The Oviposition Inhibitory Neuron is a potential hub of multi-circuit integration in the Drosophila brain

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Abstract Understanding how neural circuits integrate sensory and state information to support context-dependent behavior is a central challenge in neuroscience. Oviposition is a complex process during which a fruit fly integrates context and sensory information to choose an optimal location to lay her eggs. The circuit that controls the oviposition sequence is known, but how the oviposition circuit integrates multiple sensory modalities and internal states is not. We investigated the neural circuitry underlying complex processing related to oviposition using the Hemibrain connectome. We identified the Oviposition Inhibitory Neuron (oviIN) as a key hub in the oviposition circuit and analyzed its inputs to uncover potential parallel pathways that may be responsible for computations related to decision-making. We applied graph-theoretic analyses to the subconnectome of inputs to the oviIN to identify clusters of neurons that may constitute novel circuits. Our findings indicate that the inputs to oviIN form multiple parallel pathways through the unstructured neuropils of the Superior Protocerebrum, a region implicated in context-dependent processing and decision-making.

Introduction

With a central nervous system consisting of just under 140,000 neurons (***Dorkenwald et al., 2024***), the fruit fly, *Drosophila melanogaster*, exhibits the capacity for complex behavior driven by neuronal circuits that carry out sophisticated brain computations (***Scheffer and Meinertzhagen, 2021***; ***Griffith, 2013***; ***Oram and Card, 2022***; ***Haberkern and Jayaraman, 2016***). Context-dependent computations, which conditionally process or integrate inputs, endow an animal with behavioral flexibility (***Hindmarsh Sten et al., 2021***; ***Roemschied et al., 2023***; ***Devineni et al., 2019***). For instance, hungry flies persistently track a food odor despite repeated failure to find food, showing that need is selectively reinforced in such conditions (***Sayin et al., 2019***). Context can modulate egg-laying preference for acetic acid, despite the otherwise strong avoidance for this as food (***Joseph et al., 2009***). Additionally, circadian rhythms influence egg-laying, making oviposition more likely around the onset of night (***Cury et al., 2019***; ***Allemand, 1976***; ***Riva et al., 2024***). Processed sensory inputs integrate with context, memory, and state, in what we term high-level processing, to produce complex behavior.

Oviposition is a prime example of a behavior that relies on high-level processing (***Cury et al., 2019***; ***Yang et al., 2008***; ***Churchill et al., 2021***; ***Vijayan et al., 2023***; ***Cury and Axel, 2023***). Following mating, the female fly assesses a variety of factors such as the firmness (***Zhang et al., 2020***), taste

(*Vijayan et al., 2022*; *Joseph et al., 2009*; *Yang et al., 2008*), and spatial attributes of a substrate (*Schwartz et al., 2012*), before deciding whether to deposit an egg. While sampling and evaluating multiple sensory inputs, the fly computes a relative value for each encountered substrate (*Vijayan et al., 2023*; *Yang et al., 2008*, *2015*; *Azanchi et al., 2013*). The fly considers social contexts and physical environment to optimize survival of the egg (*Rockwell and Grossfield, 1978*; *Shelly, 1999*; *Schwartz et al., 2012*; *Bailly et al., 2023*). Computations such as these contribute to the complex decision-making process inherent in oviposition.

The circuit responsible for commanding the oviposition sequence and the cell types involved are known (*Wang et al., 2020*). Within each hemisphere, a set of Oviposition Descending Neurons (oviDN) are directly downstream of a single Oviposition Excitatory Neuron (oviEN) and Oviposition Inhibitory Neuron (oviIN) (*Figure 1*A). Mating status is conveyed to the oviDN via the oviIN by way of a subset of pC1 neurons. The pC1 cluster of neurons is a sexually dimporhic cell type that promotes persistent aggressive behaviors (*Deutsch et al., 2020*; *Schretter et al., 2020*) and sexual receptivity (*Zhou et al., 2014*). After a mating experience, sex peptide inhibits the Sex Peptide Sensory Neurons (SPSN) and Sex peptide Abdominal Ganglia (SAG) neurons (*Wang et al., 2020*; *Feng et al., 2014*; *Laturney et al., 2023*). The pC1a and pC1b neurons downstream of SPSN and SAG are silenced by the reduced input which in turn silences the oviIN. The result is the disinhibition of the oviDN which elicit the oviposition motor action (*Wang et al., 2020*). While this neural pathway is responsible for encoding and conveying mating status (*Yang et al., 2009*; *Wang et al., 2020*), it does not account for other computations that influence oviposition, such as the evaluations of substrate firmness and nutrient content, which depend on sensory inputs. The neural correlates of these computations are not yet well-defined, but multiple circuits may converge onto the oviposition circuit to influence decision-making and eventually egg-laying behavior.

Identifying the circuits responsible for high-level processing within the vast network of the fruit fly brain presents a significant challenge (*O'Leary and Marder, 2014*). The recent advent of partial and full-brain connectomes provides a powerful resource for this investigation. The currently available connectome datasets derive from electron microscope reconstructions of the central brains of two female fruit flies (*Scheffer et al., 2020*; *Dorkenwald et al., 2024*). The connectomes comprehensively trace and annotate all the neurons in a given volume. They provide an extensive amount of morphological detail about individual neurons as well as cell type labels (*Schlegel et al., 2024*; *Scheffer et al., 2020*). In particular, these connectomes provide abundant detail about the locations and strengths of synapses among these neurons. Publicly accessible datasets provide the most comprehensive map of the connectivity in the fruit fly brain currently available, although there is some variability between connectomes (*Lin et al., 2024*).

The Hemibrain data has primarily been used to study well-characterized regions of the brain such as the central complex (CX) (*Hulse et al., 2021*) and the Mushroom Body (MB) (*Li et al., 2020*). The cell types and connectivity profiles of the CX circuits that compute abstract internal representations of angular orientation, maintained by self-motion signals and visual cues, have been cataloged by *Hulse et al.* (*2021*). Furthermore, they found that the output types of the CX project to various parts of the brain, including premotor and sensory areas, and that they also return to the CX, evoking a deep recurrent network. The MB supports associative learning, enabling flies to pair odorants with positive and negative experiences (*Handler et al., 2019*; *Aso et al., 2014a*,b). Investigations using the connectome and in vivo calcium imaging have led to a detailed understanding of how the anterior paired lateral neuron (APL) normalizes Kenyon cell signals (*Prisco et al., 2021*). The connectome has also aided researchers in discovering new MBON cell types that project outside of the MB lobes (*Li et al., 2020*) and that receive visual inputs as well as odor information (*Ganguly et al., 2024*; *Li et al., 2020*). While great advances have been made in understanding these well-studied areas of the brain, there remains significant potential to leverage this dataset to investigate less well understood regions (*Scheffer and Meinertzhagen, 2021*; *Lin et al., 2024*; *Ito et al., 2014*).

In our present study, we investigated the inputs to the oviposition circuit to begin an exploration

of the high-level circuits that influence oviposition decision-making. Our initial analyses revealed that the oviIN is a highly interconnected neuron in the oviposition circuit and in the brain. We find that the inputs to oviIN predominantly come from the Superior Neuropils of the Protocerebrum (SNP) which have been known to support high-level processing for taste and water-seeking behavior (***Landayan et al., 2021***; ***Kim et al., 2017***), locomotion and olfactory responses (***Marquis and Wilson, 2022***), interactions between hunger and thirst (***Jourjine et al., 2016***), and persistent internal states in female flies (***Deutsch et al., 2020***). There is currently no comprehensive map of functional circuits in the SNP, thus, we employed a graph-theoretic approach to identify community structure among the inputs to the oviIN. Our analysis revealed parallel pathways from diffuse regions of the central brain that impinge on the oviIN. Ultimately, determining the organization of oviIN's inputs will facilitate the discovery of novel circuits involved in high-level processing.

Results

oviIN is an outlier in the oviposition circuit

We queried the Hemibrain for the oviposition cell types from ***Wang et al.*** (***2020***) (***Figure 1***A) and found the quantity of presynaptic and postsynaptic connections made by each of the oviposition cell types (***Figure 1***B). The left instances are sometimes truncated and less complete than the right instances, thus left instances of oviposition cell types were excluded from the results. We were immediately struck by the oviIN because it makes far more synaptic connections than the other oviposition cell types (***Figure 1***B). Particularly relevant is the quantity of input connections to oviIN. The right oviIN has 21,624 synaptic input connections which is nearly 10 times as many inputs as the other oviposition neurons.

The oviIN makes abundant connections within the oviposition circuit (***Figure 1***C), particularly with SMP550 which is presumed to be the Oviposition Excitatory Neuron (oviEN; see ***Nojima et al.*** (***2021***)). The oviIN and oviEN are strongly, reciprocally connected. As expected, oviIN is presynaptic to all subtypes of oviDN (including the putative additional pair of oviDNa that are labeled as SLP410 in the Hemibrain; see ***Nojima et al.*** (***2021***)) where it inhibits the initiation of the oviposition sequence. Among the pC1 neurons, oviIN receives inputs from all subtypes a-e and also sends outputs to subtypes b-e, forming a small recurrent motif. In accordance with whole-cell recordings from ***Wang et al.*** (***2020***), the SAG neurons strongly contact the pC1a-b neurons. SAG neurons also make strong connections to pC1c in the Hemibrain data. The pC1a-b neurons make moderate connections onto the oviIN. Interestingly, the oviIN provides some feedback connections to the pC1b neurons (the 1 synaptic connection to one of the pC1a neurons was below the synaptic strength threshold for ***Figure 1***C). The remaining pC1 neurons are not among the subtypes that directly convey mating status to the oviposition neurons, but as noted elsewhere, the pC1 neurons are recurrently connected and may share mating status information received by the pC1a-b neurons (***Wang et al., 2020***; ***Deutsch et al., 2020***). We note that pC1c sends relatively weak connections to oviIN but receives strong feedback from oviIN. The pC1d-e neurons send strong connections to oviIN which are stronger than those from pC1a-b. Although the pC1d-e neurons receive weak input from SAG neurons, they were not found to be responsive to SAG activation by ***Wang et al.*** (***2020***). The pC1d-e neurons also receive moderate feedback from oviIN.

