A connectome of the Drosophila central complex reveals network motifs suitable for flexible navigation and context-dependent action selection

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ABSTRACT [150 words]:
Flexible behaviors over long timescales are thought to engage recurrent neural networks in deep brain regions, which are experimentally challenging to study. In insects, recurrent circuit dynamics in a brain region called the central complex (CX) enable directed locomotion, sleep, and context- and experience-dependent spatial navigation. We describe the first complete electron-microscopy-based connectome of the Drosophila CX, including all its neurons and circuits at synaptic resolution. We identified new CX neuron types, novel sensory and motor pathways, and network motifs that likely enable the CX to extract the fly's head-direction, maintain it with attractor dynamics, and combine it with other sensorimotor information to perform vector-based navigational computations. We also identified numerous pathways that may facilitate the selection of CX-driven behavioral patterns by context and internal state. The CX connectome provides a comprehensive blueprint necessary for a detailed understanding of network dynamics underlying sleep, flexible navigation, and state-dependent action selection.

INTRODUCTION

Many animals are capable of selecting their actions based not just on immediate sensorimotor information, but also on internal state, past experience and future goals. This dynamic process of combining diverse streams of information from the present with knowledge gathered from past experience to determine context-appropriate patterns of actions into the future is at the heart of flexible, goal-oriented behavior. On shorter timescales, the brain must transform information from sensors into a reference frame suitable for motor output to appropriately direct an animal's actions (Buneo et al., 2002; Huston and Jayaraman, 2011; Huston and Krapp, 2008; Pouget et al., 2002). However, initiating and maintaining a series of actions (or maintaining a period of inaction) over a longer timescale than just the immediate present requires animals to ignore distractions. In such situations, it is not enough for the brain to directly transform sensory information into motor commands. Flexible, longer timescale behaviors that are robust to transient changes in sensory information are thought to rely on more abstract internal representations, not just in animal brains (Moser et al., 2008), but also in artificial agents (Banino et al., 2018; Cueva and Wei, 2018). Such representations, which persist in the absence of direct sensory input, are typically generated by recurrent neural circuits in deep brain regions rather than just those at the sensory and motor periphery. A major challenge in understanding the dynamics and function of such circuits is that—in contrast to early sensory circuits—their inputs and outputs are usually difficult to identify and characterize. Further, the internally generated attractor dynamics (Knierim and Zhang, 2012) and vector computations (Bicanski and Burgess, 2020) that characterize many of these circuits, particularly in the context of flexible navigation, rely on structured connectivity between large populations of neurons, which is difficult to determine, at least in large-brained animals. Insects, with their identified neurons and smaller brains (Haberkern and Jayaraman, 2016), present an excellent opportunity to obtain a detailed understanding of how neural circuits generate behavior that unfolds flexibly and over long timescales.

Insects maintain a specific pattern of action selection over many minutes and hours during active behaviors like foraging or migration, and they maintain a prolonged state of inaction during passive behaviors like quiet wakefulness or sleep, when they become relatively insensitive to sensory stimulation (Hendricks et al., 2000; Shaw et al., 2000). Both active and passive behaviors are initiated and modulated based on environmental conditions (for example, humidity, heat and the availability of food) and an insect's internal needs (for example, sleep drive and nutritive state) (Griffith, 2013). The context-dependent initiation and control of many such behaviors is thought to depend crucially on a highly conserved insect brain region called the central complex (CX) (**Figure 1, Figure 1—figure supplement 1, Figure 2**) (Helfrich-Forster, 2018; Pfeiffer and Homberg, 2014; Strauss, 2002; Turner-Evans and Jayaraman, 2016). In Drosophila, this highly recurrent central brain region, which is composed of ~3000 identified neurons, enables flies to modulate their locomotor activity by time of day (Liang et al., 2019), maintain an arbitrary heading when flying (Giraldo et al., 2018; Warren et al., 2018) and walking (Green et al., 2019; Turner-Evans et al., 2020), form short- and long-term visual memories that aid in spatial navigation (Kuntz et al., 2017; Liu et al., 2006; Neuser et al., 2008; Ofstad et al., 2011), use internal models of their body size when performing motor tasks (Krause et al., 2019),

track sleep need and induce sleep (Donlea et al., 2018), and consolidate memories during sleep (Dag et al., 2019).

The precise role of CX circuits in generating these behaviors is an area of active investigation. Neural activity in the region has been linked to sensory maps and directed actions using electrophysiology in a variety of different insects (el Jundi et al., 2015; Guo and Ritzmann, 2013; Heinze and Homberg, 2007; Heinze and Reppert, 2011; Stone et al., 2017; Varga and Ritzmann, 2016). In the fly, CX neurons have been shown to track the insect's angular orientation during navigation in visual environments and in darkness (Fisher et al., 2019; Green et al., 2017; Kim et al., 2019; Seelig and Jayaraman, 2015; Turner-Evans et al., 2017). Many computational models have been proposed to explain how the CX generates such activity patterns during navigation (Arena et al., 2013; Cope et al., 2017; Kakaria and de Bivort, 2017; Kim and Dickinson, 2017; Kim et al., 2019; Stone et al., 2017; Su et al., 2017; Turner-Evans et al., 2017). However, a key untested assumption in most computational and conceptual models of CX function is the connectivity of CX circuits. Importantly, connectivity and circuit structure, in turn, can inspire models of function.

Although the anatomy of the CX (**Figure 1A-E**) and the morphology of its neurons have been examined in a wide variety of insects using light-level microscopy (el Jundi et al., 2018; Hanesch et al., 1989; Heinze and Homberg, 2008, 2009; Homberg, 2008; Lin et al., 2013; Pfeiffer and Homberg, 2014; Strausfeld, 1999; Williams, 1975; Wolff et al., 2015; Wolff and Rubin, 2018), the synaptic connectivity of CX neurons has mainly been estimated indirectly from the light-level overlap of the bouton-like processes of one neuron and the spine-like processes of another. GFP-reconstitution-across-synaptic-partners (GRASP) (Xie et al., 2017) and trans-Tango (Omoto et al., 2018), methods that are limited in accuracy and reliability (Lee et al., 2017; Talay et al., 2017), have also been used to infer synaptic connectivity in the fly CX. Optogenetic stimulation of one candidate neural population and two-photon imaging of the calcium responses of another (Franconville et al., 2018) has allowed estimations of coarse functional connectivity within the CX, but this technique currently lacks the throughput to comprehensively determine connectivity at the single-neuron (rather than neuron-type) level, and also cannot easily discriminate direct from indirect synaptic connectivity. There have also been efforts to establish the synaptic connectivity of neurons in the CX and associated regions with electron microscopy (EM) in the bee and locust (Held et al., 2016; Homberg and Muller, 2016), and coarser-scale EM has been used to infer connectivity in the sweat bee (Stone et al., 2017). Most recently, the reconstruction of a small set of CX neurons within a whole-brain volume acquired using transmission EM (TEM) (Zheng et al., 2018) was used to examine the relationship between circuit structure and function in the head direction system (Turner-Evans et al., 2020).

Here we analyzed the arborizations and connectivity of the ~3000 CX neurons in version 1.1 of the 'hemibrain' connectome—a dataset with 25000 semi-automatically reconstructed neurons and 20 million synapses from the central brain of the fly (Scheffer et al., 2020). For most of the analyses, interpretations and hypotheses in this manuscript, we built on a large and foundational body of anatomical and functional work on the CX in a wide variety of insects.

Not all parts of the dataset have been manually proofread to exactly the same level of completeness (measured as the percentage of synapses associated with neural fragments that are connected to identified bodies). In the CX, some substructures were proofread more densely and to a higher level of completion than the others. Comparing the connectivity maps obtained after different extents of proofreading indicated that synaptic connectivity ratios between CX neuron types were largely unchanged by proofreading beyond the level applied to the full CX. This analysis reassured us that our analyses and conclusions were not significantly compromised by the incompleteness of the connectome.

We identified multiple, parallel sensory pathways from visual (polarized and unpolarized light) and mechanosensory areas into the ellipsoid body (EB), a toroidal structure within the CX (**Figure 1B**). Neurons within each pathway make all-to-all synaptic connections with other neurons in their type and also onto "compass neurons" known to represent the fly's head direction, creating a potential neural substrate within the EB for extracting orientation information from a variety of environmental cues.

The compass neurons are part of a recurrent sub-network with a topological and dynamical resemblance to theorized network structures called ring attractors (Ben-Yishai et al., 1995) that have been hypothesized to compute head direction in the mammalian brain (Hulse and Jayaraman, 2019; Kim et al., 2017b; Turner-Evans et al., 2017; Turner-Evans et al., 2020; Xie et al., 2002; Zhang, 1996). A key connection in the sub-network is from the compass neurons to a population of interneurons in a handlebar-shaped structure called the protocerebral bridge (PB) (**Figure 1B**). The profile of synapse counts across this connection suggests that they ensure that the head direction representation is maintained in a cosine-shaped "bump" of population activity before it is transferred to multiple types of so-called "columnar" neurons. In addition to receiving head direction information, many of these columnar neurons likely receive input related to the fly's self-motion in paired structures known as the noduli (NO) (**Figure 1B**), which may independently tune the amplitude of cosine-formatted activity bumps in the left and right PB. Some classes of columnar neurons project from the PB back to the EB, while others project to localized areas within a coarsely multi-layered and multi-columnar structure called the fan-shaped body (FB) (**Figure 1B**). The layers and columns of the FB form a rough, two-dimensional grid.

FB columnar neurons convey activity bumps from the left and right PB to the FB using neuron type-specific phase shifts, similar to those used to update the head direction representation in the EB (Green et al., 2017; Turner-Evans et al., 2017; Turner-Evans et al., 2020). Each FB columnar type contacts characteristic interneuron types whose individual neurons collectively tile FB columns but with neuronal morphologies that either form horizontal arborizations with specific phase jumps (forming connections across columns) or vertical arborizations (connecting layers within a column). The many FB interneuron types form a densely recurrent network with recurring motifs and phase shifts that seem ideal for vector computation. They also provide the major input to canonical CX output types. The phase shifts of the output columnar types between the PB and the FB are well suited to generate goal-directed motor commands based

on the fly's current angular orientation. Together, the architecture and connectivity of the FB indicate a capacity for coordinate transformations necessary for a variety of vector-based navigational computations, and, consequently, also a potential for behavioral complexity in the fly.

In addition to the columnar neurons and interneurons, the FB receives layer-specific synaptic inputs from multiple regions, including the superior medial protocerebrum (SMP). In turn, some of these inputs link the FB to a brain region called the mushroom body (MB), which is involved in associative memory and is the subject of a companion manuscript (Li et al., 2020). These layer-specific inputs reinforce an existing view of the FB as a center for context-dependent navigational control: a structure that enables different behavioral modules to be switched on and off depending on internal state and external context. Although the FB provides the scaffold for computations within layers and across columns, information within the structure largely flows from ventral to dorsal regions.

The dorsal regions of the FB have also been associated with sleep-wake cycles in the fly (Donlea et al., 2011). We found that this region of the FB contained an unexpectedly high number of neuron types, including input from two types of dopaminergic neurons (DANs). We also found direct connections from the dorsal FB to sleep centers in the EB.

Finally, we identified multiple novel output pathways from both the EB and ventral and dorsal layers of the FB to the lateral accessory lobe (LAL), superior medial protocerebrum (SMP) and crepine (CRE) (**Figure 1—figure supplement 1**), regions that feed several descending neurons that project to motor centers in the ventral nerve cord (VNC). A remarkable feature of the CX's output pathways is the number of collaterals that are fed back into the CX at each stage.

In summary, our analysis revealed remarkable patterning in the connections made by the hundreds of neuron types that innervate different structures of the CX. In the Results sections that follow we describe this patterned connectivity in some detail. In the Discussion section, we synthesize these findings and explore what this patterned circuit structure may imply about function, specifically in the context of vector-based navigation and sleep.

