The lPNs and lvPNs project to MB and LH via the iACT pathway. The vPNs project to the LH only via the mACT pathway. Lateral horn (LH) is marked by the black arrowhead, and mushroom body (MB) is marked by the black arrow.

Notably, the DA2 glomerulus stood out because it has only lPNs with laterally located cell bodies that project to both MB and LH (Figure 4). Projections from DA2 PNs form two branches, innervating the dorsal and ventral edges of the LH without apparent overlap with other glomerular PNs that innervate central LH regions.

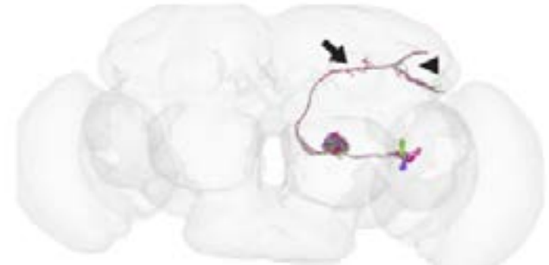


Figure 4. 3D visualization of projections from DA2 glomerular PNs to lateral horn (LH) and mushroom body (MB) neuropils. Each of the 6 ventral PNs (vPNs) has a distinct color and projects via the iACT pathway to the MB (black arrow) and LH (black arrowhead).

The projection patterns of PNs to MB and LH relate closely to the function of olfactory circuits. Simultaneous inputs from dopaminergic (DA) and olfactory neurons to MB Kenyon cells mediate associative learning between odors and rewards or punishments (Marin et al., 2002). The LH, on the other hand, integrates olfactory inputs to mediate

stereotyped behavioral responses to specific odors (Jefferis et al., 2007). Interestingly, PN innervation of the LH maintains the medial to lateral segregation in the antennal lobe, with attractive pheromone-detecting PNs projecting to more dorsolateral LH and repulsive odor-detecting PNs projecting to more ventromedial LH (Figure 5). PN projections to the MB were less spatially segregated and more sparse compared to the LH. We also found that pheromone-detecting PNs innervate more MB neurons, which may support greater behavioral learning and plasticity for courtship behaviors (Table 1).

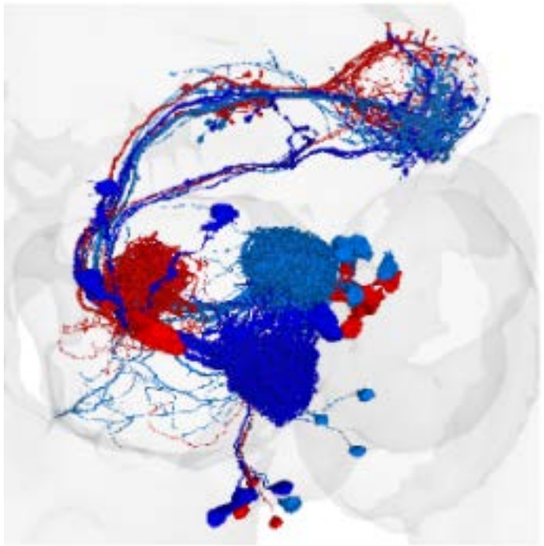


Figure 5: 3D visualization of attractive pheromone-detecting versus repulsive odor-detecting projection neurons for each olfactory circuit. On the right hemibrain, all pheromone-detecting PNs (DA1, VA1v, VA1d) are blue, and all odor-detecting PNs (DA2, DC4) are red. Pheromone-detecting PNs project to the ventrolateral region of the LH, whereas repulsive odor-detecting PNs project to the dorsomedial region of the LH.