While oviIN forms strong synaptic connections with many of the oviposition cell types, overall connections with the oviposition neurons make up a very small proportion of oviIN's synapses (***Figure 1***D). Fewer than 3% of synapses on oviIN are from other oviposition neurons. It is clear from the Hemibrain data that oviIN makes a vast number of connections outside of the oviposition circuit.

oviIN is an outlier in the brain

Given that oviIN's extensive connectivity makes it an outlier in the oviposition circuit, we next sought to determine whether it is an outlier among the rest of the neurons in the Hemibrain with

an eye towards other large inhibitory neurons. We found that the oviIN is one of the most synaptically endowed neurons in the Hemibrain. The right oviIN is the 6th highest ranking neuron for total synaptic connections (50,898 synapse weights; ***Figure 2***A, ***Figure 1***B). The Anterior Paired Lateral (APL) neuron has the most synaptic connections (230,088 synapse weights) out of all the 21,739 traced, uncropped neurons in the Hemibrain dataset. The APL has a role in normalizing the activity of odor-encoding Kenyon cells (***Prisco et al., 2021***). In the synaptic connection ranking, it is closely followed by the right Dorsal Paired Medial (DPM) neuron which is involved in odor memory and associative learning (***Waddell et al., 2000***; ***Keene et al., 2004***, ***2006***), and is sleep-promoting (***Haynes et al., 2015***). The local Lateral Neurons (lLNs) of the antennal lobes also rank highly for synaptic connections. Notably, these highly synaptically-endowed neurons are large inhibitory neurons (***Haynes et al., 2015***; ***Pitman et al., 2011***; ***Chou et al., 2010***). The average neuron in the Hemibrain makes a total of 1,565 synaptic connections. When considering the extent of oviIN's inputs separately, it ranks 13th for synaptic input connections (21,624 synapse weights), making it an outlier in terms of the amount of input it receives.

The right oviIN ranks 7th for the number of total synaptic partners (pre and post) with 4,548 distinct partnering neurons (***Figure 2***B), which is well above the mean number of partners for neurons in the Hemibrain (348.9), and which surpasses the other large inhibitory neurons. APL ranks 21st with 3,464 partners, while the DPMs and lLNs have 3,030.5 and 3,553.5 on average, respectively. However, when considering only presynaptic partners, oviIN ranks 14th while APL ranks 2nd. The oviIN receives inputs from 2,524 neurons in the Hemibrain while the average neuron in the Hemibrain only receives inputs from 216 neurons.

The oviIN distinguishes itself from these other large inhibitory neurons with the number of cell types that it interacts with. With 1,379 cell types among its synaptic partners, oviIN ranks 15th in the Hemibrain on that metric (***Figure 2***C). Despite a high ranking for the total number of synaptic connections, APL only ranks 442nd in terms of the number of cell types among its direct connections with 493 cell types. The DPMs and lLNs connect with 192.0 and 296.5 cell types on average, respectively, while the average neuron in the Hemibrain makes direct connections with 139.2 cell types (***Figure 2***D). Notably, the oviIN ranks 5th when only taking into account the number of cell types that are presynaptic to it. The oviIN receives inputs from 968 cell types while neurons in the Hemibrain on average receive inputs from 82 cell types. Compared to the average neuron in the Hemibrain which makes connections in 4.8 primary neuropils, oviIN connects to about 3 times as many. The right oviIN makes synaptic connections in 14 out of the 63 primary neuropils in the Hemibrain. Most of those connections are in the superior neuropils of the central brain.

"Hub-and-bespoke" structure among inputs to oviIN

In light of oviIN's prodigious connectivity, we wondered whether it is a hub that integrates information from multiple circuits. It seems unlikely that all or most of the cell types that send inputs to oviIN are part of one large circuit that happens to span multiple brain areas, but the extent to which oviIN's inputs are composed of multiple parallel channels is unknown.

One could imagine that oviIN's extensive connectivity might be a result of it receiving small amounts of input from many different neurons. On the contrary, we find that oviIN's strongest presynaptic partners also favor oviIN as a postsynaptic partner. The oviIN is the primary target for the majority of cell types that strongly synapse onto it (≥100 synapse weights) (***Figure 3***A). As might be expected, this primacy decreases for cell types that do not send strong connections to oviIN, indicating that those cell types prioritize making connections with other neurons besides oviIN. As a point of comparison, a small portion of the strong inputs to the APL primarily send their synapses to APL (4.3%). Only the LHMB1 and LHCENT5 cell types are primary strong inputs to APL. This finding confers with APL's divisive normalization function where it samples from many neurons to obtain a population-level average of activity. As such, the oviIN's role does not appear to be divisive normalization. Rather, the primacy of the strong inputs to oviIN is suggestive of dedicated pathways of information flow.

The primary strong inputs to oviIN lay the foundations for potential pathways to oviIN from other circuits, and their identities may be crucial for determining which circuits they funnel information from. However, the majority of cell types connecting to oviIN are uncharacterized, meaning they have either not been studied before being identified in the Hemibrain or they have not yet been identified from existing scientific literature. The Hemibrain provides generic labels to uncharacterized cell types that indicate the neuropil in which the bulk of their synapses are found (***Scheffer et al., 2020***). Neurons that have similar morphology and significant overlap in their synaptic connection patterns are assigned to the same cell type in the Hemibrain. A large proportion of the inputs to oviIN, including its primary strong inputs, are cell types with Hemibrain-provided generic cell type labels that indicate that they mainly reside in the Superior Medial Protocerebrum (SMP). However, oviIN's strongest input comes from the FS1A cell type. The FS1A neurons are a columnar output cell type of the Fan-shaped Body (FB) (***Hulse et al., 2021***). The FS1A neurons mainly receive FB inputs from layers 2-6 and leave the FB to project outputs to the SMP and Crepine. The pC1d and pC1e are also among the strongest inputs to oviIN. The pC1d has been shown to promote aggressive behavior in the female fly (***Schretter et al., 2020***), while activation of pC1d-e together drives a persistent response of several minutes indicative of an internal state encoding (***Deutsch et al., 2020***).

Among the primary strong inputs to oviIN, some of the cell types form highly reciprocal connections with oviIN while others have more of a feedforward relationship with oviIN (***Figure 3***B). The FS1A, SMP544, LAL134, and VES047 cell types hardly make any feedback connections to oviIN. In contrast, the SMP179, SMP386, SMP153, SMP237, SMP550, SMP007, and IB017 cell types are strongly reciprocal with oviIN. These findings imply that FS1A, SMP544, LAL134, and VES047 neurons directly convey information from other circuits while the information conveyed by oviIN's reciprocally connected primary strong inputs, which are primarily uncharacterized SMP types, can be modulated by oviIN.

Clustering the inputs to oviIN

The FS1A and pC1 neurons are not known to have any overlapping functions, suggesting that oviIN integrates information from multiple distinct circuits. However, the right oviIN receives inputs from 968 cell types leading us to think that many of those cell types might be associated with the same circuit. Furthermore, there is the problem of what to make of the hundreds of uncharacterized cell types among the inputs to oviIN. To begin to identify the circuits projecting to oviIN, we used a network community detection method. We employed a machine learning algorithm, RenEEL (see Methods and Materials), to find clusters of densely connected neurons from an undirected network where the nodes are neurons and the links are weighted synaptic connections (***Guo et al.*** (***2019***)).

Modularity is a measure of the excess of within-group connections compared to a randomly-connected network (***Newman, 2006***). The goal of maximizing modularity is to find an optimal partitioning of the network into modules (i.e. clusters) with higher within-group than between-group connectivity. Generalized Modularity analysis is a multi-resolution method that can find community structure at various scales by adjusting a resolution scale parameter. At a low resolution, the coarse structure of the network is returned as a small number of modules with many neurons in each. As the resolution is increased, the network is partitioned into a greater number of smaller modules. The clusters of densely connected neurons identified in this way may form the basis of a hierarchical circuit structure. Previous studies identified community structure in the whole Hemibrain connectome across multiple resolutions by maximizing Generalized Modularity Density [eqn.1] (***Kunin et al., 2023***). This unsupervised analysis recovered the gross structure of the brain by returning modules that reflect the neuropil super-structures. At higher resolutions, known fine structures, such as the sub-circuits of the layered FB, were recovered.

In the current study, we limit our analysis to coarse modularity of the network of inputs to the right oviIN. Since this network is a part of the larger connectome, we refer to it as a subconnectome. The subconnectome of inputs to oviIN contains all of oviIN's presynaptic partners and the

connections among them, and it excludes all other connectome neurons. Our goal was to identify community structure that may otherwise be subsumed by the superstructure of the neuropils when analyzing the full Hemibrain connectome. The modular structure we uncover depends on the relative connection strengths within and between groups; thus, by eliminating neurons that do not form direct connections to oviIN we can hope to identify densely-connected communities that potentially span multiple brain areas. RenEEL returned 7 modules containing from 199 to 347 neurons each for the subconnectome of inputs to oviIN (Supplementary Table 1). Each module contains between 111 and 199 unique cell types.

The modules divide the primary strong inputs to oviIN

The most prominent inputs to oviIN are spread out among the different modules (***Figure 4***A), indicating that they represent parallel pathways that funnel distinct information to oviIN. The FS1A cell type is clustered into the same module as the other FB cell types that send strong inputs to oviIN (FC2B and FC2C; module 4) along with select, uncharacterized SMP cell types. The pC1d-e are clustered together in module 3. The primary strong inputs to oviIN are also spread among the modules (see primary bar in ***Figure 4***B), supporting the notion that if oviIN is a hub, the primary strong inputs define the spokes that convey information from distinct circuits.

Although most of the cell types in these modules are uncharacterized, there are a few characterized cell types that corroborate the potential functional basis of the modules. The aIPg1-3 cell types were previously found to elicit aggressive behavior from female flies and to form a recurrent network with the pC1d-e (***Schretter et al., 2020***; ***Deutsch et al., 2020***). The aIPg cell types make moderate connections to oviIN, and they are clustered together with the pC1d-e in module 3 (Supplementary Table 1). We note that pC1a-b are clustered into module 7 with the oviEN (SMP550) while the pC1c are in module 1. Module 7 has other *fru*+ (fruitless-expressing) neurons that, along with the pC1a-b neurons, are broadly implicated in female receptivity. These include vpoDN, SAG, aSP-f2, and a non-fruitless cell type, SMP286 (a.k.a. pCd-2; ***Imoto et al.*** (***2024***)). Also in module 7 is the SMP029 which is a candidate for the aDN (***Matsliah et al., 2023***) which have a role in site selection during oviposition (***Nojima et al., 2021***).