RESULTS

In contrast to many previous EM-based circuit reconstruction efforts, which have relied on sparse, manual tracing of neurons (Eichler et al., 2017; Helmstaedter et al., 2013; Turner-Evans et al., 2020; Zheng et al., 2018), the hemibrain connectome was generated using a combination of automatic, machine-learning-based reconstruction techniques (Januszewski et al., 2018; Li et al., 2019; Scheffer et al., 2020) and manual proofreading (Scheffer et al., 2020). This semi-automatic process allowed us to reconstruct a large fraction of most neurons that project to the CX. This, as we explain below, aided our efforts to classify and name CX neurons. Of course, a complete CX connectome would contain the complete reconstruction of all neurons with processes in the CX, the detection of all their chemical and electrical synapses, and an identification of all pre- and post-synaptic partners at each of those synapses. The resolution of the techniques used to generate the FIBSEM connectome did not permit the detection of gap

junctions. Nor does the connectome reveal neuromodulatory connections mediated by neuropeptides, which are known to be prevalent in the CX (Kahsai and Winther, 2011). Glial cells, which perform important roles in neural circuit function (Allen and Lyons, 2018; Bittern et al., 2020; De Pittà and Berry, 2019; Ma et al., 2016; Mu et al., 2019), were not segmented. In addition, although the hemibrain volume contains the core structures of the CX —the entire PB, EB, and FB, and both the right and left NO and asymmetric bodies (ABs)— it does not include all brain structures that are connected to the CX. Specifically, for many brain structures associated with the CX that are further from the midline, the hemibrain volume only contains complete structures within the right hemisphere (Scheffer et al., 2020). Thus, the hemibrain does not contain the LAL, CRE, WED, VES, Gall, and BU from the left hemisphere, and CX neurons whose arbors extend into these excluded brain structures are necessarily cut off at the borders of the hemibrain volume (their status is indicated in the connectome database, see Materials and Methods). Even for structures within the right hemisphere, such as the LAL, CRE and PS, some neural processes that connect these structures to each other were not captured in the volume, sometimes making it impossible to assign the orphaned arbors to known neurons. Nevertheless, we were able to identify the vast majority of CX neurons and many neurons in accessory regions as well.

CX neuron classification and nomenclature

Historically, Drosophila CX neurons have been typed and named by using data from light microscopy (LM) (Hanesch et al., 1989; Lin et al., 2013; Wolff et al., 2015; Wolff and Rubin, 2018; Young and Armstrong, 2010b). Light-level data, often acquired from GAL4 lines that genetically target small numbers of neuron types (Jenett et al., 2012; Nern et al., 2015; Pfeiffer et al., 2010; Wolff et al., 2015; Wolff and Rubin, 2018), reveals neuronal morphology in sufficient detail to classify neurons into types and subtypes. However, only neurons targeted by existing genetic lines can be identified through light-level data, and morphologically similar neuron types can be hard to distinguish. For example, the PEN1 and PEN2 types (now called PEN_a and PEN_b; see Materials and Methods), which seem identical at the light level (Wolff et al., 2015), were initially differentiated by their functional properties (Green et al., 2017). The hemibrain dataset provides much higher resolution maps of CX neurons than light-level data and enables a distinction to be drawn based on connectivity between neuron types that are morphologically identical in light-level samples. EM reconstructions confirm that PEN_a and PEN_b neurons are indeed strikingly different in their synaptic connectivity (Scheffer et al., 2020; Turner-Evans et al., 2020).

However, LM data offer far greater numbers of neurons per neuron type across different brains. To date, just this one fly CX has been completely analyzed at synaptic resolution with EM. Thus, for neuron types that are represented by just one neuron per hemibrain, at best two fully traced neurons are available for analysis (one per side), and only one if the arbor of the second extends outside the hemibrain volume. The inherent variability of individual neurons of a single type is not yet clear. It is likely that arbors in some neuropils exhibit a greater degree of variability than other neuron types; for example, the number and length of branches in a larger neuropil such as the LAL is likely to be less tightly regulated than in a small neuropil, leading to

greater variability in morphology and perhaps also in synaptic connections with upstream or downstream partners in that neuropil.

The full spectrum of the morphology of a neuron type will likely not be verified until multiple single neurons are analyzed in a split-GAL4 line known to target just that one neuron type, or until multiple brains are analyzed at EM resolution. In the meantime, EM and LM datasets contribute both partially overlapping as well as unique anatomical insights useful for classifying and naming neurons. Both datasets were therefore used to assign names to previously undescribed neurons of the CX, the overwhelming majority of which are FB neurons (see **Nom Tables 1** and **2** for all new CX neuron types, with numbers for each type). All CX neurons have been given two names, a short name that is convenient for searching databases and that is used as a shorthand abbreviation throughout this manuscript, and a longer name that provides sufficient anatomical insight to capture the overall morphology of the neuron. The long anatomical names have their roots in both the EM and LM datasets, emphasize overall morphology and attempt to define neuron types based on features that we anticipate can be distinguished at the light level, ultimately in split GAL4 lines (see details in Materials and Methods). The short names are derived primarily from hemibrain connectivity information. Our overall method for connectivity-based neuron type classification of CX neurons was described in (Scheffer et al., 2020), but see Materials and Methods for a short summary.

Validation of CX connectome

The manual proofreading procedure we used is labor-intensive and time consuming. For this reason, it was not be performed to the same extent on the entire connectome. In general, completeness within the core CX structures is higher than those of peripheral CX structures (Scheffer et al., 2020). We therefore performed a series of validation analyses to examine how estimates of connectivity within CX regions might be affected by the percentage of known synapses that are assigned to known neuronal bodies rather than to unidentified neural fragments (partially reconstructed neural processes) within the region.

<u>Neuron-to-neuron connectivity before and after dense proofreading for the same neurons:</u>
To assess the sensitivity of network connectivity estimates to the completeness level of the CX connectome, we compared both the pre- and postsynaptic connectivity of a selection of neurons (see Methods) arborizing in a specific brain structure, the EB, before and after it was subjected to focused proofreading (**Figure 3A**). Proofreading increased the number of pre and post synapses for which the synaptic partner could now be identified (see increase in percentage of identified synaptic partners for most neuron types in **Figure 3Aii**), mainly by merging small dendritic fragments onto known neurons. Importantly, however, the *relative* synaptic contributions that each neuron received from its various upstream partners remained largely the same before and after dense proofreading (example fit for the EPG neuron of **Figure 3Ai** in **Figure 3Aiii**, slopes from regression analyses of all neurons shown in **Figure 3Aiv**). Indeed, with the exception of some FB neurons with minor projections in the EB, (green points in **Figure 3Aiv**, right), input and output vectors of most neurons did not significantly change (**Figure 3Aiv**, see also **Figure 3—figure supplement 1**).

Neuron-to-neuron connectivity at different completion percentages within the same brain region:

In addition to comparing connectivity for the same neurons at different completion levels, we also examined connectivity in different sub-regions that were proofread to differing levels of completeness. Parts of the PB (glomeruli L4 and R3) and FB (the third column) were also intentionally proofread to a denser level of completeness than other areas within those structures. In addition, although we did not perform an analysis of these differences, the right NO was more densely proofread than the left NO (Scheffer et al., 2020). These differences in level of completeness must be kept in mind when interpreting synapse counts in these regions.

In the PB, we compared connectivity within the densely proofread R3 and L4 glomeruli to their less densely proofread mirror symmetric glomeruli, L3 and R4, respectively. For a meaningful comparison, we focused our connectivity analysis on neurons with arbors restricted to single PB glomeruli (**Figure 4Ai**, for example, shows examples of PFNa neurons that innervate L3 and R3, respectively). The impact of dense proofreading in the PB was evident in the increased percentage of synapses from or to identified partners of neurons of the same type in the densely proofread glomeruli, L4 and R3 (**Figure 4Aii**, for presynaptic partners, left, and postsynaptic partners, right). However, as with the EB comparison before and after dense proofreading (**Figure 3A**), we found that the relative contributions of different identified partners onto the selected types remained nearly unchanged across the two glomeruli (**Figure 4Aiii, 2Aiv for an example regression**), both for inputs and outputs. As a control, we also performed the same analyses on two additional pairs of mirror-symmetric PB glomeruli, L5 and R5, and L6 and R6 (**Figure 4—figure supplement 1**), finding no more differences in relative contributions than we found when comparing the L3-R3 and L4-R4 pairs (**Figure 4A**).

Finally, we also selected a columnar region within the medial part of the FB ('C3') for focused proofreading (**Figure 5Ai**). As with the EB and PB, this process led to an increase in the percentage of synapses with identified partners (**Figure 5Aii**) without significant changes in connectivity (**Figure 3 Val Ciii**). Because FB columns do not have sharp borders and thus cannot be cleanly defined, a similar comparison between columns was not possible. While many neuron types did show significant differences in their connectivity between columns (**Figure 5Aiv**), we believe this to be a result of true inhomogeneities across vertical sections of the FB, as we discuss further in the section on the FB.

Assessing the relative importance of different synaptic inputs

Morphologically, many fly neurons feature a single process that emanates from the soma, which usually sits near the brain surface, and this process then sends branches out into multiple brain structures or substructures. Although there is usually one compartment with mainly postsynaptic specializations, the other compartments are typically "mixed", featuring both pre- and postsynaptic specializations. This heterogeneity and compartmentalization make it challenging to evaluate the relative weight of different synaptic inputs to the neuron's synaptic output. Even for fly neurons that spike, action potential initiation sites are largely unknown (although see (Gouwens and Wilson, 2009; Ravenscroft et al., 2020)). Furthermore, spiking

neurons may perform local circuit computations involving synaptic transmission without action potentials. In this study, we will evaluate the relative contributions of different presynaptic neurons keeping in mind the compartment in which the synapses are made, and, where possible, we will assume a polarity for neurons based on compartments in which they are mainly postsynaptic. For spiking neurons, these "dendritic" areas would be expected to play a more significant role in determining the neuron's response, even if the neuron displays mixed pre- and postsynaptic specializations in other compartments. Consider, for example, this rule applied to a much-studied olfactory neuron, the projection neuron (PN). PNs receive most of their inputs in the antennal lobe (AL) and project to regions like the mushroom body (MB) and lateral horn (LH), where they have mixed terminals. Our rule would lead to the AL inputs being evaluated separately and being considered stronger contributors to a PN's spiking outputs than any synaptic inputs in the MB and LH. As discussed further in a subsequent section, we will use this logic to define the 'modality' of most ring neurons, which innervate the BU and EB, by the AOTU input they receive in the BU rather than by the inputs they receive in the EB. This logic also applies to the tangential neurons of the FB, which receive mainly inputs in regions outside the CX and have mixed terminals inside the FB. Note that some CX neuron types may not rely on spiking at all, and that our assumptions may not apply to such graded potential neurons. The situation is also somewhat different for some interneuron types, such as the PB-intrinsic $\Delta 7$ neurons, which have multiple arbors with post-synaptic specializations.

CX synapses are not all of the 't-bar' type that is most common in the insect brain (Frohlich, 1985; Meinertzhagen, 1996; Trujillo-Cenoz, 1969). As discussed later, several CX neurons make 'e-bar' synapses (Shaw and Meinertzhagen, 1986; Takemura et al., 2017), which we do not treat any differently in analysis. In addition, although many synapses are polyadic, with a single presynaptic neuron contacting multiple postsynaptic partners (**Methods Figure 1A**), it is notable that some neurons, such as ring neurons make rarer convergent synapses in which multiple presynaptic ring neurons contact a single postsynaptic partner (**Methods Figure 1B**) (Martin-Pena et al., 2014). The function of such convergences is, at present, unknown.

Visual, circadian, mechanosensory and motor pathways into the EB

Although the hemibrain volume did not permit us to trace pathways from the sensory periphery all the way into the CX, we tried to identify as many inputs as possible, using previous results from light microscopy as our guide to the earliest stages that we had access to. We began with identifying components of a prominent pathway from the fly eyes to the EB.