Dopaminergic Neurons –

Our examination of dopaminergic cells gave insight into the intricate connections between glomerular projection neurons (PNs) and various brain regions. All five glomerular PNs project to numerous mushroom body (MB) Calyx cells, which converge onto a dorsal paired medium (DPM) neuron that subsequently projects to PAM08 dopaminergic neurons (Keene et. al., 2006). Additionally, DA1, DC4, and VA1d glomerular PNs project to an anterior paired lateral (APL) neuron, an MB interneuron that also project to the PAM08 dopaminergic neuron in the protocerebral anterior medial (PAM) region (Prisco et. al., 2021), which primarily mediates positive reinforcement (Owald and Waddell, 2015). The VA1v PNs exhibit a unique projection pattern, connecting to a superior medial protocerebral (SPM) neuron, which in turn projects to an MB output neuron (MBON) (Li et. al., 2020). This MBON then extends to the PAM12 dopaminergic neuron (Table 2).

The connections between PNs and dopaminergic cells via the lateral horn (LH) are equally complex. DA1, DC4, VA1v, and VA1d PNs project to DSKMP3 dopaminergic neurons through LH neurons. DA1 and DC4 also reach DSKMP3 neurons via superior lateral protocerebrum (SLP) neurons. DA2 PNs have a more intricate pathway, projecting to DSKMP3 dopaminergic neurons through multiple layers of numerous LH neurons. DC4, VA1v, and VA1d PNs also connect to PAM01 dopaminergic neurons via LHCENT3 neurons, which are centrifugal neurons providing feedback from higher-order neurons to the LH and MB (Schlegel et al., 2021). DA1 PNs uniquely project to PAM01 dopaminergic neurons through both MB and LH neurons. Lastly, DA2 PNs project to many LH and SLP neurons, which in turn are connected to PAM01 dopaminergic neurons via a GABAergic lateral horn centrifugal (LHCENT) neuron (Table 3).

Serotonin Neurons –

For each glomerular circuit, PNs projecting to MB and LH neurons connect to distinct populations of serotonergic neurons. We found that all glomeruli except for DC4 have projections to PFNm neurons through MB and/or LH neurons. Specifically, DA1 PNs project to PFNm serotonergic neurons via MB Kenyon cells and LH.CRE neurons that synapse onto MBON neurons (Table 2). In addition, DA2 and VA1v PNs project to PFNm serotonergic neurons via MB neurons to lateral accessory lobe-crepine-posterior nodulus (LCNOp) neurons. Similarly, VA1d PNs project to PFNm serotonergic neurons via both MB and LH neurons, which both project to LCNOp neurons. In contrast, DC4 PN pathways to PFNm

Glomerulus	# of ORNs	# of PNs	# of LNs
DA1 (Or67d)	66 - ACH (43) - SER (5) - N/A (18)	11 - ACH (10) - GABA (1)	38 - GABA - SER - N/A
DA2 (Or56a)	22 - ACH (13) - SER (1) - N/A (8)	6 - ACH (6)	18 - GABA - SER - ACH - GLUT
DC4 (Ir64a)	11 - GABA (3) - ACH (1) - N/A (7)	4 - GABA (3) - ACH (1)	63 - GABA - ACH - SER - N/A
VA1d (Or88a)	46 - ACH (4) - GABA (1) - N/A (41)	5 - ACH (4) - GABA (1)	31 - GABA - ACH - GLUT - SER
VA1v (Or47b)	42 - ACH (5) - GABA (2) - N/A (35)	7 - ACH (5) - GABA (2)	45 - GABA - SER - ACH - N/A

Table 1: Quantification of neuronal types and receptors associated with each glomerulus in the female *D. melanogaster* connectome. The table details the number of olfactory receptor neurons (ORNs), projection neurons (PNs), and local interneurons (LNs) connected to each glomerulus, along with the neurotransmitter type released by each type of neuron and the odor receptor expressed by the ORNs. Neurons with cell bodies on the right side of the brain connectome were quantified and traced. Each glomerulus exhibits distinct odor receptors linked to various functions: Or67d (detects cVA from males, suppresses courtship in males, and increases courtship receptivity in females), Or56a (triggers repulsive behavior), Ir64a (detects acids, mediates avoidance, and is expressed in the coeloconic sensilla ORNs), Or88a (attraction), Or47b (mediates copulation).

or LB1b serotonergic neurons appear convoluted with many nodes or with weak synaptic connections, suggesting that the DC4 circuit does not have strong connections to PFNm serotonergic neurons (Table 2).