These examples are only a handful of neurons and cell types from among the hundreds of neurons in their respective modules and therefore it would not be reasonable to ascribe a specific function to the entire module based on the minority of cell types that happen to be characterized. However, the grouping of functionally similar neurons into the same module, based purely on connectivity, does suggest that our method is capable of identifying functional circuits. Furthermore, the relationships between characterized neurons and the primary strong inputs to oviIN in their module may reveal clues about the functions of those uncharacterized primary strong inputs.

The only clock neurons that directly connect to oviIN are two LPN and two instances of the LNd cell type which correspond to the E1 subtype of evening cells (***Shafer et al., 2022***). These neurons are clustered together in module 1 with aMe24, a cell type from the accessory medulla, an area involved in circadian rhythms (***Helfrich-Förster et al., 2007***). Module 1 is also where the left oviIN was found. The E1 LNds have reciprocal connections with the SMP520 which is a primary strong input to oviIN that was clustered into module 1 (***Figure 4***B). Whether or not the SMP520 carries information about circadian timing to oviIN remains to be seen because the LNd inputs (indeed, any clock inputs) to SMP520 are not among its strongest inputs. However, three other primary strong inputs from module 1, SMP237, SMP051, and SMP052, make a small, strongly recurrent network with the aMe24 and pC1c.

Modules 5 and 6 are peppered with mushroom body output neurons (MBONs). The three MBON cell types in module 5 (MBON27, MBON33, MBON35) are atypical MBONs. MBON27 is particularly specialized for visual inputs (***Li et al., 2020***), and it is implicated in a small network connecting CX to MB along with oviIN and mALD1 which appeared in module 5 as well (***Hulse et al., 2021***). The sole primary strong input to oviIN from module 5, IB017, makes relatively weak reciprocal connections with mALD1 and barely connects to the MBON27, indicating that it is not part of that

CX to MB circuit. However, IB017 does form moderate connections with MBON35 and MBON33. The MBONs in module 6 are a mixture of typical and atypical types (MBON01, MBON04, MBON05, MBON12; atypical: MBON26, MBON31, MBON32). The connectivity profile of the MBON12 suggests that it has a role in water-seeking behavior, and MBON26 receives a significant amount of thermo-hygrosensory input (***Li et al., 2020***). The SMP112, the only primary strong input to oviIN in module 6, does not receive strong inputs from MBON12 and MBON26 but it does receive moderate input from MBON31 and MBON32 which are the strongest inputs to MBON26.

As mentioned, the FB cell types that input to oviIN were mainly clustered into module 4. The FS1A, FC2B, and FC2C form a strongly recurrent network. The SMP cell types in module 4 that are primary strong inputs to oviIN make very sparse connections to these FB cell types, but the SMP386, SMP153, and SMP007 receive moderate to strong inputs from the FB cell types, indicating that the flow of information goes from the FB neurons to the recurrently connected network of oviIN, SMP386, SMP153, and SMP007. In particular, oviIN and FS1A are the strongest inputs to SMP386. FS1A is the strongest primary input to oviIN, but the SMP386, SMP153, and SMP007 are also primary strong inputs to oviIN and they may form a secondary pathway through which FB information can reach the oviIN, or they may form a circuit that performs a distinct computation on FB inputs before conveying the results to the oviIN.

Anatomical organization of the modules

RenEEL was run 30 times on the same subconnectome data with different random seeds and it largely returned the same modules. While this gives us confidence in the RenEEL algorithm's ability to detect fairly consistent communities within the network of oviIN's inputs, it does not on its own confirm that RenEEL has uncovered biologically-relevant brain structure. To determine whether there is any spatial organization inherent in the modules, we visualized the input synapses to oviIN on its wire skeleton (***Figure 4***C,D; ***Figure 4—figure Supplement 1***). The input synapses on oviIN are shown as dots and each dot is colored according to the module of the presynaptic neuron. Neurons from modules 1, 3 and 7 mostly synapse onto oviIN in the dorsal tuft of processes within the SMP where most of oviIN's output sites also reside. Neurons from modules 4 and 6 also form most of their synapses onto oviIN in the dorsal tuft, but they also have a large portion of synapses in the inferior neuropils along the posterior and anterior part of the Crepine, respectively. Module 5 neurons predominantly form their synapses to oviIN along the anterior Crepine, but also occupy portions of the dorsal tuft. Neurons from module 2 mainly form their connections along the ventral portion of the oviIN intersecting with lateral complex (LX), ventromedial (VMNP) and inferior neuropils (INP). The modules do not simply reflect the boundaries of the neuropils that intersect with oviIN because all of the module synapses intersect with a mixture of neuropils and they all intersect to some extent with the SNP (***Figure 4—figure Supplement 2***). Nonetheless, the anatomical organization of modules indicates that RenEEL found biophysically meaningful clusters of neurons. The organization along the distinct branches of oviIN suggests that neurons work together with the other neurons in their module to influence the activity within different regions of this large neuron. It is possible that the motifs within these modules act as local processing units to drive, summate, or shunt activity from other local processing units.

It is reasonable to wonder whether the anatomical organization of the modules is a reflection of the neuropil organization of the brain more broadly, rather than the neuropil boundaries of the synapses on oviIN. In other words, has RenEEL clustered oviIN's inputs according to the neuropils that they "belong to"? Strictly speaking, neurons cannot easily be classified as belonging exclusively to one neuropil because they often make connections in multiple neuropils, although researchers sometimes classify a neuron based on the neuropil where it makes the majority of its synaptic contacts (***Scheffer et al., 2020***; ***Schlegel et al., 2024***; ***Ito et al., 2014***). To determine the various brain regions that are represented among the inputs to oviIN, we queried the Hemibrain for the neuropils in which oviIN's presynaptic partners make synaptic connections with any neuron, including but not limited to oviIN (***Figure 5***A). We found that RenEEL did not partition oviIN's

input connectome according to the neuropils where those neurons form their connections. In all but modules 2 and 5, the neurons form the majority of their synapses in the SNP. We had already determined that, collectively, the majority of input connections to oviIN are made in the SNP (***Figure 3***B), and that a large proportion of oviIN's input connectome contains neurons that are labeled as SNP neurons. ***Figure 5***A shows that RenEEL has identified SNP sub-groups that may pertain to distinct SNP circuits. Neuropil designations are primarily driven by anatomical considerations and do not necessarily demarcate functional circuits (***Ito et al., 2014***; ***Yu et al., 2013***). Thus, the modules may represent circuit structure that draws from SNP together with other neuropils. For example, the most prominent cell type input to oviIN, FS1A, is in module 4. The FS1A neurons receive most of their input connections in the FB neuropil of the CX, but they make most of their output connections in the SMP (of the SNP) and CRE (of the INP) (***Hulse et al., 2021***). Cell types such as this may form a bridge from one part of the brain to the SNP, INP, LX, or VMNP neuropils where they interact with oviIN.

Functional organization of the modules

We next wondered whether the partitions found for oviIN's input connectome reflect those previously found for the full Hemibrain connectome. The coarse modules found in the full Hemibrain connectome were roughly aligned with the neuropil structure of the brain, indicating that RenEEL identified macroscale brain structure. Even though we only consider neurons which make direct synaptic contact with the right oviIN in the present study, we might expect strong alignment with the full Hemibrain modules if the brain's super-structure is still present within the subconnectome of oviIN's inputs. We compared our results to the modules identified by ***Kunin et al.*** (***2023***), and we did not find strong alignment. The portion of oviIN's input connectome that resides in full Hemibrain module 3 – which corresponds mostly to SMP and Superior Intermediate Protocerebrum (SIP) – is spread out among all of the subconnectome modules (***Figure 5***B). In other words, large portions of the neurons in the subconnectome modules all share a common module in the full Hemibrain clustering - namely the module that encompasses the medial and intermediate superior neuropils. Full Hemibrain module 1 corresponds mainly to the VMNP, and the portion of oviIN's connectome that is from that area is dominated by subconnectome module 2. Likewise, full Hemibrain module 2 (which corresponds to FB) is mainly represented in subconnectome module 4. However, all of the subconnectome modules are composed of a combination of full Hemibrain modules. In particular, every cluster identified in oviIN's connectome has significant overlap with the full Hemibrain cluster that encompasses the superior intermediate and superior medial neuropils (SIP, SMP). This suggests that, in the full Hemibrain, the connections within SNP are stronger overall than connections between SNP and other neuropils, and that connections within potential circuits that include SNP neurons combined with other cell types are dense enough to be detected in the absence of the connections with the broader network of each neuropil.

To determine whether the coarse modules of oviIN's input connectome correspond to mid-resolution modules of the full Hemibrain, we computed the Jaccard similarity [eq.3] between the coarse clustering of the subconnectome of oviIN's inputs and the clusterings of those same neurons in the full connectome at various resolutions. A high Jaccard similarity (close to 1) is an indication that many pairs of neurons are partitioned into a module together in both the subconnectome clustering and the full connectome clustering at a particular resolution. We found low similarity between the subconnectome clustering and the full connectome clustering (***Figure 5***C). For all resolutions of the full connectome clustering, Jaccard similarity with the subconnectome clustering was less than 0.2. However, Jaccard similarity was always slightly higher than a random baseline in which the module labels for the subconnectome were shuffled. Thus, applying RenEEL to a subconnectome of the brain, as we have done for oviIN's input connectome, does not partition the neurons in the same way as for the full connectome, either at the coarse or higher resolutions. Our results suggest that the modules correspond to a microscale organization at the level of oviIN's inputs – which may correspond to distinct circuit pathways and which may have functional signifi-

cance for oviIN.