<u>The anterior visual pathway: compartmentalization within the AOTU</u>

The anterior visual pathway brings visual information from the medulla into the smaller subunit of the AOTU, and thence to the BU's ring neurons (Hanesch et al., 1989), which deliver more processed information to the EB (Omoto et al., 2017; Timaeus et al., 2020) (**Figure 6A,B**). The ring neurons, which are called TL neurons in some insects (Homberg et al., 1999; Muller et al., 1997), are fed by multiple, developmentally distinguishable types of visually responsive neurons from the small unit of the AOTU. Together these TuBu neurons compose the first part of the anterior visual pathway that we can access in the hemibrain volume. The pathway comprises neurons that project from the photoreceptors to the medulla, the medulla to the AOTU, the

AOTU to the BU, and the BU to the EB (**Figure 6A,B**) (Homberg et al., 2003; Omoto et al., 2017; Sun et al., 2017). Across insects, some types of TuBu neurons (also called TuLAL1 neurons in some insects) are known to be tuned to polarized light e-vector orientations and visual features (el Jundi et al., 2014; Heinze et al., 2009; Heinze and Reppert, 2011; Omoto et al., 2017; Pfeiffer et al., 2005; Sun et al., 2017), properties that they likely inherit from their inputs in the AOTU (Hardcastle et al., 2020; Omoto et al., 2017; Sun et al., 2017). In the fly, some TuBu neurons are known to respond strongly to bright stimuli on dark backgrounds (Omoto et al., 2017) or the orientation of polarized light e-vectors (Hardcastle et al., 2020), consistent with the idea that these neurons may be part of a sky compass pathway, as in other insects (el Jundi et al., 2018; Homberg et al., 2011).

The hemibrain volume (light blue shaded region in **Figure 6A**) does not include areas of the optic lobe that would permit an unambiguous identification of AOTU inputs from the medulla (together called the anterior optic tract), thus only two broad subclasses of medullar columnar neurons can be distinguished (MC64 and MC61; **Figure 6C**). However, following the schema employed in recent studies, we used the innervation patterns of different medullar columnar neuron types to delineate two distinct zones in the small unit of the AOTU, or AOTUsu (Figure 6Di), which closely match previously characterized regions that receive input from different types of medullar columnar neurons (Timaeus et al., 2020). In other insects, some AOTU-projecting medullar columnar neurons (MeTus) are known to be tuned to polarized light e-vector orientations (Heinze, 2014), and such information is known to be present in the Drosophila medulla as well (Weir et al., 2016). A more recent study has confirmed that some classes of fly AOTU neurons, as well as their downstream partners, respond to polarized light stimuli much like in other insects (Hardcastle et al., 2020)—we believe these to be the TuBu01 and TuBu06 neuron types (**Figure 6C, Dii**).

TuBu neuron types arborize in subregions of the AOTU that respect the boundaries defined by medullar columnar inputs (AVP Fig 1Dii). Further, TuBu neurons form columns along the dorsal-ventral axis of their respective AOTU subregion (**Figure 6Dii**) (Omoto et al., 2017; Timaeus et al., 2020). Both the medullar columnar neurons and the TuBu neurons tile each AOTUsu zone (**Figure6 Bi, E**), consistent with the TuBu neurons inheriting a retinotopic organization from their columnar inputs (Timaeus et al., 2020). On average, there is a 40:1 convergence from medulla columnar neurons onto TuBu neurons (**Figure 6—figure supplement 1**), potentially increasing the size of the receptive fields of TuBu neurons compared to MeTu neurons. Although this tiling is well-organized for TuBu neurons that receive inputs from MC61 medulla neurons (**Figure 6Ei**), it becomes more diffuse for TuBu neurons that receive inputs from MC64 medulla neurons (**Figure 6Eii**), consistent with LM-based anatomical analysis (Timaeus et al., 2020). This differentiation of TuBu neuron types is also maintained in their downstream projections to the BU (next section). Note, however, that multiple TuBu neuron types can also receive their inputs from the same AOTUsu zone, as, for example, TuBu01 and TuBu06 do (**Figure 6Dii**). It is possible that these different TuBu neuron types receive different medullary inputs in the same zone of the AOTU, but they are, regardless, easily distinguished both by the additional inputs they receive in the BU, as well as by their downstream partners in that structure (discussed below).

The anterior visual pathway: convergence and divergence largely within BU zones

The BU is a dedicated input structure for the CX: nearly every neuron type that receives most of its input in the BU has presynaptic specializations within one or more core CX structures (**Figure 7A**). Most neurons innervating the BU have spatially restricted, glomerular arborizations (Trager et al., 2008), such as the TuBu neuron types and ring neurons (except ER6), but some arborize widely within the structure, such as the AOTU046 and ExR neurons (**Figure 7B**). A recent study combining lineage-based anatomy and functional imaging has suggested that the BU may be organized into zones with similarly tuned TuBu neurons (Omoto et al., 2017). Consistent with this functional segregation, TuBu neurons originating from different parts of the AOTU do indeed segregate into different zones within the BU (superior, inferior and anterior BU, **Figure 7B, Ci**), with MC61-fed TuBu neurons innervating the superior BU and MC64-fed TuBu neurons targeting the inferior BU, thereby maintaining some separation of visual information from the AOTU (**Figure 6C**). We used synapse locations of different types of TuBu neurons and their downstream partners, the ring neurons, to partition the BU into different zones (**Figure 7C**), each with numerous microglomeruli where TuBu neuron types make synapses onto different types of ring neurons. Ring neurons that receive their input in the BU send their dendrites into a single zone of the BU.

The type-to-type mapping from TuBus to ring neurons is largely one-to-one, with most ring neuron types receiving synaptic inputs from only one TuBu type each, for example, TuBu01 to ER4m and TuBu06 to ER5 (**Figure 7D**, see yellow and blue boxes). However, some TuBu types feed multiple ring neuron types, for example, most TuBu02 neurons project to both ER3a_a and ER3a_d ring neurons, and also to ER3m ring neurons (**Figure 7D**). Interestingly, although the segregation of TuBu types is largely maintained at the type-to-type level, there is significant divergence at the level of individual TuBu neurons, with most TuBu neurons feeding several ring neurons of a given type (**Figure 7D**, **Figure 7—figure supplement 1B**). Most TuBu02 neurons, for example, make synapses onto multiple ER3a_a, ER3a_d and ER3m neurons (**Figure 7**, **Figure 7—figure supplement 1B**). There is also significant convergence at the single neuron level, with many ring neurons receiving inputs from more than one TuBu neuron (**Figure 7D**, **Figure 7—figure supplement 1A**). This combination of convergence and divergence patterns likely underlies the degree of retinotopy observed in the responses of various ER neurons to visual features (Omoto et al., 2017; Seelig and Jayaraman, 2013). However, the level of divergence varies between types.

An interesting contrast is between the mapping of TuBu01 to ER4m, which is strictly one-to-one, preserving receptive fields (but not polarotopy (Hardcastle et al., 2020)), and TuBu06 to ER5, where multiple TuBu06 neurons contact a single ER5 neuron and single TuBu06 neurons project to multiple ER5 neurons (**Figure 7D,E**, **Figure 7—figure supplement 1B,C**). This is noteworthy, as TuBu01 and TuBu06 receive input in the same region of the AOTU, but their downstream partners, ER4m and ER5 neurons, are known to have different functions: ER4m is tuned to polarized light (Hardcastle et al., 2020) and likely involved in visual orientation whereas ER5 neurons are involved in sleep (both will be discussed in more detail in later sections).

Overall, this combination of divergence from individual TuBu neurons to multiple ring neurons and convergence from multiple TuBu neurons to individual ring neurons strongly suggests that we should expect receptive fields in the anterior visual pathway to expand across BU synapses. Finally, it is important to note that there are several neuron types with wide arborizations within the BU that likely also influence processing in the structure. These include the ExR neuron types (ExR1, ExR2, ExR3 and ExR5) and the AOTU046, which are discussed below.

The anterior visual pathway: contralateral influences

Thus far, we have characterized the anterior visual pathway as a largely feedforward pathway of neurons with spatially localized arbors projecting from the early visual system to the AOTU and on to the BU. However, the connectome suggests that other, widely arborizing neuron types likely also determine responses of neurons at different stages of the pathway (**Figure 8A, B**). Indeed, although many TuBu neuron types in the superior BU are known to display prominent ipsilateral visual receptive fields consistent with ipsilateral inputs in the AOTU (Omoto et al., 2017), there is also strong functional evidence suggesting that many of these neurons receive large-field inhibitory input from the contralateral hemisphere, creating the potential for stimulus competition across the left and right visual fields (Omoto et al., 2017; Sun et al., 2017).

The inter-hemispheric TuTuB_a neurons (**Figure 8Ai**), which pool medullary input from one visual field and synapse onto a subset of TuBu neurons on the other side, are a likely candidate to provide such inhibition —note that at least one of these TuTuB neurons is known to be tuned to polarized light e-vector orientation (Hardcastle et al., 2020). The inter-hemispheric AOTU046 neurons, which receive input from multiple brain regions including both AOTUs, the BU (although they primarily send outputs there), the FB, and the ipsilateral (to their soma) IB and SPS (**Figure 8Aii,C-E**), are the major candidate for such inhibition, since both neuron types receive input from large areas of the AOTU (**Figure 8C**) and since ring neurons show stronger signatures of contralateral inhibition than TuBu neurons (Sun et al., 2017).

We could not, however, find any obvious candidates that could confer TuBu neurons of the inferior BU with small-object-sized receptive fields in the contralateral hemisphere as has recently been suggested for a subset of these neurons (Omoto et al., 2017; Shiozaki and Kazama, 2017). Based on the connectome, one possibility is that these reported responses were caused not by small-field feature detectors related to the AOTU or BU, but by other input from the ExR3 neurons (**Figure 8Aiii,D**). The ExR3 neurons receive synaptic input from a variety of neurons in areas that are known to respond to optic flow and the fly's own movements (**Figure 8F**), both of which may have contributed to the reported response properties. A second possibility is that these responses were observed in the subset of ring neurons that form glomeruli in the BU, but that also receive additional inputs in the LAL (ER3a_a and ER3a_d, **Figure 7A**, **Figure 10—figure supplement 5**).

The widely arborizing neurons —ExR1, ExR2, ExR3, ExR5 and AOTU046 (Figure 8A)— have somewhat overlapping arbors in the BU (**Figure 8D**) and are selectively interconnected in the

region (**Figure 8B**). The bilaterally projecting AOTU046 neurons receive ipsilateral inputs from ExR2 and ExR5 neurons and contralateral input from the ExR3 neurons (**Figure 8B**), and themselves feed ExR1 neurons in the same hemisphere (**Figure 8B**). Importantly, the widely arborizing neurons are selective in their TuBu and ring neuron type connections within the BU: the AOTU046 neuron type, for example, provides presynaptic input to the TuBu01, but not to the TuBu06 (**Figure 8—figure supplement 1A**), the pair of neuron types whose dendrites appear to arborize in similar AOTU zones (**Figure 6Dii**). TuBu1 neurons, for example, receive significant input from ExR5 and AOTU046 neurons in the BU, but TuBu6 neurons do not (**Figure 8B**). The ExR3 neurons appear to be recurrently connected to TuBu02, TuBu03 and TuBu04 neuron types (**Figure 8B**). Thus, most TuBu neuron types that appear to receive input from similar zones in the AOTU can be discriminated by their BU inputs from widely arborizing neuron types. The function of these external inputs is not yet known.

Ring neurons that receive circadian input

In contrast to all the ring neuron types described above, most of which have been considered as bringing visual input into the EB, we note that the ER5 neurons have been primarily associated with conveying a signal related to sleep homeostasis (Donlea et al., 2018; Liu et al., 2019; Liu et al., 2016a; Raccuglia et al., 2019). These neurons are known to receive input from TuBu neurons (Guo et al., 2018; Lamaze et al., 2018; Liang et al., 2019; Raccuglia et al., 2019). The connectome allowed us to identify these TuBu neurons as being TuBu06 neurons (**Figure 7D,E, Figure 8B**), which, in turn, receive input from the interhemispheric TuTuB_b neurons that receive clock circuit input through the DN1pB neurons (Guo et al., 2018; Lamaze et al., 2018; Liang et al., 2019) (**Figure 6C**). This information alters and refines the picture for sleep and circadian circuits within the CX, which we discuss in more detail in a later section. Consistent with related suggestions from other insects, it is also likely that circadian input from DN1pB neurons to the polarized light e-vector pathway, involving the TuBu01 neurons that project to the ER4m neurons (**Figure 6C**), could allow for polarized light responses to be adjusted based on the time of day to compensate for changes in the polarization pattern as the sun moves across the sky (Sauman et al., 2005).