We also found that all five glomerular PNs project to LB1b neurons via LH and GNG neurons. Specifically, DA1, VA1v, and VA1d PNs project to LB1b serotonergic neurons via two nodes of LH and gnathal ganglion (GNG) neurons (Table 3). Similarly, DA2 and DC4 PNs project to LB1b serotonergic neurons via three nodes of LH, LH.SLP, and GNG neurons. Yet, the pheromone-detecting PNs appear to have fewer nodes between the PNs and LB1b neurons compared to the odor-detecting PNs (Table 3).

Insulin Neurons –

The projection patterns of olfactory projection neurons (PNs) to insulin-producing cells show distinct pathways through both the mushroom body (MB) and lateral horn (LH). Through the MB pathway, DA1 and DA2 PNs project to insulin-producing cells via APL neurons, which are connected to the ventral median (VM) OA cluster (OA-VPM) neurons, and subsequently to bilateral T-shaped OA-VUM neurons. Notably, DC4, VA1v, and VA1d PNs do not appear to have direct projections to insulin-producing cells through this pathway (Table 2).

Through the LH pathway, different PNs exhibit varied connectivity patterns. DA1 PNs connect to insulin-producing cells via LH neurons to SLP

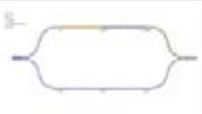
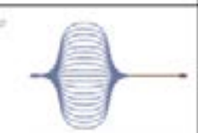
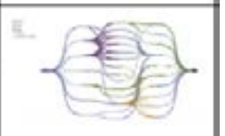
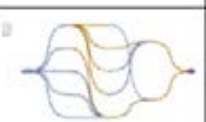
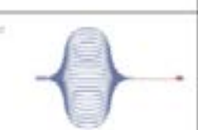
Glomerulus	Dopamine (DA)	Serotonin (SER)	Insulin	Clock
DA1 (Or67d)				
DA2 (Or56a)				
DC4 (Ir64a)		N/A	N/A	N/A
VA1d (Or88a)			N/A	N/A
VA1v (Or47b)			N/A	N/A

Table 2: Pathways from PNs to Target Cells via Mushroom Body. The table shows the connectivity pathways from each of the five glomeruli (DA1, DA2, DC4, VA1d, VA1v) to each type of target cell (DA, SER, insulin, and clock neurons) via intermediate neurons that include MB neurons. The type of neurotransmitter used in the synapse between two neurons corresponds to the color of the pathway between two nodes, and is indicated by the key in the upper left corner of each diagram. The number of synapses connecting two neurons is written as a number on each pathway between two nodes. In each diagram, the projection neuron and target cell correspond to the far left and far right nodes, respectively.

neurons, while DA2 PNs extend this pathway further, projecting via LH neurons to SLP neurons and then to DNpe neurons in the superior medial protocerebrum (SMP). DC4 and VA1d PNs take a different route, projecting to insulin-producing cells through LHAV neurons in the LH to DSKMP3 dopaminergic neurons. VA1v PNs follow a similar pattern, connecting insulin-producing cells via LH neurons to DSKMP3 dopaminergic neurons (Table 3).

Clock Neurons –

For each olfactory circuit, we found that projection neurons (PNs) had more direct pathways to clock neurons through the LH compared to the MB. For pathways connecting to clock neurons through the MB, we found that DA1 PNs project to s-CPDN3C clock neurons via MB calyx neurons to VL1 PNs, connecting to VP1 PNs in the left AL, ultimately projecting to the s-CPDN3C circadian clock neurons in superior medial protocerebrum (SMP). DA2 PNs project to I-LNv clock neurons via MB calyx neurons, diverging to DN1a (dorsal neuron group 1 anterior) neurons in the posterior lateral protocerebrum, followed by connections to medulla tangential neurons that ultimately synapse on the PDF-expressing large ventrolateral clock neurons in the tangential and accessory medulla regions. However, DC4, VA1d, and VA1v did not have clear projections to clock neurons via MB neurons (Table 2).