To verify that these modules are representative of distinct circuits feeding into oviIN, we once again considered their connections within the full Hemibrain. We computed the similarity of inputs from anywhere in the brain to each pair of neurons in oviIN's input connectome (see Methods and Materials). On average, the similarity of within-module pairs exceeds those of between-module pairs (***Figure 6***A). In other words, presynaptic partners of oviIN have more of their inputs in common with other neurons in their same module than with neurons in a different module. Moreover, comparison to a randomly partitioned network reveals that within-module pairs are more similar than would be seen by chance (***Figure 6***B). Along the diagonal of ***Figure 6***B, the mean input similarity among neurons in the same module (red dashed line) is higher than the mean input similarity among neurons from random modules and lies outside of the distribution of similarities. In off-diagonal plots, the mean input similarity between neurons from two different modules (red dashed lines) tends to be within the similarity distribution for neurons from random modules or much lower than the distribution mean. The mean similarity between modules 1 and 3 is an exception because it is slightly higher than chance. Taken together, these results support the idea that the modules approximate parallel streams of input from different circuits.

Discussion

The complexity inherent in the decision-making and behavior of *Drosophila* makes studying its tiny brain a worthwhile endeavor because isolating the circuits that drive high-level processing is within reach. The *Drosophila* brain has a hierarchical organization where peripheral sensory inputs undergo multiple stages of higher order processing, evident in both the adult (***Jeanne et al., 2018***; ***Kunin et al., 2023***) and larval brain (***Winding et al., 2023***; ***Betzel et al., 2024***). Researchers have made progress studying the sensory periphery and progressively discovering higher-order neurons and circuits (***Walker et al., 2024***; ***Jacobs et al., 2024***; ***Matsliah et al., 2024***), but such an approach is inadequate for discovering the circuits that drive the complex computations involved in context-dependent decision-making. Behaviors like oviposition depend on nearly every sensory modality, and starting at the periphery to discover the high-level circuits that integrate sensory inputs, state, and memory would require an expansive search. The connectome now offers another way to discover the neural circuits that are responsible for high-level processing.

In our approach, we analyzed the inputs to the oviposition circuit to study the neural correlates for the highest level computations that drive oviposition. We first observed that the oviIN is an outlier in the oviposition circuit and in the brain due to its abundant connectivity within and beyond the oviposition circuit. Given that the vast majority of inputs to oviIN are uncharacterized cell types, our goal was to identify the structural pathways into the oviposition circuit so that future work could determine the sensory and functional bases for these input pathways. We found that oviIN is the primary target of multiple cell types rather than solely collecting small amounts of input from neurons that primarily send outputs elsewhere. This pattern of connectivity is more aligned with a multi-circuit integrator role than a divisive normalizer. After applying a community-detection analysis, we found that the primary strong inputs to oviIN are distributed across multiple modules indicating that those primary strong inputs form the backbones of multiple distinct pathways to oviIN. Furthermore, we show that our modularity results do not reflect the same scope of anatomical organization that was found in the full Hemibrain modules by ***Kunin et al.*** (***2023***). Instead, maximizing modularity for the subconnectome containing oviIN's inputs reveals an anatomical organization at a biophysically relevant scale for oviIN that is reminiscent of dendritic processing. Finally, we show that there is higher similarity among the inputs to neurons within a module than across modules, which further supports our interpretation of the modules as partitions around neuronal circuits. Together, these findings suggest that the inputs to oviIN represent multiple parallel pathways from high-level processing circuits.

Our study is limited to a partial brain connectome of a single specimen. Variability among individual female fruit flies may hinder the generality of our results, as well as the missing connec-

tions with neurons on the far left side of the central brain in the Hemibrain data. Additionally, the Hemibrain connectome only reconstructs classical chemical synaptic connections. Although electrical synapses and neuropeptides play an important role in the computations and functions of the *Drosophila* brain, gap junctions and neuropeptide release sites are not included in the current connectome data. Future iterations of *Drosophila* connectomes may include these additional features, enabling us to build upon the results we present here.

High-level processing in the SNP

The majority of oviIN's inputs come from the SNP -– a mysterious region that includes the Superior Lateral Protocerebrum (SLP), SIP, and SMP neuropils and that lacks a topological organization (***Ito et al., 2014***). This area is rife with high-level processing potential. MBONs carry odor information and send projections to the SNP at convergence zones where they meet the dendrites of dopaminergic neurons which respond to the valence of a stimulus (***Aso et al., 2014a***,b). Thus, the SNP is a likely site for circuits that are responsible for classical conditioning. Sensory and interoceptive inputs to the subesophageal zone are relayed to the SMP where they regulate water and food seeking behavior (***Jourjine et al., 2016***; ***Landayan et al., 2021***). Taste projection neurons that project to the SLP are essential for conditioned taste aversion whereas those that bypass the SLP are not (***Kim et al., 2017***). With regards to oviposition, dopaminergic neurons that project to the SLP influence the sucrose preferences of egg-laying flies (***Yang et al., 2015***). The sexually dimorphic pC1 neurons have a role in persistent states related to courtship and aggression (***Deutsch et al., 2020***; ***Zhou et al., 2014***). They are presynaptic to oviIN, and according to the Hemibrain, they make most of their synapses in the SNP as well. Despite these behaviorally relevant functions, the SNP remains *"terra incognita"* as far as its organization (***Scheffer et al., 2020***; ***Ito et al., 2014***).

Without a pre-existing map of the SNP or a comprehensive wiring logic for it, our results point to clusters of mainly uncharacterized cell types that may form high-level circuits that do computations that are relevant for oviposition. To be clear, we do not claim to have identified complete circuits since our approach intentionally limited our analysis to the connectivity among the neurons that directly input to oviIN. Rather, our coarse clustering casts a wide net around the primary strong inputs to oviIN to dredge up clues about the regions and circuits that are associated with its inputs. Future experimental and computational work will be needed to validate the potential circuits indicated by our analysis.

We observed that the FS1A neurons form recurrent connections among themselves and with other FB types, and they project to a recurrent network of SMP neurons that includes SMP386, SMP153, and SMP007. The FS1A neurons also project directly and strongly to oviIN. Thus we found two pathways along which FB information may flow to oviIN: a direct pathway via FS1A, and an indirect pathway from FS1A through a recurrently connected network of uncharacterized SMP neurons which themselves make strong, direct connections to oviIN. This suggests that the recurrently connected SMP neurons that are targeted by FS1A perform a distinct computation on the FB information received. Furthermore, since these SMP neurons also form recurrent connections with oviIN, they may be involved in a computation of FB input that is itself modulated by oviIN. It is possible that the SNP is organized into recurrently connected microcircuits such as this that perform high-level computations.

Selective application of modularity analysis for structural scope

Network community detection methods generally fall into two classes: spectral methods which cluster nodes by leveraging the spectral properties of the graph to embed the network into a low-dimensional space (***Chung et al., 2021***), and modularity-based methods which partition nodes to maximize the difference between observed intra-cluster connections and those expected under a random graph model. In this study, we used a modularity-maximization approach using the ReNEEL machine learning scheme (***Guo et al., 2019***) because of its ease of interpretation. Its emphasis on intra-connectedness of the node communities (***Newman, 2006***; ***Newman and Girvan, 2004***; ***Guo***

et al., 2023; ***Kunin et al., 2023***) reveals assortative groups of neurons and cell types. By forming densely connected modules within the subconnectome of oviIN's inputs, our modularity-based method offers insight into the structural organization of this network and provides predictions for the functions of the cell types that are clustered together.

In our study, we chose to apply RenEEL to a subnetwork of the connectome to control the scope of the structure we were probing. RenEEL was previously applied to the full Hemibrain connectome by ***Kunin et al.*** (***2023***) and had recovered gross anatomical structure that reflects the neuropil organization of the brain, as well as finer structure such as the layers of the FB. While such macroscale anatomical structure often underlies functional circuit structure (***Betzel et al., 2013***), it might obscure mesoscale and microscale circuit structure that is not necessarily contained within a given neuropil (***Betzel et al., 2018***; ***Betzel, 2023***). For example, the FS1A and the SMP386 neurons are from different neuropils and they are clustered into separate modules for the full Hemibrain connectome; however, they are clustered into the same module when RenEEL is applied to the subconnectome of oviIN's inputs. These neurons are separately partitioned in the full connectome because the connections they make with other neurons from their own neuropil are denser than any connections made with neurons outside of their neuropils. When the scope of the network is limited only to neurons that are involved in a particular function, such as oviposition, the interactions between these neurons becomes more apparent. By applying RenEEL to a subconnectome consisting of the inputs to oviIN, we limited our analysis to finding partitions at a mesoscale and microscale level of relevance.

Generalized modularity [eqn.1] offers an established method with which to probe mesoscale and microscale structure. The resolution parameter can be adjusted to retrieve more modules containing fewer neurons. A previous study demonstrated that generalized modularity reveals the hierarchically nested anatomical structure of the brain when applied to the full Hemibrain network (***Kunin et al., 2023***). The modules identified at higher resolutions were generally subsets of the coarsest modules, though this partitioning was not always strict. Even still, the high resolution modules of the full Hemibrain did not correspond to the coarse modules that we obtained for the subconnectome consisting of the inputs to oviIN (***Figure 5***B,C). This demonstrates that classical (i.e. coarse) modularity on a subconnectome does not produce the same results as generalized modularity at higher resolutions on a full connectome. Although our study did not set out to investigate the subtleties between classical and generalized modularity analysis, we propose that applying classical modularity to a subconnectome targets a different scope of brain structure than applying generalized modularity on the full connectome. Future work is needed to gain a comprehensive understanding of how community-detection tools can be honed to reliably retrieve circuit structure in the *Drosophila* brain.

Methods and Materials

Accessing and working with the Hemibrain connectome data

All analyses presented were performed using the publicly available Hemibrain connectome data released by Janelia (***Scheffer et al., 2020***). The data are from an EM reconstruction of the right hemisphere and partial left hemisphere of a single female fly brain. Morphological data for the 3D reconstruction was accessed, along with tabular data of the neuron annotations and connectivity, using the Neuprint python package released by Janelia (https://github.com/connectome-neuprint/neuprint-python). The data accessed via Neuprint is Hemibrain version 1.2.1. For the brain-wide rankings (***Figure 2***), we relied on the most recent table of traced neurons made available by Janelia (https://storage.cloud.google.com/hemibrain/v1.2/exported-traced-adjacencies-v1.2.tar.gz) which is version 1.2, and which contains only neurons that are traced and uncropped.