Mechanosensory (wind) input to ring neurons

Although the anterior visual pathway has been the focus of many studies in flies and other insects, extracellular recordings from the cockroach CX have demonstrated that the compass system also receives directional mechanosensory input (Bender et al., 2010; Guo and Ritzmann, 2013; Ritzmann et al., 2008). In the fly, recent work has shown that such information is carried by ER1 and ER3a ring neurons (Okubo et al., 2020). Unlike the majority of ring neurons that bring sensory information to the EB and receive visual input in the BU, these ring neurons gather much of their input from the LAL and wedge (WED) (**Figure 9A,B**), a structure known to receive significant mechanosensory input from the antennal Johnston's organ (Patella and Wilson, 2018; Suver et al., 2019). The CX connectome revealed that both of these neuron populations consist of multiple types. There are two ER1 types, ER1_a and ER1_b, and four ER3a types (**Figure 10—figure supplement 5**). Of the four ER3a types, ER3a_a and ER3a_d primarily receive inputs in the BU, wheras ER3a_b and ER3a_c receive inputs in the LAL (**Figure 10—figure supplement 5**). We found that only ER3a_b and ER1_b neurons are postsynaptic to

neurons that we believe to be WPN neurons (**Figure 9A-C**) —neurons that have been characterized as being wind-direction-sensitive (Suver et al., 2019). These putative WPN neurons are themselves downstream of APN neurons (**Figure 9A,C**), which are known to be mechanosensory (Patella and Wilson, 2018; Suver et al., 2019). The ER1_b neurons also receive input in the LAL from the WLL neurons (Franconville et al., 2018; Okubo et al., 2020), which, recent work has suggested, tonically inhibit the ER1_b neurons (Okubo et al., 2020). We note that this study also showed evidence for WLL neurons being gap-junction-coupled to ER1_b neurons, which the hemibrain volume lacks the resolution to confirm. Note that in contrast to the ER1_a neurons, whose processes are distributed across the dorsal-ventral axis of the LAL (**Figure 9Di,Ei**), the ER1_b neurons receive inputs, especially from ExR7 neurons (discussed in later sections) in relatively distinct clusters arranged along the dorsal-ventral axis (**Figure 9Dii,Eii**). The latter spatial segregation is consistent with physiological reports of their distinct but overlapping tuning to wind direction (Okubo et al., 2020). Our analysis suggests that directional information from wind stimuli is conveyed to the EB by ER1_b and ER3a_b neurons. Functional properties of major inputs to the other LAL ring neurons have not been characterized and it is therefore unknown what sensory information is conveyed by these types.

It is important to note that ER1_a, ER1_b and ER3a_b neurons receive the majority of their input from unidentified neurons in the LAL, WED and —particularly in the case of the ER1_b and ER3a_b— also the CRE (**Figure 9B**). Thus, the directional mechanosensory information that these neurons receive may well be combined with other sensorimotor information from their many presynaptic partners (see, for example, presynaptic partners of the ER1_b neurons (**Figure 9—figure supplement 1**).

The EB: architecture for a flexible, multimodal compass representation

The EB or Central Body Lower (CBL), as it has been called in other insects (Muller et al., 1997), is one of the most distinctively shaped brain structures in the fly CX. Behaviorally, the EB has long been considered to play a key role in locomotion and navigation (Bausenwein et al., 1994; Kong et al., 2010; Kottler et al., 2019; Kuntz et al., 2017; Liang et al., 2019; Neuser et al., 2008; Ofstad et al., 2011; Pan et al., 2009; Strauss, 2002; Wang et al., 2008). More recently, functional studies have demonstrated that the population activity of EPG (compass) neurons, each of which arborizes in single EB sectors (wedges), represents the fly's compass direction (Seelig and Jayaraman, 2015). This activity, which localizes to a contiguous 'bump' spanning the processes of EPG neurons in neighboring sectors, is required for flies to stably maintain arbitrary headings as they walk or fly (Giraldo et al., 2018; Green et al., 2019). We will not describe fly compass function in detail here, and instead refer readers to recent reviews on the topic (Green and Maimon, 2018; Hulse and Jayaraman, 2019). Broadly, numerous experimental and computational studies suggest that the compass-like dynamics of the EPG population, which resemble those of a theorized network structure called a ring attractor (Kim et al., 2017b; Turner-Evans et al., 2020), are generated by the interaction of several neuron types (Cope et al., 2017; Fisher et al., 2019; Green et al., 2017; Kakaria and de Bivort, 2017; Kim et al., 2019; Turner-Evans et al., 2017; Turner-Evans et al., 2020). The neural compass relies on input from

the anterior visual pathway to remain tethered to the fly's visual surroundings (Fisher et al., 2019; Hardcastle et al., 2020; Kim et al., 2019; Turner-Evans et al., 2020) and from mechanosensory ring neurons to tether to the direction of wind (Okubo et al., 2020). This allocentric representation of the fly's head direction is also updated by self-motion cues (Green et al., 2017; Turner-Evans et al., 2017) and persists even when the fly is standing still in darkness (Seelig and Jayaraman, 2015; Turner-Evans et al., 2020). Not surprisingly, the EB receives input from a large number of canonical ring neuron types. It also receives inputs from numerous columnar neurons that together bidirectionally link the EB to the PB, NO, Gall and LAL, and to the ExR neurons, which connect the EB to a large number of brain structures outside the CX (**Figure 10A**).

The anatomical organization of the EB

All the ring neurons send their processes around the entire circumference of the EB, but different types occupy rings at specific depths within the anterior-posterior axis (Hanesch et al., 1989; Lin et al., 2013; Young and Armstrong, 2010b), and make synapses within type-specific annuli along the radial axis. **Figure 10B**, for example, shows the locations of ER4m synapses (see **Figure 10—figure supplements 1-3** for synapse locations of other ring, columnar and ExR neuron types respectively). Different ring neuron types vary in the spatial extent of their arbors, which overlap to differing degrees with the arbors of other ring neuron types. **Figure 10Ci** displays this overlap through the location of synapses in the anterior-radial plane; see **Figure 10—figure supplement 4A** for anterior-radial cross-sections along different parts of the EB). However, when clustered by the subregions of structures outside the EB where they receive their inputs, synapses of different ring neuron classes appear to tile the anterior-radial cross-section of the EB (sample cross-section shown in **Figure 10Cii**). Most columnar neurons, which are so-named because they each only innervate single glomeruli in the PB and single sectors ("tiles" and "wedges" (Wolff et al., 2015)) of the EB torus, spread their synapses across fairly large fractions of the anterior-radial cross-section of the structure (**Figure 10D**, **Figure 10—figure supplement 4B**). In particular, the population of EPG and EL neurons, which were called EB.w.AMP.s-Dga-s.b in (Wolff et al., 2015) and have never been characterized physiologically, make synapses across most of the EB (see EPG synapse locations in **Figure 10—figure supplement 1**). The ExR neuron types are similar to ring neurons in arborizing across the entire circumference of the EB. However, in contrast to most ring neurons, they make synapses across fairly large fractions of the anterior-radial cross-section of the EB (**Figure 10E**, **Figure 10—figure supplement 3C**, **Figure 10—figure supplement 4C**). The connectivity patterns between these different neuron types in the EB may play an important role in generating neural dynamics that maintain the fly's head direction representation and control sleep (**Figure 10F**).

Ring neurons bring type-specific information into the EB

Most ring neurons are thought to be GABA-ergic (Hanesch et al., 1989; Homberg et al., 1999; Isaacman-Beck et al., 2019; Turner-Evans et al., 2020) and therefore inhibitory. They bring diverse sensory information into the EB (see **Table 3**, neuron renderings in **Figure 10—figure supplement 5**). Visually tuned ring neurons with ipsilateral receptive fields (ER2a-d, ER4d) and ring neurons tuned to polarized light (ER4m) are well characterized and the fly's head direction system is known to tether to such cues (Fisher et al., 2019; Hardcastle et al., 2020; Kim et al.,

2019). ER1_b and ER3a_b are tuned to wind direction, but only ER1_b appears to directly influence the head direction representation (Okubo et al., 2020). Less is known about the tuning and function of other ER3 neuron types. Pan-neuronal imaging has suggested that a subset of ER3 neurons in the aBUi responds to contralateral visual information and motor actions (likely some of the ER3a types), but many ER3 neurons have been reported to be unresponsive to visual stimuli (Omoto et al., 2017; Shiozaki and Kazama, 2017). Based on their connectivity with ExR3 and ExR1, we hypothesize that ER3d ring neurons are involved in sleep maintenance (see Sleep section for details). One other ring neuron type, ER5, has been explicitly linked to the control of sleep homeostasis (discussed further in a later section). Finally, the unusual ring neuron type —the ER6—receives most of its non-EB input in the Gall (discussed in more detail in a later section), where it contacts EPG and PEG neurons.

Generating an allothetic head direction representation from diverse sensory cues

Sensory information from a variety of ring neurons provides external references for the fly's allothetic head direction representation. Using the complete EB connectivity information of the hemibrain connectome, we found that most —but not all— ring neuron types from both hemispheres make direct synaptic contacts with EPG neurons from both sides of the brain (**Figure 11A**, third and fourth column of the connectivity matrix) (see also (Turner-Evans et al., 2020)). The number of synapses that various visual and mechanosensory ring neurons make onto EPG neurons provide clues about the influence that these different sensory cues exert on the EGP compass. The observation that ER3a_b neurons, which respond to wind direction, do not directly impact EPG dynamics (Okubo et al., 2020) is well accounted for by their lack of direct synaptic connections to EPG neurons (**Figure 11A**). The other ER3a neurons also make few connections to EPG neurons. Note that although the unusual ER6 neuron type does not synaptically contact EPG neurons in the EB (top two row blocks in **Figure 11A**), these neurons —and most of the ER3a as well as ER5 neurons— do make strong contacts with the EL columnar neuron types (first and second column in **Figure 11A**,**B**). The functional significance of EL neurons and of these connections is at present unknown.

The diversity of sensory cues that the head direction representation tethers to indicates that the fly compass is flexible enough to function in a variety of multisensory settings. This flexibility of tethering the EPG compass to different cues is thought to be achieved by experience-dependent plasticity acting on the synaptic weights between ring neurons and EPG neurons (Fisher et al., 2019; Kim et al., 2019). These studies suggested that the strength of a visual ring neuron's synapses onto EPG neurons would, through sculpted inhibition, localize EPG activity to a specific sector of the EB for a given head direction in a specific visual setting (schematized in **Figure 11C**). Considering the strong correlation between synapse counts and the area of synaptic contact between neuron pairs in at least larval *Drosophila* (Barnes et al., 2020), and assuming that the functional strength of synapses depends on synaptic surface area (Holler-Rickauer et al., 2019), we asked whether we could detect any signatures of this sculpting in synapses between ring and EPG neurons. Specifically, we examined the connectivity matrix between a variety of ring neurons —ER1_a, ER1_b, ER2_a-d, ER4d, and ER4m— and EPG neurons for any consistent non-uniformities. We conjectured that if synapse counts or relative weights reflect functional synaptic strengths, we might expect to see specific patterns in the

connectivity between ring neurons and EPG neurons. Specifically, we might expect that the ring neuron synaptic profiles onto multiple EPG neurons within a wedge (that is, EPG neurons that share the same head direction tuning) would be more similar than their synaptic profiles onto EPG neurons with very different tuning (that is, those from angularly distant parts of the EB) (**see Figure 11C**). We found no such correlations or modularity for most ring neuron types (**Figure 11—figure supplement 1**) (**Table 4**). There were two exceptions: the polarization-tuned ER4m neurons (**Figure 11A,D**, **Figure 11—figure supplement 1Cviii**), and the ER1_a neurons (**Figure 11—figure supplement 1Ci**). Thus, if synaptic weights from sensory ring neurons onto EPG neurons vary in consistent patterns as a result of a fly's experience, as we expect they must, this is not reflected in synapse counts for the vast majority of ring neuron types (note, however, that we know little of the sensory experience of this fly). The absence of obvious structural signatures of synaptic strength is perhaps unsurprising in the context of plasticity that acts at a timescale of tens of seconds to a few minutes, as is the case here (Fisher et al., 2019; Kim et al., 2019). In the case of the polarization tuned ER4m neurons, we observed that synapse counts to EPG neurons varied smoothly along the circumference of the EB, but with mirror symmetric profiles for ER4m neurons from the left and right hemisphere, respectively (**Figure 11D**), resulting in stronger connections from the left hemisphere for EB wedges on the right half of the EB and stronger connections from the right hemisphere to the left half of the EB. Given the mirror symmetry of the represented axis of polarization (Hardcastle et al., 2020), this connectivity pattern may allow the fly to generate a complete, 360-degree head direction representation from twofold symmetric polarized light input, an idea that we return to in Discussion. Although a recent study reported wind-direction tuned responses in ER1 neurons (Okubo et al., 2020), we believe that the neurons characterized in that study to be only ER1_b neurons —whether the ER1_a neuron type is also mechanosensory is as yet unknown.