For pathways connecting to clock neurons through the LH, we found that DA1 PNs project to I-LNv clock neurons via neurons in the LH and anterior ventrolateral protocerebrum (AVLP), then to neurons spanning the AVLP and gnathal ganglia, followed

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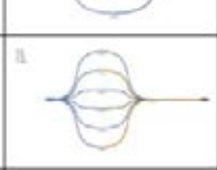
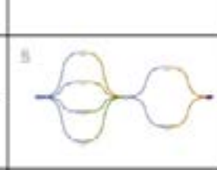
Glomerulus	Dopamine (DA)	Serotonin (SER)	Insulin	Clock
DA1 (Or67d)				
DA2 (Or56a)				
DC4 (Ir64a)				
VA1d (Or88a)				
VA1v (Or47b)				

Table 3. Pathways from PNs to Target Cells via Lateral Horn. The table shows the connectivity pathways from each of the five glomeruli (DA1, DA2, DC4, VA1d, VA1v) to each type of target cell (DA, SER, insulin, and clock neurons) via intermediate neurons that include LH neurons. The type of neurotransmitter used in the synapse between two neurons corresponds to the color of the pathway between two nodes, and is indicated by the key in the upper left corner of each diagram. The number of synapses connecting two neurons is written as a number on each pathway between two nodes. In each diagram, the projection neuron and target cell correspond to the far left and far right nodes, respectively.

by neurons in the posterior lateral protocerebrum (PLP) and medulla (ME), ultimately projecting to an I-LNv clock neuron that bilaterally projects in the ME neuropil. Similarly, DA2 PNs project to I-LNv clock neurons via the LH through AVLP neurons to neurons spanning the PLP and ME, ultimately projecting to the PDF-expressing I-LNv clock neurons in the medulla. In contrast, DC4 and VA1d PNs project to circadian clock neurons in the SMP. Specifically, DC4 adPNs project to s-CPDN3c clock neurons via LH neurons that project to SMP neurons and then to circadian clock neurons in the SMP. Similarly, VA1d adPNs project to s-CPDN3c clock neurons via LH and SLP neurons that connect to SMP neurons that then synapse in the SMP on the circadian clock neurons. Finally, VA1v vPNs project onto 5th-LNv clock neurons via LH neurons that connect to DN1a MB neurons that then connect to the visual projection circadian clock neuron in the SMP (Table 3).

Discussion

Antennal Lobe - Spatial and Functional Segregation

Our findings show distinct spatial and functional segregation between pheromone-detecting and food odor-detecting glomeruli in the antennal lobe (AL). Pheromone-detecting glomeruli (DA1, VA1v, VA1d) are localized laterally, while repulsive odor-detecting glomeruli (DA2, DC4) are positioned more medially.

DA1 exhibits the highest numbers of ORNs (66) and PNs (11), but relatively few LNs (38) among their respective categories. This suggests that there is minimal lateral inhibition and high specificity

for its ligand, cVA. This configuration supports selective attraction behaviors triggered only at low cVA concentrations. Conversely, DC4 displayed the opposite pattern, having the fewest ORNs (11) and PNs (4) but the most LNs (63). This may indicate broad sensory integration and generalized responses to acidic compounds, which rely on the increased LN connectivity. DA2, with the fewest LNs, reflected minimal sensory integration across the AL, emphasizing fast, specific geosmin detection and avoidance hardwired into the fly for survival. In other words, selective detection of geosmin may avoid interference from other olfactory circuits and may be essential for survival.

Overall, DA1 and DA2 circuits exhibit strong selectivity with minimal cross-glomerular input, enabling precise behavioral responses to critical stimuli. In contrast, DC4's LN-rich network suggests broader sensory integration for detecting and processing diverse environmental odors.