Every contiguous object in the Hemibrain is assigned a unique bodyId, and many of these are also assigned a cell type. Janelia annotated cell types based on their morphology and shared connectivity patterns. There are some traced, uncropped neurons that are not assigned a cell type.

These are labeled as "None" and were excluded from certain analyses wherever indicated. Janelia also annotates neurons with an instance label that can be used to differentiate between right and left instances of bilateral cell types. For example, there are 2 neurons in the Hemibrain of the oviIN cell type: a right and a left instance that are denoted as oviIN_R and oviIN_L, respectively. Our analyses largely focused on the right oviIN.

Neurons in the Hemibrain that match with neurons that have been empirically characterized in the literature are typically annotated with their known name, thus we denote these as characterized cell types. Neurons that have not been characterized are often given a cell type name that begins with the abbreviation for the neuropil where most of its synaptic contacts are located followed by a 3-digit number where the numbering sequence begins with 001. Neurons such as these may have been previously characterized in the literature but have not been matched in the Hemibrain. One example is SMP550 which is presumed by ***Nojima et al.*** (***2021***) to be the oviEN that ***Wang et al.*** (***2020***) identified in the FAFB volume, but the Hemibrain has not updated its cell type label to reflect that proposition. Except for this particular case, we denote generically-labeled and unnamed cell types ('None') as uncharacterized.

Drosophila synapses are polyadic, meaning that multiple postsynaptic densities can be associated with a single presynaptic T-bar. The Hemibrain provides counts of the postsynaptic density sites as well as counts of the presynaptic sites on the bodies of anything that is given a bodyId, as well as locations in 3D space of those sites. Additionally, the Hemibrain provides data for the synaptic connections among neurons in the 3D reconstruction. These are given as synaptic weights where the weight is the count of postsynaptic sites within the synapses that connect two neurons.

Primacy of inputs to oviIN

A neuron or cell type is considered a primary input to oviIN if it has oviIN as its strongest output. It is determined on a by-cell type basis by querying the synaptic outputs of all the neurons of a given cell type, summing those weights by postsynaptic cell type, and putting those aggregated weights in descending order. The position of the oviIN type in the list determines the primacy of oviIN as an output. If oviIN is in the first position of outputs from a neuron, that neuron is a primary input to oviIN (see ***Figure 4***B).

Parsing neuropil data

When fetching adjacencies between neurons or cell types in the Hemibrain, the data indicates the neuropil, or region of interest (ROI), in which the connection is located. For consistency and clarity, we merged right and left ROIs and relabel primary ROIs with their corresponding supercategory of neuropil according to ***Ito et al.*** (***2014***).

For pie chart visualizations in which the data were restricted to synapses made with oviIN (***figure Supplement 2***), the synapse weights between oviIN_R and its presynaptic partners were queried by ROI. For each module, the synapse weights from module neurons onto oviIN were summed within each ROI, thus providing the total synapse weights onto oviIN_R made within each ROI by the neurons in a given module. For the pie chart visualization in ***Figure 5***A, all synapse weights involving presynaptic partners of oviIN were queried – including synapses made with neurons other than oviIN. For each neuron, the proportion of synapses made within each ROI were calculated by totaling the synapse weights made by the neuron in that ROI and dividing by the total synapse weights made by the neuron in all the ROIs. These proportions were tallied up by ROI for each module. The pie slices represent the proportion of connections made within each ROI by all the neurons in a module, but normalized on a per neuron basis.

RenEEL method for computing generalized modularity

We used Reduced Network Extremal Ensemble Learning (RenEEL, ***Guo et al.*** (***2019***)), a machine-learning based approach for network clustering, to identify the partition $\mathcal{C}$ which maximizes the generalized modularity density [eqn.1](***Guo et al., 2023***). RenEEL first creates an ensemble of can-

didate partitions using a fast heuristic. The ensemble is iteratively updated, alternating steps of reducing and replacing. In the reducing step, each community identified by all partitions in the ensemble is combined to a single node. In the replacing step, the fast heuristic is used again on the reduced network. If the result matches one of the previous partitions, or has a lower score than any of the ensemble partitions, the ensemble size is reduced by one. Otherwise, the result replaces the ensemble partition with the lowest modularity score. This process repeats until only one partition remains in the ensemble, representing a consensus best partition. We used an implementation that has previously been shown to produce fast and accurate results for networks of the size and density we consider (***Guo et al., 2019***, ***2023***; ***Kunin et al., 2023***). The source code is available on Github: https://github.com/prameshsingh/generalized-modularity-density.

The set of neurons presynaptic to oviIN and the synaptic connections among them were converted into an undirected, weighted graph. The weight of each edge was the total synaptic weight between the two neurons in both directions. The community structure of the network was identified with the partition $\mathcal{C} = \{C_1, C_2, \dots\}$ maximizing the generalized modularity density score (***Guo et al., 2023***),

$$Q_g = \sum_{C \in \mathcal{C}} \left(\frac{m_C}{m} - \left(\frac{K_C}{2m} \right)^2 \right) \rho_C^{\chi} \tag{1}$$

The sum is taken over communities C; m is the total number of edges in the network, m_C is the total weight of edges in community C, K_C is the weight-degree sum of nodes in C (the sum of the weights of edges incident to each node in C), and ρ_C is the relative density of connections in C, defined by

$$\rho_C = \frac{2m_C}{n_C(n_C - 1)} \tag{2}$$

Where n_C is the number of nodes in community C. The exponent χ controls the resolution of the clustering. At $\chi = 0$, Q_g equals classic modularity density (***Newman, 2006***), with $\chi > 0$ resolving smaller, more densely-connected communities (***Guo et al., 2023***).

In our study, RenEEL was applied to the subconnectome of oviIN_R inputs which consists of all the presynaptic partners to oviIN_R and the connections among them. Only traced, non-cropped neurons were included in this subconnectome in keeping with the analyses done by ***Kunin et al.*** (***2023***). The results presented are from a representative run (using $\chi = 0$) that resulted in a single set of partitions, $\mathcal{C}$. RenEEL was subsequently run on the same subconnectome 30 times with different random seeds to determine that partitions were largely consistent between runs (mean Jaccard similarity: 0.89). The weak inputs to oviIN drove most of the variability in the results while the primary strong inputs to oviIN were more consistently partitioned. When analyzing and making comparisons to the modularity of the full Hemibrain, RenEEL results from ***Kunin et al.*** (***2023***) were used.

Analyses and Visualizations

All analyses and visualizations were done in Python using custom-written scripts along with various published and open-source packages. Our analysis code is publicly available on GitHub: https://github.com/orgs/Gutierrez-lab/oviIN-inputs.

Joint clustering plots

We compared the modularity results for the oviIN_R inputs subconnectome clustering and the full Hemibrain connectome clustering. We computed the proportion of nodes from a module in one clustering that are partitioned into a module from another clustering. The width and height of the boxes in ***Figure 5***B represent the joint counts of neurons within two modules over the total counts within a module of the respective dataset. If N_{H_i} is the number of nodes in module i of the full Hemibrain clustering, and N_{O_j} is the number of nodes in module j of the oviIN input clustering, and the number of nodes in both module i of the full Hemibrain clustering and in module j of the oviIN input clustering is $N_{H_i \cap O_j}$, then the width of any box is $N_{H_i \cap O_j} / N_{O_j}$ and the height of any box is $N_{H_i \cap O_j} / N_{H_i}$.

Jaccard Similarity

To evaluate the similarity between different neuron clusterings $\mathcal{A}$ and $\mathcal{B}$, we compute the Jaccard similarity. For a set of neurons $\{1, 2, 3, \dots N\}$, Jaccard Similarity considers all pairs of neurons in the set and is defined as:

$$J(\mathcal{A}, \mathcal{B}) = \frac{\sum_{i=1}^{N} \sum_{j=i+1}^{N} \mathbf{1}\{C_i^{\mathcal{A}} = C_j^{\mathcal{A}} \ and \ C_i^{\mathcal{B}} = C_j^{\mathcal{B}}\}}{\sum_{i=1}^{N} \sum_{j=i+1}^{N} \mathbf{1}\{C_i^{\mathcal{A}} = C_j^{\mathcal{A}} \ or \ C_i^{\mathcal{B}} = C_j^{\mathcal{B}}\}} \tag{3}$$

where N is the total number of neurons in the set, $C_i^{\mathcal{A}}$ and $C_i^{\mathcal{B}}$ are the cluster assignments of neuron i in clusterings $\mathcal{A}$ and $\mathcal{B}$, respectively. The indicator function $\mathbf{1}\{\cdot\}$ equals 1 if the condition inside is true and 0 otherwise.

The Jaccard similarity ranges from 0 to 1. A value of 0 indicates no overlap between the clusterings, while a value of 1 indicates perfect overlap. Intermediate values indicate that some pairs of neurons are clustered together for both clusterings. In our study, we applied this method to compare the clustering of the oviIn inputs with the clustering of the entire Hemibrain for different resolutions of RenEEL algorithm.

Input Similarity

Input similarity was computed as the cosine distance between the vectors representing the inputs to each neuron. For every neuron n, we compute the vector n_{in} of length equal to the number of neurons in the Hemibrain, with the ith entry equal to the synapse weight from neuron i to neuron n. If neuron i is not synaptically connected to neuron n, the corresponding entry is 0. For two vectors u, v the cosine distance is given by $d_{\cos}(u, v) = \langle u, v \rangle / \|u\| \|v\|$. Because all entries in n_{in} are nonnegative, cosine similarity ranges between 0 and 1. A similarity of 0 means that two neurons receive inputs from completely disjoint sets of neurons; a similarity of 1 means they receive inputs from exactly the same neurons in exactly the same proportion.

Mean similarity was computed within modules by averaging over all pairs of distinct neurons belonging to that module, and across modules by averaging over all pairs with one neuron in each cluster. For comparison, we shuffled module membership, preserving the size of each module but randomly assigning module labels to each neuron.