<u>Distributions of ring neuron synapse locations on EPG arbors differ by type</u>

The same plasticity in the EB that enables the fly's internal compass to tether to visual cues in the animal's surroundings (Fisher et al., 2019; Kim et al., 2019) likely also enables the flexible tethering of the head direction representation to other sensory cues. What if the different sensory streams do not provide a mutually consistent estimate of head direction? We next asked if clues about how such conflicts are mediated may lie in the connectivity of different ring neuron types to EPG neurons. Specifically, keeping in mind that the electrotonic distances of synapses to the spike initiation zone of a neuron is often correlated with the influence that the input can exert on the neuron's ability to fire (or not fire) a spike, we asked if we could extract an implicit hierarchy of head-direction-tethering cues conveyed by ring neurons based on the location of their synapses on their downstream EPG neuron targets.

Although little is known about spike initiation zones in fly neurons, the best estimates thus far have come from studies in olfactory projection neurons (Gouwens and Wilson, 2009). In these multipolar neurons, spikes are thought to be initiated near the base of their major input arbors in the antennal lobe. These results are also consistent with the subcellular localization of voltage-gated sodium channels in a broader class of central brain neurons (Ravenscroft et al., 2020). We thus made the assumption that spikes in the EPG neurons are initiated at the root of their arbors in the EB, near the intersection of these processes with processes that travel to the

Gall and PB (**Figure 12A**). We found that these putative spike initiation points (shown in yellow in **Figure 12A, C** (left)) tend to cluster at a certain depth and radial position of the EB, indicating that EPG neurons tend to enter the EB near a center annulus and from the posterior side. We expected that there might be some variation in synapse locations because different ring neuron types arborize at different depths and radial positions in the EB (**Figure 10—figure supplement 1C**). By grouping the ring neuron types by their sensory modality (**Figure 12B**), we see that within modality groups the locations of synapses from the ring neurons to the EPG neurons tend to occur in clusters (**Figure 12C** (right), see also **Figure 12—figure supplement 1A**). Further, an analysis of the synapses of the ring neurons in these different sensory modality groups onto the EPG neurons revealed a clear trend in their relative electrotonic distances to the root node (**Figure 12D,E**) (see Materials and Methods). Broadly, as seen in the cumulative distribution function (CDF) of an example EPG (**Figure 12D**), the electrotonic distance between different ring neuron types and the root of each of their postsynaptic EPG neurons suggests an implicit hierarchy, with the mechanosensory neuron synapses being the closest, the different visual neurons being next, and the sleep-related ring neurons potentially exerting the least impact on the EPG neuron's ability to fire a spike. When we included PEN synapses in this analysis (labeled "motor" in **Figure 12D-E**, **Figure 12—figure supplement 1A**), these self-motion inputs (discussed in later sections) were electrotonically closer to the root of the EPG arbors in the EB than any of the ring neuron inputs (compare left plot with purple region in right plot in **Figure 12D**). While there is some variability, these broad trends are consistent across types within the different modality groups (**Figure 12—figure supplement 2**). Importantly, this rank ordering is fairly consistent across EPG neurons, as can be seen by comparing the medians of the distributions of the synapse locations of ring neurons from each modality group (**Figure 12D**). If we rank order the modality groups by these medians, we see this ordering is indeed preserved with minimal variations across the entire population of EPG neurons (**Figure 12—figure supplement 1B,C**) (see Materials and Methods). A similar picture emerged for the EL neuron type, the second columnar neuron type that receives inputs from many ring neurons (**Figure 12—figure supplement 3**; the higher variability across individual ELs is likely due to a greater variation in arbor shape along the circumference of the EB relative to the EPG neurons (**Figure 10—figure supplements 4B**)).

In summary, our analysis of the electrotonic distances of synapses from different ring neuron types to EPG neurons in the EB suggests an implicit hierarchy of sensory inputs to EPG neurons. Sculpted inhibition from different sensory pathways appears largely focused on the intermediate arbors of the EPG neurons, and excitatory self-motion inputs that update the head direction representation during turns are closer to the root of the EPG neuron arbors in the EB.

<u>A hierarchy of ring neuron types defined by all-to-all inhibition</u>

Past studies have classified ring neurons based on their morphology and their developmental origins (Hanesch et al., 1989; Omoto et al., 2017; Omoto et al., 2018; Renn et al., 1999; Young and Armstrong, 2010a). The connectome permitted us to reclassify ring neurons based on their pre- and postsynaptic connectivity patterns in different brain regions (Scheffer et al., 2020). A key distinguishing feature to classify ring neurons into different types was their dense within-

type connectivity. A recent study combined optogenetics with electrophysiology to show that ring neurons inhibit other ring neurons of the same type (Isaacman-Beck et al., 2019). Although these data were collected in a single ring neuron type, the connectome suggests that within-type, all-to-all inhibition is likely a feature of nearly all ring neuron types (blocks around the diagonal in **Figure 13A**). As we outline in Discussion, such all-to-all inhibition may help to minimize the influence of noise on the compass system and effectively enhance the impact of the most prominent sensory cues on the EPG bump, that is, on its angular position in the EB.

Notably, some ring neuron types are connected all-to-all, not just to other neurons of their type, but to neurons of other types. Examples of types that are fully connected include the ER4m, ER2a-c, and ER4d_a types (off-diagonal blocks in **Figure 13A**). A different fully connected set includes the ER3p_a and ER3p_b neurons. All these ring neurons directly synapse onto the EPG neurons in the EB (**Figure 11A**, **Figure 13B**). There are also some ring neuron types, for example, the ER3a_a, ER3a_b and ER3a_c neurons, which do not synapse onto the EPG neurons, and only interact with them indirectly —in the case of the ER3a neurons, through their impact on ER3m neurons (**Figure 13A**) and EL neurons (**Figure 13B**).

To assess the potential impact of across-type connections between ring neurons, we generated a graph for connections between ring neurons, which showed that ring neuron types with similar tuning formed highly interconnected clusters (**Figure 13C**). We ranked individual types by the numbers of synapses that they make onto EPG neurons —presumably contributing to the generation and maintenance of the head direction signal (Fisher et al., 2019; Kim et al., 2019) (**Figure 13C**, see Materials and Methods). This revealed another implicit hierarchy between different types of ring neurons (**Figure 11A**, **Figure 13B**). Prominent among these ring neuron types are those that —based on their TuBu inputs and recent neurophysiological evidence (Hardcastle et al., 2020)— we believe to be associated with the sky compass pathway: ER4m, ER2_a-d, and possibly ER3w, which are all part of one of the highly interconnected clusters. Within this cluster, connections between inhibitory ring neurons define a suppression hierarchy. For example, the ER4m neurons, which likely provide polarized light e-vector orientation information (Hardcastle et al., 2020), make a significant number of synapses onto all other neuron types that are part of the sky compass pathway (**Figure 13A,C**), and do not receive nearly as many in return (as indicated by the size of the dot representing the neuron type), placing this neuron type at the top of the hierarchy within this cluster. The ER2_c type appears next, inhibiting ER2_a and ER2_d neuron types. ER2_c neurons also make a small number of synapses onto ER4d neurons (**Figure 13C**), which likely convey information about a broader range of visual features (Seelig and Jayaraman, 2013), but these neurons are not inhibited by any of the other ring neuron types and make a large number of synapses onto EPG neurons themselves. Other high-ranking ring neuron types include the ER3p_a, which is one of the primary target types of the EL neurons in the EB (**Figure 13B, Figure 13—figure supplement 1B**), and the ER1_a neurons (**Figure 12B**), which are mechanosensory and bring information about wind direction to the EB (Okubo et al., 2020). We refer readers to **Figure 13C** for the entire implicit hierarchy of all ring neuron types in terms of the relative strength of their inputs to EPG neurons, and, thus, of their relative contributions to tethering the EPG compass.

We note that a few ring neuron types appear to be privileged in also receiving feedback from the EPG neurons (**Figure 13—figure supplement 1B**). This privileged set of ring neuron types includes, once again, the ER4m neurons, which carry polarized light e-vector information. This feedback may serve to amplify their impact on tethering the head direction representation to sensory input, while potentially reducing the control of ring neurons that carry other types of sensory information.

Ring neuron connectivity with other neuron types

A few ring neuron types synapse onto many other neuron types in the EB. The ER6 type, already noted above as contacting EL rather than EPG neurons in the EB (**Figure 11A**), also make a large number of synapses onto PEG and PEN_b (formerly called PEN2) neuron types around the entire circumference of the EB (**Figure 13—figure supplement 1A**). In addition, ER1_a and ER1_b neurons, which are known to provide localized mechanosensory information (Okubo et al., 2020), contact PEN_a (formerly PEN1) neurons from both sides of the brain although those connections are rather weak (**Figure 13—figure supplement 1A**). Finally, it is important to note that some ring neuron types receive presynaptic input in the EB from columnar neurons (**Figure 13—figure supplement 2B**). The ER4m neurons receive input from EPG and PEN_b neurons, with the latter also providing some input to ER2 neurons. The ER1_a and ER1_b neurons receive input from the PEN_a neurons, which —as we noted— are their only non-EPG columnar targets in the EB. The ER6 neurons receive a large number of synapses from the columnar PEG neurons, which, in turn, receive synapses from ER6 neurons. And, finally, the EL neurons, which themselves receive input from several ER neuron types, in turn make synaptic contacts onto most ring neuron types, most strongly to ER3p neurons. We do not yet know the function of these columnar-to-ER connections.

'Head direction' versus 'heading direction'

In contrast with mammals (Hulse and Jayaraman, 2019; Rubin et al., 2014; Taube et al., 1990a, b), the function of the fly compass has not yet been monitored in head-free animals. This motivated us and others to refer to the representation as being of 'heading direction' rather than 'head direction' until the issue was conclusively resolved with direct evidence. However, considering that the anterior visual pathway tethers the compass representation to visual cues in the fly's surroundings (Fisher et al., 2019; Kim et al., 2019; Turner-Evans et al., 2020), that mechanosensory cues sensed by the Johnston's organ on the fly's antennae are used as a reference by the compass (Okubo et al., 2020) and may have the potential to suppress self-motion input from the PEN neurons in the EB, that high-contrast visual cues control the movement of the EPG bump more than self-motion cues from the fly rotating its body (Seelig and Jayaraman, 2015), that diverse sensory cues are all communicated to the EB in head-centered coordinates, and that neurons innervating the FB may be anatomically configured to transform head direction into body direction (see Discussion), we will now employ the term, 'head direction' to refer to the EPG population representation. Note that this is consistent with the terminology that has long been used in studies of the CX in many other insects (Homberg, 2004; Varga and Ritzmann, 2016), but awaits experimental confirmation in those animals as well.