PN Projections to LH and MB - Functional Segregation and Pathway Specialization

Our findings highlight that DA2 exhibits unique projection patterns compared to other olfactory circuits. Unlike DA1, VA1v, VA1d, and DC4, which project through both the inner antennocerebral tract (iACT) and medial antennocerebral tract (mACT), DA2 PNs bypass the mACT pathway entirely. This exclusive use of the iACT suggests that DA2 is functionally segregated from other olfactory circuits, reflecting its specialized role in detecting geosmin even at low concentrations.

DA2 PNs target both dorsal and ventral LH regions while avoiding central LH zones. This bifurcated projection may enable the integration of parallel avoidance pathways, enhancing sensitivity to geosmin. In contrast, PNs from other glomeruli project to inner LH regions, emphasizing distinct spatial coding of pheromonal and food-related odors in the LH.

DA2 also exhibits spatial segregation in the MB, which may reinforce its functional segregation. While DA1, VA1v, VA1d, and DC4 exhibit overlapping MB projections, the DA2 circuit's sparse innervation was limited to specific MB zones, reinforcing its role in hardwired avoidance rather than associative learning. In contrast, the broader MB innervation, especially by DA1, likely supports memory and courtship learning. These differences reflect the DA2 circuit's prioritization of rapid, innate avoidance with minimal sensory interference, while other glomeruli balance innate and learned behavioral responses through broad, overlapping projections to both LH and MB.

Lateral Horn (LH) - Behavioral Response Integration

The LH plays a central role in mediating innate and immediate behavioral responses to olfactory stimuli by integrating PN inputs into specific behavioral outputs (Jefferis et al., 2007). PN projections retained the antennal lobe's medial-lateral spatial organization, with pheromone-detecting PNs targeting lateral LH regions and food odor-detecting PNs targeting medial regions.

Pheromone-detecting PNs from DA1, VA1v, and VA1d project to the ventrolateral LH, consistent with their role in mediating attraction. In contrast, food odor-detecting PNs from DA2 and DC4 project more dorsomedially, aligning with their roles in mediating avoidance. Notably, DC4 exhibits broad, diffuse LH innervation which supports general responses to acidic compounds, enabling vast sensory input integration. Conversely, DA2 has highly localized LH innervation, reflecting its high selectivity for geosmin and its hardwired avoidance response.

Thus, our findings suggest that spatially distinct LH innervation patterns encode behavioral valence. Pheromone-specific circuits drive attraction through localized lateral LH innervation, while repulsive food-odor circuits rely on medial LH targets, showing spatially segregated wiring for survival-critical responses.

Mushroom Body (MB) - Associative Learning and Plasticity

The MB mediates associative learning by integrating PN inputs with modulatory signals from dopaminergic (DA) neurons that encode reward or punishment (Marin et al., 2002). Compared to the LH, PN innervation of the MB appears much sparser, likely reflecting the MB's role in flexible, learned behavioral responses rather than innate, reflexive behaviors.

PNs from all five glomeruli sparsely target overlapping regions of the MB. This overlap may enable MB Kenyon Cells (MBNs) to integrate inputs from multiple glomeruli, promoting odor discrimination and memory formation. MB output neurons (MBONs) likely combine MBN activity in unique ways depending on the circuit, supporting distinct learned behavioral responses for different olfactory cues.

Interestingly, pheromone-detecting PNs from DA1, VA1v, and VA1d show broader and denser MB innervation than food-detecting PNs. This suggests enhanced associative learning for courtship-related stimuli. In contrast, the more restricted MB input from food-detecting PNs, especially DA2, reflects limited associative learning for aversive stimuli greater reliance on hardwired avoidance mechanisms rather than learning. Future research should investigate how these innervation patterns shape memory formation, learning plasticity, and circuit modulation.