Acknowledgments

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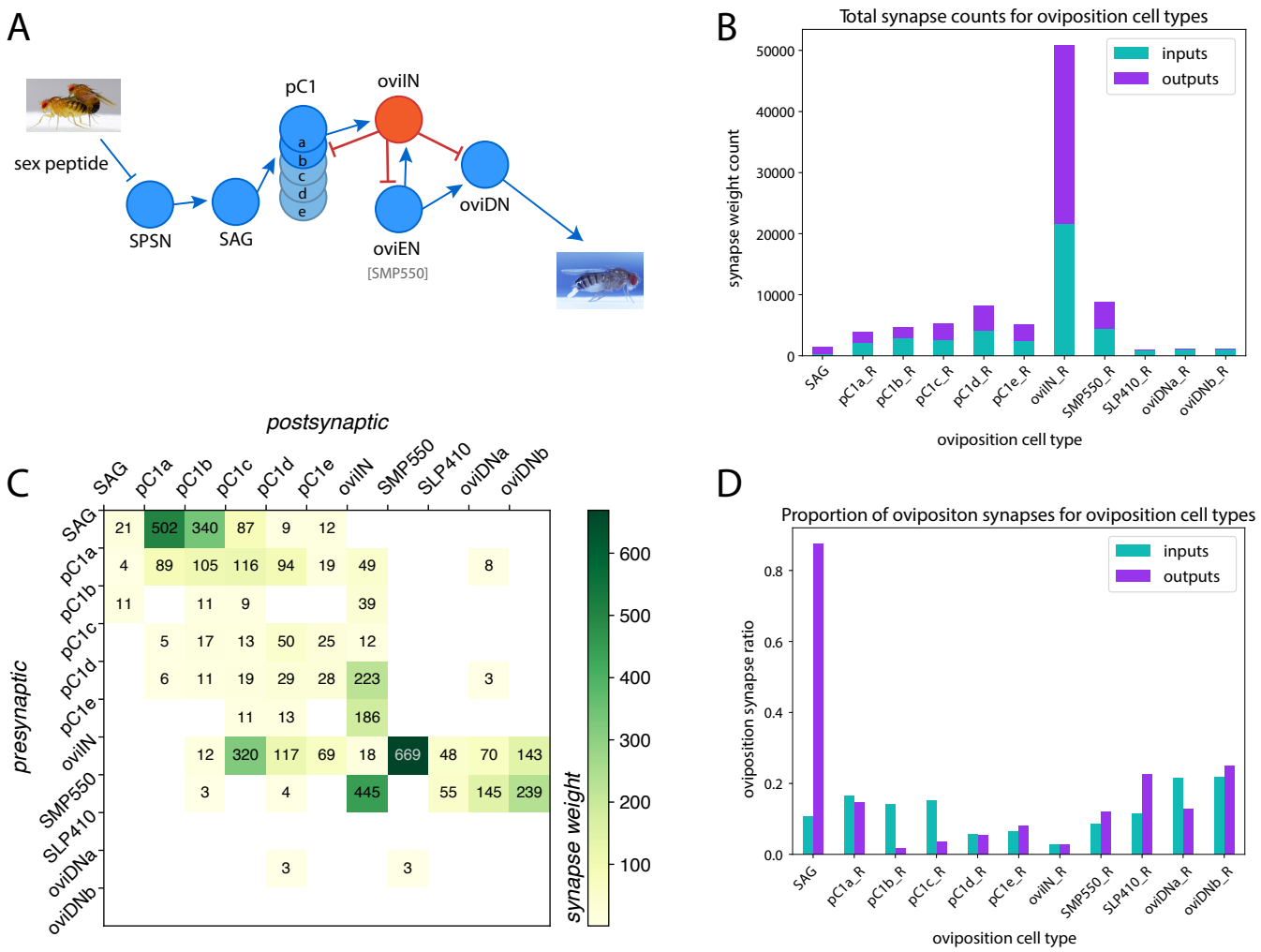

Figure 1. The connectivity profile for the oviposition circuit in the Hemibrain connectome.
A. Schematic circuit diagram of the oviposition circuit from ***Wang et al.*** (***2020***) that conveys mating status to the Oviposition Descending Neurons (oviDN) which command the oviposition motor sequence. The oviIN (red) is an inhibitory neuron in this circuit. [*Note: the photos are placeholders and will be replaced by open-source photos or illustrations]
B. Total synapse counts for the oviposition cell types in the Hemibrain connectome. Counts are based on the number of presynaptic (inputs; teal) and postsynaptic (outputs; purple) connections by weight. Only right instances of the oviposition cell types are included because left instances are often truncated. The mean counts are presented for the SAG and SLP410_R because they each have two instances. The SMP550 are presumed to be the oviEN, and the SLP410 are believed to be an additional pair of oviDNa (***Nojima et al., 2021***).
C. Connectivity matrix for the oviposition cell types (including right and left instances). Connection weights less than 3 are excluded. Synapse counts are the aggregated synaptic weights from all neurons of one cell type to all neurons of another cell type.
D. The proportion of synapses that are made with other oviposition neurons. The inputs ratio (teal) for a given cell type is the proportion of synapses received from presynaptic oviposition partners relative to synapses from all presynaptic partners. The outputs ratio (purple) is the proportion of synapses to other oviposition neurons relative to synapses to all postsynaptic partners.

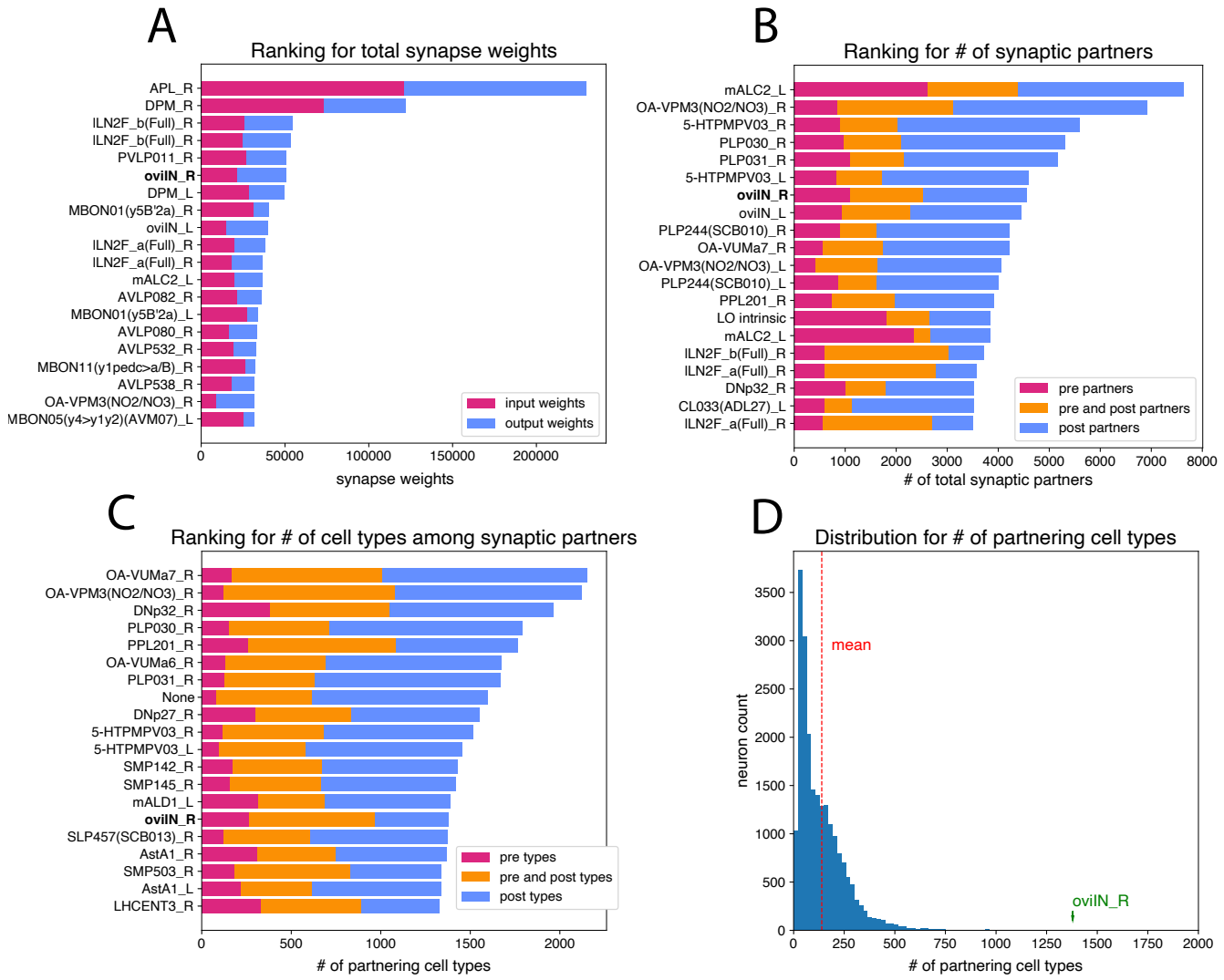

Figure 2. The oviIN is an outlier in the Hemibrain.

A. Out of the 20,000+ neurons in the Hemibrain, the top 20 neurons with the highest total synaptic connections are ranked in descending order and labeled by their instance label from the Hemibrain. The right oviIN (oviIN_R) ranks 6th with 50,898 synaptic connections. Input weights (magenta) are counts of postsynaptic sites on the indicated neuron that are paired with a presynaptic site. Output weights (blue) are counts of postsynaptic sites that are paired with a presynaptic site that is on the indicated neuron.

B. The top 20 neurons in the Hemibrain with the greatest quantity of unique neuron partners are ranked in descending order and labeled by instance. Counts of partner neurons that are exclusively presynaptic are shown in magenta, exclusively postsynaptic partners shown in blue, and counts of neurons that are both presynaptic and postsynaptic to the neuron are shown in orange. The oviIN_R ranks 7th with 4,548 total partners (2,524 presynaptic and 3,441 postsynaptic neurons).

C. The top 20 neurons in the Hemibrain with the greatest quantity of distinct cell types among their direct synaptic partners are ranked in descending order and labeled by instance. Counts of cell types that are exclusively presynaptic are shown in magenta, exclusively postsynaptic cell types shown in blue, and counts of cell types that are both presynaptic and postsynaptic to the neuron are shown in orange. The oviIN_R ranks 15th with 1,379 total cell types among its partners (968 presynaptic and 1,112 postsynaptic cell types).