ExR neurons connect the EB with numerous other brain regions.

In addition to the ring neuron types, which – with the notable exception of ER6 – all provide input from non-CX regions to the EB, there is another class of neurons termed extrinsic ring neurons or ExR that form ring-shaped arbors along the circumference of the EB (Hanesch et al., 1989). In contrast to most of the ring neurons, ExR neurons are comprised of only one or two neurons per hemisphere and arborize in multiple regions outside of the central complex (**Figure 14A**). Only a subset of the ExR neurons has been described before and, with few exceptions, little is known about their function. We identified 8 types of ExR neurons, each type targeting distinct sets of brain regions (**Figure 14A**, **Figure 14—figure supplement 1**). Correspondingly, the inputs and outputs of different ExR types are largely distinct (**Figure 14B**, **Figure 14—figure supplement 2A**). While all ring neurons serve as inputs to the CX, only a subset of the ExR neurons appear to be inputs while most types have mixed polarity, and two types are CX output neurons (**Figure 14A**, bottom). The CX input neurons are ExR1 and ExR4 bring, which information primarily from the BU, LAL and Gall (**Figure 14A**, **Figure 14—figure supplement 2B**). For a more detailed descriptions of ExR connectivity in the BU, see **Figure 7B**. The CX output neurons, ExR7 and ExR8, will be discussed in more detail in the output section.

In the EB, ExR neurons make connections with a number of different neuron types (**Figure 10F**). Most ExR neurons arborize in the dorsal part of the EB, with the exception of ExR1 and ExR3, which target more anterior shells of the EB and form a connectivity cluster with sleep-related ring neurons (**Figure 10E,F**). Indeed, ExR1 neurons, also called Helicon cells, have been linked to the control of sleep homeostasis (Donlea et al., 2018). We will cover ExR1 and ExR3 neurons in more detail in the sleep section. All ExR neurons make connections to columnar EL and EPG neurons, suggesting a role in modulating the function of the fly's head direction representation **Figure 14Ci**, **Figure 14—figure supplement 3A**). The connectivity with PEG and PEN neurons is sparser, with ExR4 providing strong input to PEG and both PEN types, and ExR6 providing input to PEN_a (**Figure 14Ci**, **Figure 14—figure supplement 3A**). Columnar neurons also feedback onto ExR neurons (**Figure 14Cii**, **Figure 14—figure supplement 3B**).

One function for ExR neurons might be to connect the EB circuitry with various other brain regions. However, it appears that ExR neurons do not merely establish EB input and output pathways, as many ExR neurons make connections to the same partner types in multiple brain regions. We set out to quantify this observation by measuring the prevalence of connectivity motifs between ExR and other EB neurons, comparing connections within the EB to direct and indirect output pathways from the EB (**Figure 15A**). In our analysis we restricted ourselves to downstream partners of ExRs and focused on three motifs: Parallel connections from ExR to partners in the EB and outside of the EB, canonical feedback connections from partners back onto ExRs, and linked targets (connections between ExR partners). Most ExRs types show high prevalence of parallel connection motifs (**Figure 15B**). Exceptions to this pattern are ExR1 and ExR8, the former being largely an input neuron and the latter making no direct or indirect downstream connections to EB neurons. A closer look at the EB neuron partners of ExRs that contribute to the observed motifs, reveals that motifs are formed through different partner types in different ring neurons (**Figure 15C**), suggesting that different ExR types are involved in modulating different subsets of CX circuitry. ExR3, for example, forms parallel connections and

linked targets motifs exclusively through ring neurons (**Figure 15C,D**), while the same motifs in ExR5 are established through FB tangential neurons (**Figure 15C,E**), and in ExR6 through EB columnar neurons (**Figure 15C,F**). In contrast, canonical feedback motifs are exclusively formed through ExR neurons, and all linked target connections involved at least one ExR neuron (**Figure 15D-G**, some data not shown).

ExR2 neurons (**Figure 15G,H**) are known to be dopaminergic from the PPM3 cluster and have been linked to ethanol-induced hyperactivity and the control of circadian activity peaks (Kong et al., 2010; Liang et al., 2019; Nassel and Elekes, 1992; Omoto et al., 2018). ExR2 neurons receive input in the LAL and EB, (**Figure 14 Cii, Fig 14—figure supplement 3B**), and send outputs primarily to the BU, where they target visually-tuned BUs neurons and the interhemispheric AOTU046 neurons (**Figure 8B**). ExR2 neurons may modulate the function of the fly's head direction system through their outputs in the BU and their parallel connections in the EB (**Figure 15G**), but exactly how they impact the fly's motor activity is currently unknown.

A ring attractor network formed by recurrent loops between the EB and PB

A series of experimental studies have identified network motifs that likely underlie the generation, maintenance and updating of the fly's head direction system (Green et al., 2017; Green and Maimon, 2018; Hulse and Jayaraman, 2019; Turner-Evans et al., 2017; Turner-Evans et al., 2020). These motifs, and the network's dynamics and responses to perturbation, strongly suggest that it implements a type of ring attractor (Kim et al., 2017b; Turner-Evans et al., 2017; Turner-Evans et al., 2020). In this section, we describe the interconnectivity of the network's core components: four classes of PB-EB columnar neurons —neurons that arborize in single glomeruli in the PB and in localized regions of the EB, while also sending processes to a third structure (either the Gall or the NO). Together, these neurons recurrently link the EB and PB.

<u>EB-PB columnar neurons: recurrent partners that maintain and update the compass</u>

As shown in **Figure 16A**, each EPG neuron connects an EB wedge with a single glomerulus in the PB, while also innervating the Gall (discussed in later sections). The PB is mirror symmetric in appearance, with a left half and a right half. The EPG wedges in the EB alternate so that half go to the right PB while half go to the left. However, the wiring of the PB is not mirror-symmetric, as is evident from the pattern of mapping of the EB ring on each side of the PB (**Figure 16B**). The EPG neurons bring the head direction signal to the PB from the EB. A bump of activity in the EPG neurons in the EB will therefore manifest as two bumps in the PB, with one on either side. The remaining three types of columnar neurons linking the EB and PB —the PEN_a (PEN1), PEN_b (PEN2) and PEG neurons— are mainly postsynaptic in the PB and complete direct and indirect recurrent loops with the EPG neurons in the EB (Green et al., 2017; Green and Maimon, 2018; Hulse and Jayaraman, 2019; Turner-Evans et al., 2017; Turner-Evans et al., 2020).

<u>Updating head direction by integrating angular velocity input</u>

We recently used EM reconstruction to identify the network motifs underlying the interaction of the PB-EB columnar neurons (Turner-Evans et al., 2020). However, this study, which was based primarily on FAFB data (Zheng et al., 2018), relied on manual reconstruction of only a fraction of the circuit. The completeness of the hemibrain connectome allowed us to more

thoroughly examine the compass sub-network and exposed additional structure-function relationships within the compass circuit, which we discuss below.

The angular velocity input that moves the EPG activity bump around the EB in either direction when the fly turns is thought to primarily come from the PEN neurons (Green et al., 2017; Turner-Evans et al., 2017; Turner-Evans et al., 2020). We believe that head movements are also likely to update the EPG bump, although this has yet to be established. There are two types of PEN neurons, PEN_a (PEN1) and PEN_b (PEN2), both of which are synaptically connected in recurrent loops with EPG neurons (Turner-Evans et al., 2020), are conjunctively tuned to head direction and angular velocity (Green et al., 2017; Turner-Evans et al., 2017; Turner-Evans et al., 2020), and likely receive angular velocity inputs in the NO (discussed in a later section). These recurrent loops have either a clockwise (CW) or counterclockwise (CCW) offset (schematized in **Figure 17A**). That is, PEN neurons that receive their head direction inputs from a given EPG neuron in the PB send their own projections to an EB tile that is offset CW or CCW from the EB wedge innervated by the EPG neuron that is presynaptic to the PEN in the PB. This offset is in one direction for PEN neurons with arbors in one side of the PB, and in the other direction for PEN neurons that project to the other half of the PB (see sample images of EPG and PEN_a neurons in **Figure 16A**, **Figure 17A**; connectivity matrices in **Figure 17B,C**). Considering that the EPG head direction representation spans 360° of angular space, we can consider these offsets to be "anatomical phase shifts" of the PEN population relative to the EPG population. The functional consequence of this phase shift is that the phase (angle) of the PEN bump is likely to be shifted relative to the phase (angle) of the EPG bump. As we will discuss in later sections, PB-FB neurons also have anatomical phase shifts.

A key assumption behind fly compass models is that recurrent loops involving EPG and PEN neurons allow activity to propagate all the way around the EB and in either direction. Indeed, PEN neurons from every PB glomeruli contact EPG neurons in every wedge of the EB (**Figure 17B**). EPG neurons then complete the loop by synapsing onto PEN neurons in the inner glomeruli in the PB. There is an apparent disconnect in connectivity at the edges of the PB, but this disconnect may be addressed by an additional cell type, as will be discussed in more detail below). Importantly, even without this cell type, EPG and PEN_a neurons are connected so densely to each other within the EB that a force-directed network graph of their subnetwork forms a ring (**Figure 17D**; see Materials and Methods). Note that while the PEN_as and PEN_bs have similar connectivity to the EPG neurons in the EB and PB, they have other distinct partners in those regions (**Figure 17—figure supplement 1**).

The PEN neurons in the outermost (9th) glomeruli do not receive any EPG input from the EPG neurons that project to the 8th glomerulus. Instead, a key neuron type arborizes in that outer glomerulus and appears to fill the 'gap', the EPGt neurons (Wolff et al., 2015) (**Figure 18A**). While the EPGts have very similar connectivity to the EPG neurons in the PB (**Figure 18B**), they receive far fewer synaptic inputs in the EB (**Figure 18C**), with a striking sparsity of ring neuron inputs in particular (EB-PB Figures 3D). However, they do receive PEN input in the EB. The wedges at the bottom of the EB, which are innervated by both the EPGts and the EPG neurons

that project to glomerulus 1, therefore map to both glomerulus 1 and 9 in the PB to potentially complete the loop (**Figure 18E**).

This connectivity pattern can also be observed with the PEG neurons. The PEG neurons form a recurrent loop with the EPG neurons, as reported previously (Turner-Evans et al., 2020). Briefly, they receive input from the EPG neurons in the PB (**Figure 17C**, right columns) and synapse onto the PEN_b neurons in the EB (**Figure 17B**, top rows, 2nd group of columns to the right). The PEN_b neurons synapse onto the EPG neurons in the EB, thereby completing the loop (**Figure 17B**, 2nd group of rows from the top, first group of columns). Although some PEG neurons do synapse directly onto EPG neurons in the EB, these connections are sparse and feature only a few synapses. By contrast, the PEG-to-PEN_b and PEN_b-to-EPG connections are both strong and consistent around the ring.

There are 9 PEG neurons on each side of the PB (one for each glomerulus). Each connects to one of the 8 PEN_b neurons in the EB. PEG neurons from glomerulus 1 and glomerulus 9 both connect to PEN_b neurons from PB glomerulus 2. This connectivity pattern matches the connectivity of EPG neurons from glomerulus 1 and EPGt neurons from glomerulus 9 to PEN_b neurons from glomerulus 2. Thus, although the PB is composed of 9 glomeruli on either side, the compass is composed of 8 discrete computational units, with the combination of EPG and EPGt neuron types as well as the PEG neurons effectively merging the PB's innermost and outermost glomeruli (1 and 9) into one computational unit. The FB also appears to effectively have 8 computational units, as is discussed in a later section.

The PB: reshaping the compass signal for navigational computations

The handlebar-shaped PB is conserved across arthropods (Strausfeld, 1976; Strausfeld, 2012), including species as distant as locusts (Homberg, 1991), flies (Lin et al., 2013; Phillips-Portillo, 2012; Wolff et al., 2015) and crayfish (Sandeman et al., 1990). The structure has been associated with a wide range of locomotory behaviors (Harley and Ritzmann, 2010; Krause et al., 2019; Poeck et al., 2008; Strauss et al., 1992). Neural activity in the structure is known to carry the head direction signal (Bockhorst and Homberg, 2015; Giraldo et al., 2018; Green et al., 2017; Heinze and Homberg, 2007; Pegel et al., 2018; Turner-Evans et al., 2017; Turner-Evans et al., 2020; Zittrell et al., 2020)—indeed, the PB is where the first sensory-driven compass-like activity was reported in the insect brain (Heinze and Homberg, 2007).