DA Cell Pathways - Reinforcement and Sexual Behavior Modulation

Drosulfakinin (DSK) dopaminergic signaling has been implicated in promoting female sexual behavior (Wang et al., 2022). In line with this, DA1, VA1v, and VA1d PNs project to DSKMP3 dopaminergic neurons via a single relay from LH neurons, potentially facilitating immediate, pheromone-driven sexual receptivity. In contrast, the DA2 circuit's indirect, multisynaptic pathway to DSKMP3 neurons suggests weaker or more diffuse modulation of avoidance responses to geosmin. Thus, pheromone-detecting circuits may leverage DSK signaling to promote stereotyped, innate attraction behaviors.

All five circuits project to PAM dopaminergic neurons through MB calyx cells converging onto MB output neurons. Because PAM neurons mediate positive reinforcement (Owald and Waddell, 2015), this shared connectivity suggests that even avoidance mediating glomeruli (DA2, DC4) can engage reward pathways, possibly reinforcing adaptive responses such as avoiding toxins or learning about aversive stimuli. However, DA1, VA1v, and VA1d PNs also accessed PAM neurons via additional intermediaries like APL and SPM neurons, suggesting that pheromone-detecting circuits receive stronger positive reinforcement. This could enhance learning and memory formation linked to sexual attraction, allowing flies to integrate pheromone cues with internal states and past experiences.

SER Cell Pathways - Neuromodulatory Integration and Behavioral Context

PFNm serotonergic neurons, associated with the fan-shaped body and protocerebral bridge, influence navigational behaviors, context-dependent action selection, and wakefulness (Hulse et al., 2021; Tomita et al., 2021). All glomeruli except DC4 project to PFNm neurons through MB and/or LH neurons, indicating that pheromone (DA1, VA1v, VA1d) and geosmin-detecting (DA2) circuits may recruit serotonin for modulating behaviors under varying contexts. This could include fine-tuning attraction or avoidance based on internal states or environmental cues. DC4's lack of strong PFNm connectivity suggests that broad acid detection may rely less on neuromodulatory adjustments, maintaining more hardwired avoidance strategies.

Additionally, LB1b serotonergic neurons are linked to bitter taste detection (Sung et al., 2017). All examined circuits project to LB1b neurons via LH and gnathal ganglion (GNG) neurons, although pheromone-detecting pathways had more direct connections than DA2 and DC4. This arrangement hints that pheromone circuits may integrate taste and olfactory cues more efficiently, while food-odor circuits process bitter signals through additional intermediaries. The functional implications of this less direct connection remain an open question, potentially influencing how gustatory and olfactory signals are integrated for avoidance or other behaviors.

Insulin Cell Pathways - Metabolic State and Olfactory Drive

Insulin-producing cells (IPCs) in the pars intercerebralis (PI) regulate metabolism, feeding,

and circadian rhythms (Bisen et al., 2024). Insulin signaling is also required for cVA-mediated attraction (Lebreton et al., 2015), suggesting that DA1 projections to IPCd could form a positive feedback loop. When flies are well-fed and insulin levels are high, the DA1 circuit's excitatory input may strengthen cVA detection and facilitate reproductive behaviors.VA1v, VA1d, and DC4 have dopaminergic inputs via DSKMP3 neurons to IPCs, potentially linking attraction or avoidance circuits to metabolic cues. For instance, the pheromone-driven attraction might be modulated by insulin levels that reflect energy availability, while DC4's broader avoidance could be modulated by metabolic states to optimize foraging and dietary choices. The interplay between insulin signaling and olfaction thus may enhance adaptive decision-making by coupling internal energy balance with olfactory-driven behaviors.

Clock Cell Pathways - Circadian Modulation of Olfactory Responses

Clock neurons in Drosophila's circadian network regulate daily rhythms, influencing behavior timing. I-LNv neurons, producing pigment-dispersing factor (PDF), synchronize other clock neurons and slow circadian pace (Li et al., 2014). Both DA1 and DA2 PNs project to I-LNv neurons via LH, and DA2 PNs also project via the MB to I-LNv neurons. This suggests that both attraction (DA1) and avoidance (DA2) circuits may modulate circadian timing, potentially adjusting activity patterns in response to pheromone availability or toxin presence.