D. Histogram showing the distribution of the number of cell types among synaptic partners for all neurons in the Hemibrain. The average neuron partners with 213 cell types while oviIN_R makes direct connections with 1,379 cell types.

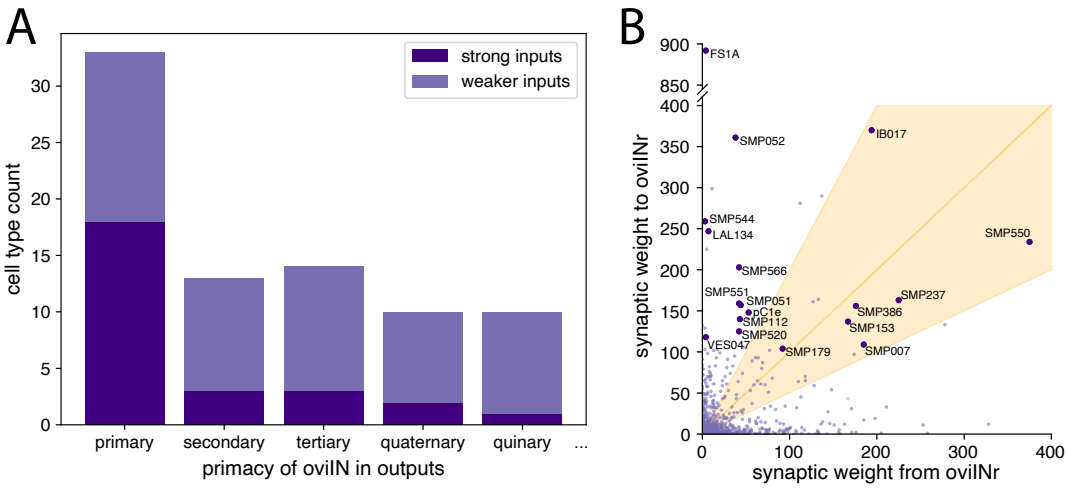

Figure 3.
A. Cell types that have oviIN as their primary output also tend to be strong inputs to oviIN. There are 33 cell types that have oviIN as their primary output. Among those, 18 cell types make 100 or more synaptic connections to oviIN. Cell types making strong connections to oviIN make up a smaller proportion of cell types that most strongly output to another neuron besides oviIN.
B. Each point on the graph represents a cell type that is a direct synaptic partner of oviIN_R. The combined synaptic weight from oviIN_R to a cell type is plotted along the x-axis while the combined synaptic weight to oviIN_R from a cell type is plotted along the y-axis. The 18 primary strong inputs to oviIN (dark purple points) are annotated with their cell type label. A subset of the primary strong inputs to oviIN_R occupy the zone of high reciprocity with oviIN_R (orange) where connections in the stronger direction are no more than twice the strength of connections in the weaker direction.

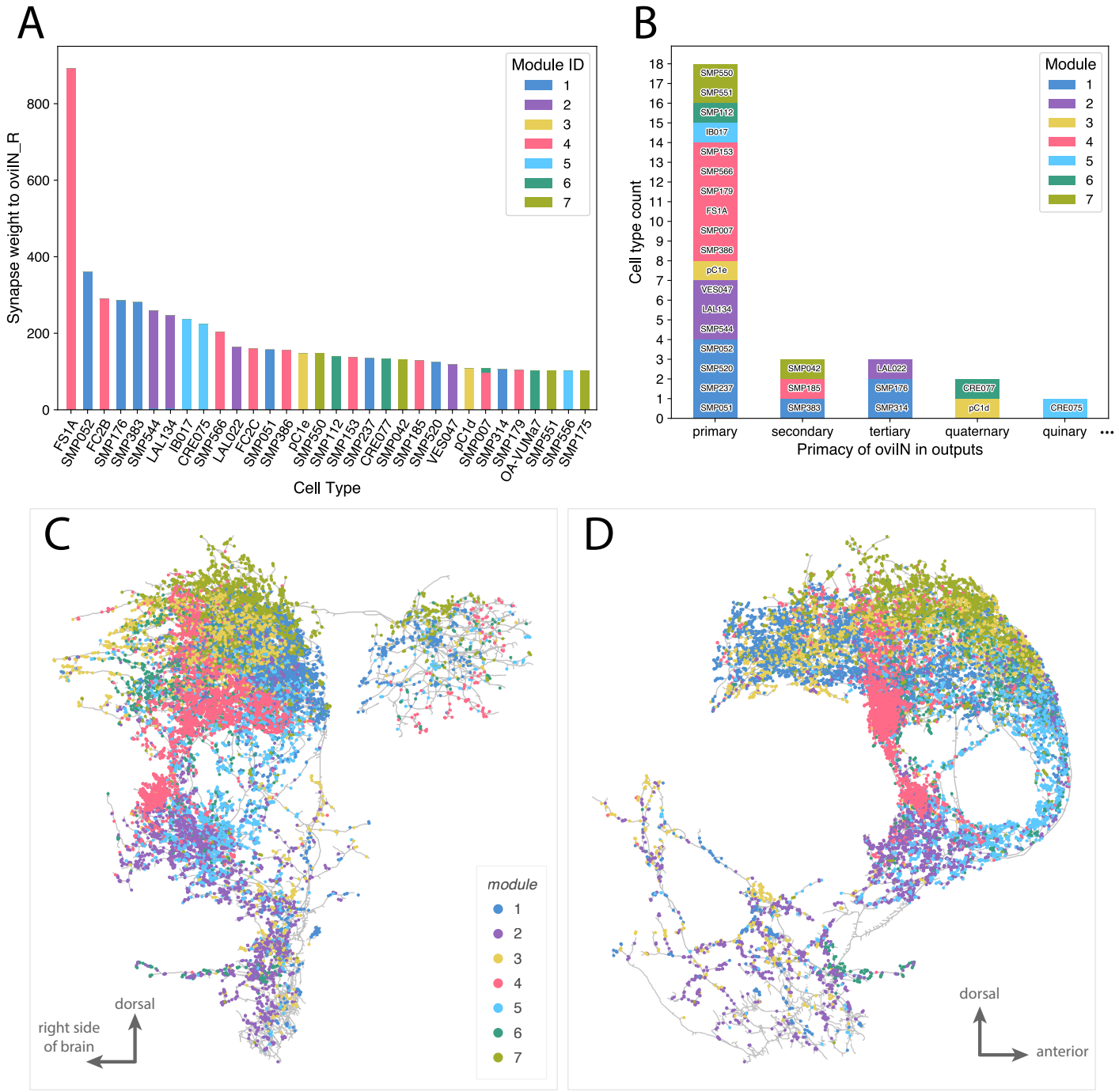

Figure 4. Modularity analysis reveals mesoscale and microscale connectome structure among inputs to oviIN.

A. The strongest inputs to oviIN_R. Neuronal cell types that make an aggregated synapse count of at least 100 onto oviIN_R are shown along the x-axis. The bars indicating synapse counts are color-coded by the module(s) into which the neurons of a type are grouped. Sometimes, neurons of the same type are clustered into different modules. For example, while all instances of FS1A are in module 4, three instances of SMP007 are grouped into module 4 and the other instance is grouped into module 6 (there are four instances of SMP007, all on the right side of the brain).

B. Most of the strongest inputs to oviIN_R are primary inputs, meaning that it primarily sends synaptic outputs to oviIN_R. Only cell types with aggregated connection strength > 100 onto the oviIN_R are shown. Primacy indicates oviIN_R's ranking among the cell type's strongest outputs. Each cell type is colored according to the module where most of its instances are clustered.

C. Front view of synaptic input sites on oviIN_R colored by module. The wire skeleton of the right oviIN from the Hemibrain is outlined in gray. Each dot on the body of oviIN_R is a postsynaptic site. These connection sites are colored according to the module of the presynaptic neuron. Synapses from neurons without a cell type label (annotated as 'None') are not shown, but results are qualitatively the same when they are included.

D. Lateral view (from the right side of the brain) of synaptic input sites on oviIN_R colored by module.

Figure 4—figure supplement 1. Synaptic inputs sites on oviIN_R colored by module and shown separately for each of the 7 modules. Left panel: front view; Right panel: lateral view.

Figure 4—figure supplement 2. Super neuropil representation in the oviIN_R's presynaptic partners. The counts of synapses to oviIN_R within a neuropil are used to compute the neuropil proportions. The pie chart wedges are calculated based on connection weights within a neuropil relative to the total connections in a module.

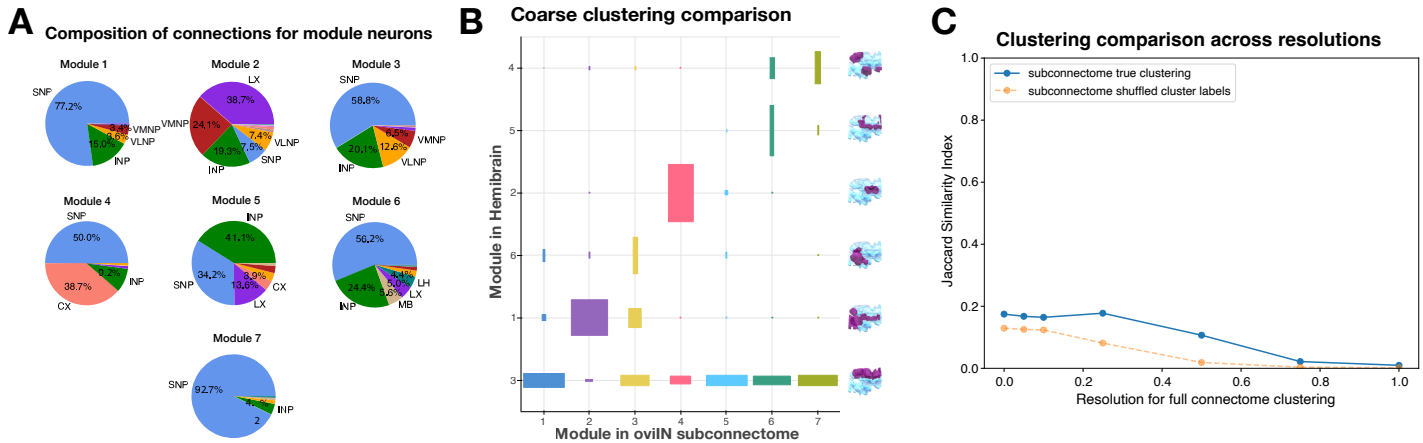

Figure 5.
A. The proportions of connections made in the various super neuropils by the neurons in each module, collectively, are represented as pie slices. The neurons in most of the modules make a large portion of their synapses in the SNP. The super neuropil hierarchy used is defined by ***Ito et al.*** (***2014***).
B. Modularity provides partitions of the oviIN input subconnectome that are distinct from partitions for the full Hemibrain connectome. The seven modules that we found for the subconnectome of oviIN's inputs are shown along the x-axis. The modules for the full Hemibrain found by ***Kunin et al.*** (***2023***) are on the *y*-axis, and an anatomical rendering is provided for each of those modules on the righthand side of the plot. The colors correspond to the subconnectome clustering. The width and height of each box is an indication of the amount of nodes that appear in a given module of the subconnectome clustering and a given module of the full connectome clustering.
C. Jaccard similarity between the coarse clustering of the subconnectome of oviIN's inputs and the full connectome at various clustering resolutions (solid line), from coarse (0.0 resolution) to fine (1.0 resolution). Jaccard similarity for a random baseline is plotted in orange (dashed line) where the cluster labels for the subconnectome were randomly shuffled before being compared to the full connectome clustering.