Above we discussed the EB-PB recurrent network, which supports the function of the compass through the interactions of EPG, EPGt, PEG, PEN_a and PEN_b neurons. EPG neurons bring columnar input from the EB into the PB, and the other neuron types take information from the PB (and accessory regions) into the EB (**Figure 19A**). The PB also connects to other brain regions. Consistent with descriptions based on light microscopy (Lin et al., 2013; Wolff et al., 2015), the hemibrain connectome shows that the PB receives much of its non-CX input from three accessory structures, the IB, SPS, and PS (**Figure 19Ai**). The PB also hosts the interactions of a variety of columnar neurons that project to the FB. Although most PB neurons are columnar and arborize in only single PB glomeruli (**Figure 19B**), the region also contains several multi-glomerular neuron types (**Figure 19C**), including two types of interneurons). The PB-FB

neurons serve as both inputs and outputs to the PB (depending on the type) and further connect to a variety of accessory regions (**Figure 19D**). Notably, the PFL neurons send outputs to the LAL, which is innervated by descending neurons (**Figure 19Aii,D**). The completeness of the hemibrain connectome in the CX allowed us to split a few of the previously identified PB-FB columnar neuron types into multiple new, distinct types, including some subtypes that were distinguishable less by their morphology than by their connectivity patterns.

One of the PB interneuron types, the Δ7 neurons, innervates all PB glomeruli as do the dopaminergic LPsP neurons and the octopaminergic P1-9 neurons (**Figure 19E**, see rows labeled 'multi' at right) (Wolff et al., 2015). The other multi-glomerular neuron types innervate at least four glomeruli each. The columnar neuron types, on the other hand, vary in their coverage of PB glomeruli, with populations of most types innervating contiguous subsets of glomeruli and excluding either the innermost or outermost glomeruli (**Figure 19E**, see rows labeled 'single glomerular arbors' at right), thereby sampling all eight computational units across the population.

<u>The Δ7 neurons: cosine-shaped reformatting of the head-direction signal</u>

The direct connection from EPG neurons to PEN neurons in the PB form a key part of the EB-PB recurrent loop, which updates the fly's head direction representation with self-motion input. However, this EPG input is also transformed by a multi-glomerular interneuron that provides an indirect but strong link from EPG neurons to not just the PEN neurons, but to all other columnar neurons: the Δ7 neurons (**Figure 20A**).

The PB-spanning Δ7 neurons and their homologs in other insects have long been believed to play a role in navigational computations. Their responses to polarized light e-vector stimuli suggested that they were organized in map-like, polarotopic fashion in the locust brain (Heinze and Homberg, 2007). More recently, imaging and perturbation experiments in the fly have confirmed the importance of these glutamatergic neurons to the function of the fly compass circuit (Turner-Evans et al., 2020). Although this recent work also used EM to identify synaptic connections between Δ7 neurons and EPG, PEG, PEN_a and PEN_b neurons, it reconstructed only a subset of the processes in a few Δ7 neurons. The hemibrain connectome allowed us to identify the complete set of inputs and outputs of Δ7 neurons across the entire PB. The picture that emerges from this analysis is of a neuron type that is the central hub of the PB: all neurons downstream of EPG neurons receive head direction input that is also processed by the Δ7 neurons (**Figure 20B**, **Figure 20—figure supplement 1,2**).

A key question raised by the hub-like connectivity of the Δ7 neurons is how these neurons might transform the EPG head direction signal. The clearest clues into this transformation come from the connectivity patterns of EPG neurons onto Δ7 neurons across different PB glomeruli (**Figure 20C**). The EPG to Δ7 synaptic profile is well fit by a cosine (**Figure 20D**, see Methods). This cosine-tuned connectivity profile, when combined with the projection patterns of individual Δ7 neurons that each send their outputs to specific PB glomeruli, predicts that any input from the EPG population will itself be reformatted into a cosine. Cosine-shaped activity

profiles are ubiquitous in motor systems where they are considered to be optimal to minimize errors in force production (Todorov, 2002). In combination with sine-shaped activity profiles, they are also particularly useful for vector-based computations that may underlie navigational algorithms, in which context the phase and amplitude of a sine wave can encode the angle and length of a vector (Pisokas et al., 2020; Stone et al., 2017; Wittmann and Schwegler, 1995).

We used simulations to test the prediction that EPG population activity is likely to be shaped into a cosine shape by the Δ7 neurons. We began by assuming that the EPG neurons have an activity bump, similar to experimental observations (Seelig and Jayaraman, 2015). Assuming that the EPG to Δ7 and Δ7 to EPG synapse weights are approximately proportional to their synaptic counts, we then propagated this activity across the neuron types and found that the output activity is even better fit by a cosine (**Figure 20E**). We further found that the results we obtained were relatively independent of the shape of the input activity. If we assumed an impulse (delta) function input from the EPG neurons to one PB glomerulus, for example, the resulting signal was still shaped like a cosine (**Figure 20F**). We note, however, that these calculations ignore any nonlinearities, as well as the large number of synapses between individual Δ7 neurons. Considering that the Δ7 neurons synapse onto nearly all PB neurons in only their specific output glomeruli (**Figure 20C**, **Figure 20—figure supplement 1,2**), we would expect this transformation of the bump to apply to those neurons as well. By similarly convolving the EPG-Δ7 synaptic profile with the output profile from the Δ7 neurons onto each of the other neuron types, we found that the cosine reshaping applied to nearly all columnar neurons (**Figure 20G**).

Note that just as the EPGt neuron type expands the influence of the head direction signal to the outermost PB glomerulus (discussed in an earlier section), the P6-8P9 neuron type may serve the function of extending the cosine-shaping of the signal to the entire PB (**Figure 21A**). The P6-8P9 neurons not only have a similar morphology to the Δ7 neurons, receiving input across multiple PB glomeruli while outputting in only one glomerulus (**Figure 21B**), they also have similar partners (**Figure 21C**), suggesting that they perform a similar, albeit far more localized, function.

<u>Inputs to the PB from within and outside the CX</u>

Although the Δ7 neurons shape the activity profile of columnar PB neurons, the amplitude of this activity is likely influenced by inputs to the PB (**Figure 22A**). External (non-CX) input comes from the Inferior Bridge (Ib) and Superior Posterior Slope (Sps) through the IbSpsP neurons and SpsP neurons (Wolff et al., 2015). The IbSpsP neurons are columnar and target a broad range of other columnar neurons including the PEN and PFN supertypes (**Figure 22B**). The pair of SpsP neurons, which are easily recognized by arbors that span exactly one half of the PB each, are very selective and target only the PFNd neurons (**Figure 22B**). The posterior slope is known to be innervated by descending neurons (Schnell et al., 2017) and can thus provisionally be considered a premotor area, but less is known about the Ib. We speculate in a later section about what this may suggest about the potential navigational role of these PB input neurons. The IbSpsPs inputs are varied and largely unknown (**Figure 22C**, **Figure 22—figure supplement 1**). The SpsP neurons are incomplete in the hemibrain, and thus their inputs in the Sps are at

present unknown. The PB also receives columnar, glomerulus-specific input from the PFNv neurons, which have never been characterized physiologically (**Figure 22B**). These neurons receive relatively uniform input in the NO and FB (**Figure 22D**).

Sources of neuromodulation in the PB

The PB receives neuromodulatory input from both dopaminergic neurons (LPsP, **Figure 19B**) and octopaminergic neurons (P1-9, **Figure 23A**). The dopaminergic LPsP neurons (**Figure 19C**) receive PB input from the Δ7 neurons as well as the EPG neurons and make synaptic contacts with a wide range of neurons, including the octopaminergic P1-9s neurons (Busch et al., 2009; Wolff and Rubin, 2018) (**Figure 23A**), from which they also receive synaptic input (**Figure 23B**). Unfortunately, the processes of both the P1-9 and the LPsP neurons are cut off outside of the PB, where they putatively receive most of their input (Wolff et al., 2015). The function of these two types is unknown, though they may control plasticity in the PB. Plasticity in the structure has been implicated in the fly's ability to estimate the size of gaps that it can safely cross based on its own body size (Krause et al., 2019).

Nonuniformity in PB glomerulus representation across PB columnar neurons

A representational system for a circular variable, like head direction, might be expected to have the same number of elements to represent each angle. Thus, we might expect each columnar neuron type to have the same number of neurons in each PB glomerulus. In addition to the columnar PB-EB neurons, this would also include the many types of PB-FB columnar neurons that are fed by the EPG and Δ7 neurons (**Figure 20B**). Indeed, the PEG, PFG, and PFL1 neurons have one neuron in each glomerulus in their PB domain (**Figure 24A,**B, blue boxes). However, these uniform distributions are the exception rather than the rule. Notably, any deviations from uniformity tend to be mirror symmetric across the right and left PB. The EPG neurons, for example, have three neurons in glomeruli R/L 3-7, four neurons in glomeruli R/L 8, and two neurons in glomeruli R/L 1 and 2 (**Figure 24A**, purple boxes). In contrast, the PEN neurons and PFN neurons, while also mirror symmetric, each have roughly double the number of neurons in glomeruli 4 and 6 as they have in the other glomeruli, for example, PEN_as have two neurons in R4, R6, L4, and L6, but one in each of the other glomeruli where they arborize (**Figure 24A,C**, magenta boxes). Finally, the PFL and PFR neurons each have their own unique distributions of neuron numbers across the PB (**Figure 24B**). We outline some potential origins and roles for such numeric differences in Discussion.

The NO: input for navigational computations in the EB and FB

The bilaterally symmetric NO has sometimes been regarded as a potential output structure of the CX (Currier et al., 2020), but the connectome establishes this paired structure as one in which numerous types of CX neurons —particularly PB columnar neurons— in fact receive inputs (**Figure 25A-C**). Structurally, both the left and right NO are divided into three sub-compartments: NO1, NO2 and NO3 (inset in **Figure 25B**). This anatomical structure is respected by most neuron types, as is reflected in the connectivity, which shows clusters of neurons corresponding to the NO region that they innervate (**Figure 25B**).

The NO receives inputs from LNO neuron types (see **Figure 27 B** for morphological renderings) that innervate the LAL, Gall and CRE (**Figure 25B,C**). These neurons provide input to and receive feedback from columnar CX neurons (**Figure 25B,C**). The only columnar neurons that lend some credence to the notion of the NO being an output structure of the CX are the PEN_bs (PEN2s), which provide strong inputs to the ExR8 neurons (discussed further in the CX output section) (**Figure 25B**). Finally, some FB tangential neurons, which each arborize in FB layers that span most columns of the FB, also make reciprocal connections with LNOs and columnar neurons in the NO (**Figure 25B**, **Figure 26—figure supplement 1B**) —their function is presently unknown. Thus, with the exception of the PEN_b (PEN2) neurons, the vast majority of NO outputs of CX columnar neurons are to other columnar neurons (usually of the same type), or to LNO neurons that then provide feedback to the columnar neurons.

Besides their interconnections in the NO, columnar neurons receive input in individual glomeruli of the PB and arborize in either FB columns or EB wedges, where they make the majority of their outputs and, to a lesser degree, receive further inputs (**Figure 26A,B**; **Figure 26—figure supplement 1A**). Broadly, the PEN and PFN columnar neurons are known to be sensitive to the fly's heading and to self-motion signals, likely enabling these columnar neurons to update navigational representations in the EB and FB (Currier et al., 2020; Green et al., 2017; Shiozaki et al., 2020; Turner-Evans et al., 2017; Turner-Evans et al., 2020). These neurons likely receive their heading input in the PB (see previous section and **Figure 26B**). While all columnar neurons receive similar inputs in the PB, their inputs in the NO differ significantly (**Figure 26C**). Much like the other CX structures, the NO is characterized by dense recurrence. All columnar neurons are recurrently connected to the LNOs that they receive input from (**Figure 25B**, **Figure 26B**). Additionally, most of the columnar neurons —with the notable exception of the PFNp neurons (whose function is unknown)— are also strongly recurrently connected to other neurons of their type (**Figure 25B**, **Figure 26D**).