All other glomeruli also formed linear LH pathways to clock neurons, indicating that the circadian system likely integrates multiple olfactory inputs to optimize daily behavior. The relative paucity of simple MB-to-clock pathways may limit the role of learning in circadian modulation. Instead, innate olfactory cues–such as pheromones and toxins–may directly influence internal clocks, ensuring that mating and feeding strategies align with optimal times of the day. Understanding how these circuits tweak circadian rhythms can clarify how environmental and social cues shape daily activity cycles.

Significance and Limitations

Our finding that pheromone and odor-detecting olfactory circuits are spatially segregated in the antennal lobe and LH aligns with previous studies (Jefferis et al., 2007). Because prior studies do not directly compare the five attractive pheromone and repulsive odor circuits, our studies extend these prior findings by showing that the pattern of spatial segregation in the AL and LH applies to all five glomeruli. Our findings also extend on prior studies by highlighting how olfactory inputs to DA, SER, insulin, and clock neuron pathways differ between attractive and repulsive circuits and may relate to the functions associated with these neuromodulatory cells.

However, several limitations must be acknowledged. The connectome data originates from a single female *D. melanogaster* hemibrain, potentially limiting insights into variability, plasticity, and sexual dimorphism in circuit architecture. A key example of sexual dimorphism is the DA1 circuit for cVA detection, which displays sexual dimorphism in the LH and promotes courtship behavior in female *D. melanogaster* and suppresses courtship behavior in male *D. melanogaster* (Kurtovic et al., 2007). Moreover, our analysis focused on structural connectivity rather than functional activity or synaptic strength, leaving open questions about how these networks operate in dynamic, naturalistic conditions. Together, the answers to these questions are termed the "effectome." Future research using functional imaging, genetic manipulations, and behavioral assays will be necessary to validate and refine hypotheses based on our comparative structural approach. Additionally, future research will need to map the pathways that link the brain to the ventral nerve cord (VNC) to understand the entire central nervous system (CNS).

Conclusion

The FlyWire connectome project analyzed five olfactory glomeruli (DA1, DA2, DC4, VA1d, and VA1v) in the Drosophila brain, revealing distinct neuronal compositions and connectivity patterns. We identified a higher number of glomerular PNs and a lower number of LNs for olfactory circuits with high specificity to a compound, such as the DA1 glomerulus for cVA or the DA2 glomerulus for geosmin. We also identified a spatial segregation of attractive pheromone and repulsive odor representations in both the AL and LH, with pheromones represented laterally and odors represented medially. For higher-order pathways to various neuromodulators, we found that pheromone-detecting circuits had more direct projections to dopaminergic DSK neurons promoting female sexual behavior, and a greater number of pathways to dopaminergic PAM neurons for associative learning and positive reinforcement. We found that all olfactory circuits project to PFNm serotonergic neurons for navigational behaviors and LB1b neurons for bitter taste detection, although the DC4 circuit for acid avoidance had less input to serotonin neurons and may receive less serotonergic modulation of acid avoidance. In addition, all olfactory circuits project through the LH to insulin-producing cells, whose activity has been shown to promote cVA attraction and may modulate the activity of other olfactory circuits, suggesting that pheromone-driven attraction or odor repulsion may be modulated by metabolic state. Finally, we found that all five olfactory circuits had inputs to clock neurons primarily through the LH, suggesting that behaviors driven by attractive or repulsive olfactory inputs and circadian rhythms may be mediated by neural pathways with less plasticity compared to pathways through the MB. Consequently, olfactory circuits connected with clock neurons may mediate stereotyped behaviors dependent on circadian time. Overall, our FlyWire connectome study reveals a spatial segregation of olfactory circuits mediating attraction to pheromones and repulsion to odors in the AL and LH, and distinct patterns of connectivity to LH neurons for innate behaviors, MB neurons for associative learning, and neuromodulatory neurons that may provide positive feedback to modulate olfactory-driven attraction and repulsion.

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