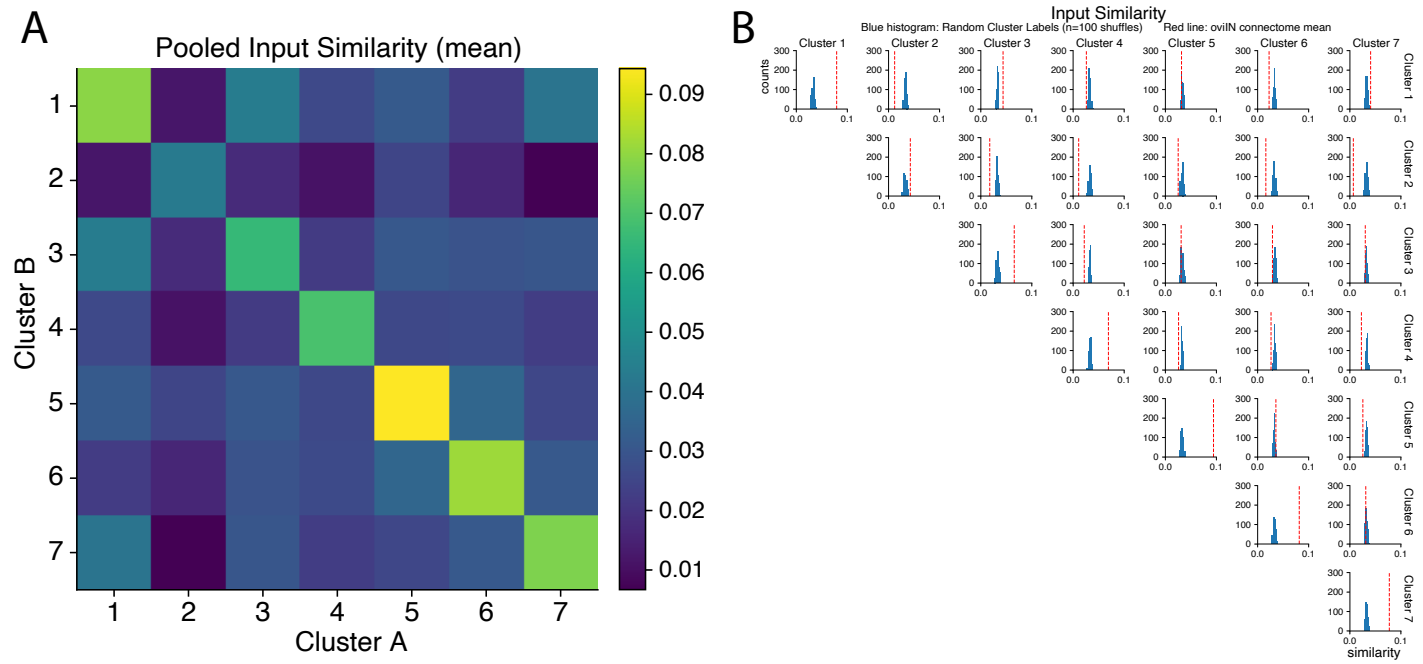

Figure 6.
A. Subconnectome neurons have higher input similarity with neurons of the same module (i.e. cluster) than with other neurons. The inputs to neurons in the subconnectome of oviIN's inputs include direct connections from neurons anywhere in the brain. Heatmap colors indicate the mean input similarity between all neurons in cluster A vs. cluster B.
B. Randomization of module labels shows decreased similarity for all self-same cluster pairs. Input similarity is shown on the *x*-axes which range from 0 to 1. The counts of similarity values for pairs of neurons is shown on the *y*-axes. The red line indicates the mean input similarity between modules as visualized in **A**.

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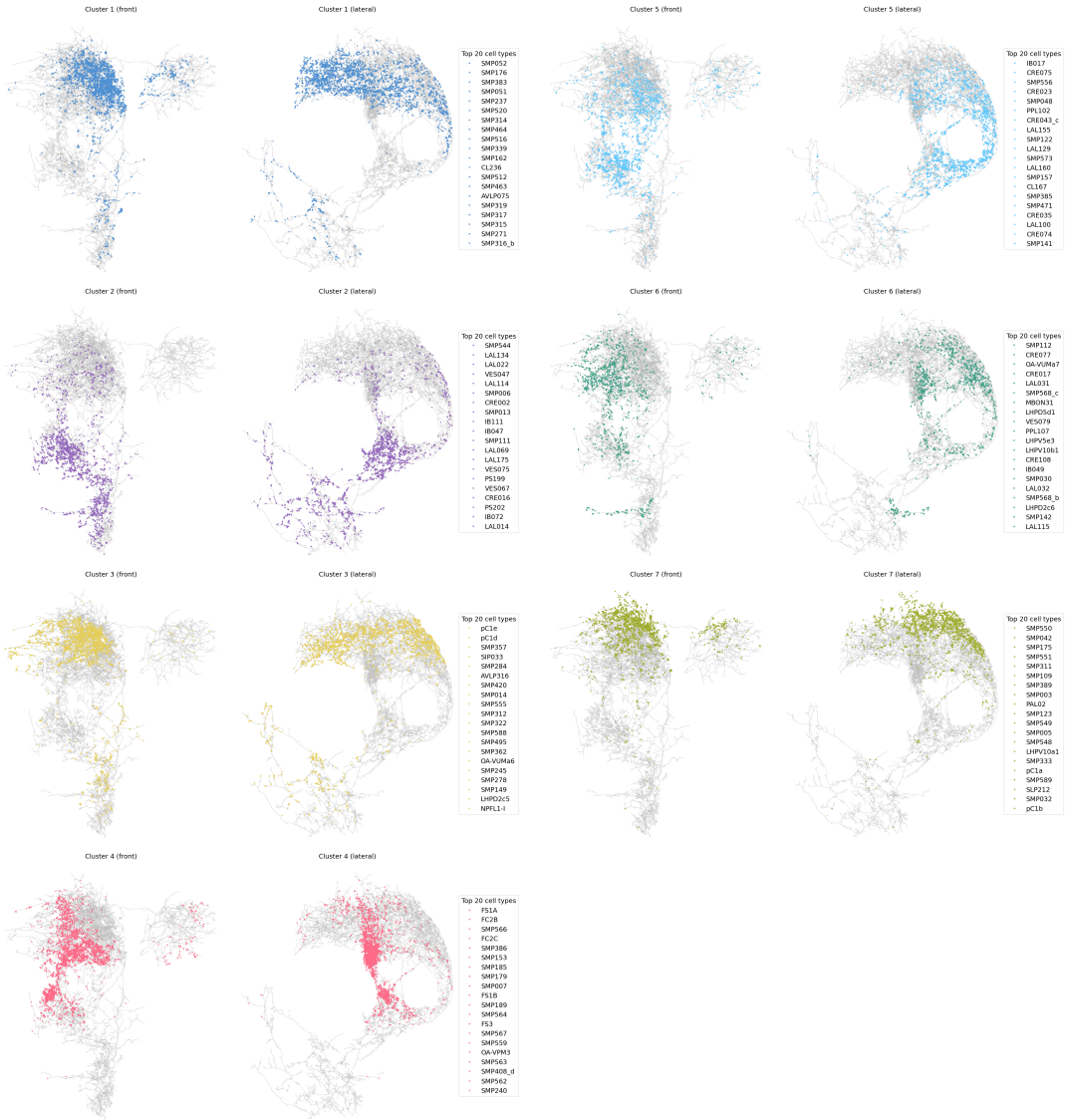

Figure 4—figure supplement 1. Synaptic inputs sites on oviIN_R colored by module and shown separately for each of the 7 modules. Left panel: front view; Right panel: lateral view.

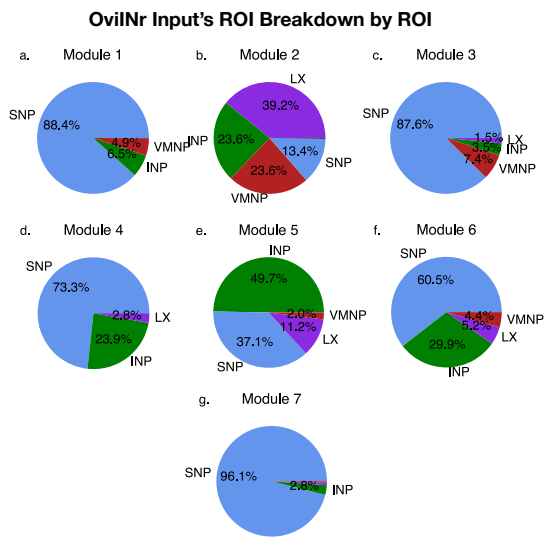

Figure 4—figure supplement 2. Super neuropil representation in the oviIN_R's presynaptic partners. The counts of synapses to oviIN_R within a neuropil are used to compute the neuropil proportions. The pie chart wedges are calculated based on connection weights within a neuropil relative to the total connections in a module.