The primary input from outside the CX to the NO is from different sub-types of LNO neurons, which, in turn, receive most of their inputs in the LAL, CRE and other accessory structures (**Figure 27A,B**). One potential organizing principle for the LNO-columnar connections is suggested by the inputs that the different types of LNOs receive: Clustering the LNOs by their inputs neatly divides them into classes that synapse onto specific groupings of columnar neurons (for example, the GLNOs that provide input to PEN neurons; LNOa_Rs that contact the PFNa_Ls, **Figure 27C**, **Figure 27—figure supplement 1A**). Note, that although a single LNO type may provide input to multiple classes of columnar neurons (**Figure 27C**), an LNO that is connected to a PE columnar neuron never contacts a PF columnar neuron and vice versa. Recordings from one LNO neuron in the sweat bee suggest that such neurons may bring self-motion information into the NO and to the columnar neurons (Stone et al., 2017). Combined with the fact that most columnar neurons receive relatively similar inputs in the PB (next section), this LNO-based segregation suggests that the self-motion information that each grouping of columnar neurons receives may largely define their response properties and may be used for different navigational computations (as discussed in the FB section and in Discussion).

Overall, the picture of the NO that emerges from these collected physiological and anatomical observations is of an important hub for self-motion information that is employed for navigational computations in the EB and FB.

The FB: a structured recurrent network for context-dependent navigation and sleep

The FB, referred to as the Central Body Upper (CBU) in other insects (Pfeiffer and Homberg, 2014; Strausfeld, 1976; Strausfeld, 2012), is the largest and most complex structure in the CX. In *Drosophila*, the FB is composed of over 200 distinct neuron types that form a dense and highly recurrent network, described in detail below. In all insects examined to date (Pfeiffer and Homberg, 2014; Strausfeld, 2012; Wolff et al., 2015), the FB is organized into columns along its medial-lateral axis and layers along its dorsal-ventral axis (**Figure 28A**).

Previous experimental work has implicated FB circuits in a variety of behaviors that require directed locomotion, including operant visual learning (Liu et al., 2006), obstacle avoidance (Harley and Ritzmann, 2010), nociceptive avoidance (Hu et al., 2018), and head optomotor responses (Akiba et al., 2020). Further, recent physiological recordings have demonstrated that PF columnar neurons convey the head direction representation from the left and right PB to the FB (Shiozaki et al., 2020), and that, similar to the PEN neurons, PFN activity is likely modulated by self-motion inputs received in the NO (Shiozaki et al., 2020; Stone et al., 2017). Sensorimotor information may also enter the FB through tangential neuron types whose activity is gated by behavioral state (Weir and Dickinson, 2015; Weir et al., 2014). Importantly, the FB is also home to a prominent class of columnar output neurons, known as PFL neurons in *Drosophila* (Wolff et al., 2015) and CPU1 neurons in other insects (Heinze and Homberg, 2007, 2008; Stone et al., 2017). These neurons, whose activity has been linked to directed movement (Skutt-Kakaria et al., 2019), send projections to the LAL, where they contact descending neurons involved in steering (Rayshubskiy et al., 2020). Consistent with this view, some CX neurons have activity that predicts an insect's future forward and rotational velocity, and electrical stimulation of the CX evokes stereotyped locomotor responses (Martin et al., 2015). Together, these studies support the view that FB circuits implement head-direction-based navigational behaviors such as straight-line orientation, long-range migration, and visual route following (reviewed in (Honkanen et al., 2019). It remains largely unknown how the FB network may support these navigational functions, but a recent study proposed a network model inspired by FB physiology and anatomy that could perform vector-based path integration (Stone et al., 2017).

Navigational functions have mostly been associated with ventral FB circuits. In contrast, more dorsal layers have primarily been studied in the context of sleep-wake control (reviewed in (Dubowy and Sehgal, 2017)). Prominent among these are a population of dorsal FB tangential neurons whose intrinsic excitability tracks sleep need and whose activation induces sleep (Donlea et al., 2011; Pimentel et al., 2016). FB tangential neurons also receive contextual input from the mushroom body (Dag et al., 2019; Li et al., 2020; Scaplen et al., 2020), an important center for learning and memory (Modi et al., 2020). Together, the above evidence suggests that context- and state-dependent action selection, including initiating periods of behavioral quiescence, may be governed by the FB.

Compared to other CX regions, much less is known about overall structure and connectivity of the FB network. Considering the sheer complexity of the structure's recurrent circuits, we devote many of the following sections to describing the FB's columnar organization before we delve into intra-FB connectivity patterns. We then describe the plethora of FB tangential neuron types that form the structure's layers and that likely provide contextual and state information to the columnar network. We end with two more focused sections, one on the sleep-wake network of the dorsal FB and the other on pathways from the MB to the FB. In Discussion, we build on this detailed structural description to propose hypotheses for the roles of different neuron types in FB circuit function.

Overview of FB structure, neuron types, and major input/output pathways

The FB is coarsely divided into columns along its medial-lateral axis by four large classes of columnar neurons: PB-FB-*, FX, vΔ, and hΔ (**Figure 28A,B**), where * and X refer to accessory regions of the CX. In addition to these ~60 columnar neuron types, ~150 types of FB tangential neurons divide the FB into 9 layers along the dorsal-ventral axis (**Figure 28A,C**). Each class of columnar neurons contains many distinct neurons types. Individual neurons within each type collectively tile all FB columns and preferentially innervate type-specific FB layers (**Figure 28D**). Based on their input from the EPG neurons and the polarity of their arbors, PB-FB-* neurons likely convey head-direction-related information from the PB to the FB (PFN, PFR, and PFG types; (Shiozaki et al., 2020)), and also from the FB to the LAL (PFL types; (Rayshubskiy et al., 2020)). Two classes of interneurons, vΔ and hΔ, are composed of neuron types whose arbors are largely confined to the FB. They relay information across layers and columns (**Figure 28B,D**). FX neurons are a heterogeneous columnar neuron class whose types primarily arborize in the FB and either the CRE or the SNP. Similar to columnar types, FB tangential neurons heavily innervate the LAL, CRE, and SNP (**Figure 28D**), but often contain additional arbors in type-specific neuropils, such as the NO, EB, or BU, which we detail in later sections.

It should be noted that FB columns are less well-defined than PB glomeruli or EB wedges/tiles, and that FB layers lack clear boundaries, much like the EB's annuli. Despite these challenges, we begin by examining the columnar structure of FB columnar types, most of which form 9 structural columns. This columnar organization allowed us to assign anatomical phase shifts to PB-to-FB projections of the PB-FB-* types (we introduced the term "anatomical phase shifts") in the previous section, Updating head direction by integrating angular velocity input)

The columnar structure of the FB as defined by PB-FB-* neuron types

Based on their FB innervation pattern, most PB-FB-* columnar types appear to divide the FB into nine structural columns (**Figure 29**). PFN neurons that innervate layer 1, such as PFNp_a, project to 1 of 9 distinct tooth-shaped structures (**Figure 29A**). This is consistent with previous light-level anatomy, which described the ventral margin of layer 1 as being composed of seven distinct teeth plus two "cryptic teeth" (Wolff et al., 2015), together accounting for the 9 clusters observed here. The two most lateral columns, C1 and C9, likely correspond to the cryptic teeth and often overlap with their neighbors in C2 or C8, but morphological renderings show a clear spatial shift between adjacent columns. Columnar types with arbors in intermediate FB layers, such as the PFNa, are less well clustered, but their innervation pattern and axon tracts are also consistent with the existence of 9 structural columns (**Figure 29A**). One neuron type, the PFGs,

has 18 neurons in total, which fairly evenly tile the FB, dividing it into 9 columns or approximately 18 "demi-columns", roughly one demi-column per neuron (**Figure 29B**; see legend for exceptions to this pattern). Moreover, individual PB-FB-* neurons have neuronal arbors whose width is slightly less than 1/9th of the layer's width (**Figure 29D**), and the distance between neurons in adjacent columns is 1/9th the layer width on average (**Figure 29E**). Variation in arbor width and spacing determines how much adjacent columns overlap. **Figure 29—figure supplement 1** contains morphological renderings and mean column locations for all PB-FB-* types, with the exceptions of PFL neurons, which we cover in later sections. One exception to this pattern is the PFNd type, which clearly forms 8 structural columns instead of 9 (**FB Figure 29 S1**). This type is also the only PFN type to receive inputs from SpsP neurons in the PB (**Figure 22**), the functional significance of which is unknown.

<u>Columnar phase shifts of PB-FB-* neurons between their PB and FB innervations</u>
The existence of 9 PB glomeruli per hemisphere and 9 FB columns suggests that PB-FB-* neurons connect single PB glomeruli to individual FB columns. To investigate the mappings from PB glomeruli to FB columns, we first focused on neurons innervating glomeruli R5 and L5. These glomeruli receive input from the same region of the EB and, therefore, have the same allocentric head direction tuning, ensuring that when a bump is centered at R5 in the right PB there will be a second bump centered at L5 is the left PB (**Figure 30Ai**; (Green et al., 2017; Turner-Evans et al., 2017)). Next, we compared the columnar position of the R5 and L5 projections in the FB (**Figure 30Ai, Aii**). Two neuron types —PFGs and PFR_a— were found to map R5 and L5 to the same FB column, C5, consistent with the absence of any phase shift. When we extended the PB-FB mapping to other members of these neuron types, we found that this projection pattern was circularly symmetric, leading to a consistent zero-degree phase shift across the populations. Thus, for PFGs and PFR_a neuron types, we would expect activity bumps carried by the left and right PB populations to overlap in the FB (see bottom of **Figure 30Ai**), regardless of bump location (**Figure 30Aiii**).

By contrast with the PFGs and PFR_a , all PFN neuron types have non-zero, contralateral phase shifts between their PB glomeruli and FB columns, much like PEN neurons in the PB-EB network. In the PFNp_a and PFNa types, for example, neurons that innervate R5 project to C6 and neurons that innervate L5 project to C4 (**Figure 30Bi-ii**). That is, PFN neurons connect PB glomeruli to FB columns using a 1-column contralateral phase shift. This phase shift implies that PFN populations from the left and right PB would generate spatially shifted activity bumps in the FB (see bottom of **Figure 30Bi**). Plotting the full PB-FB mapping revealed that these 1-column contralateral phase shifts are largely consistent across all PB glomeruli for these PFN neuron types (**Figure 30Biii**). The PFNd type also appears to use a 1-column contralateral phase shift, but the degree of this shift may vary across columns, likely due to its forming 8 structural columns, as described above. Finally, one distinct neuron type, the PFR_b, has a 1-column ipsilateral phase shift.

The above analysis establishes that PB-FB neurons link the 9 glomeruli in the left and right PB to the 9 columns in the FB, but with type-specific phase shifts. Although we chose to discretize these PB-to-FB mappings above, these projections are actually continuous in anatomical space

(**Figure 30—figure supplement 1**), and even types with the same discrete phase shift differ in the details of their PB-FB projections. For example, many PFN types innervate the lateral 8 glomeruli in the left and right PB, but as a population project to all 9 FB columns. This 8-to-9 mapping is accomplished in several ways. First, some types connect the 8 glomeruli in the left and right PB to 8 FB columns, with the lateral-most FB columns (C1 and C9) receiving input only from either the left or right PB (see orange arrow in **Figure 30Biii** top panel). Other types connect the 8 glomeruli in the left and right PB to all FB columns by having PB glomeruli 'skip' an FB column and/or by having individual glomeruli innervate more than one column (see orange arrows **Figure 30Bii** bottom panel). The potential functional significance of such subtleties is unknown.

As with the PEN neurons, whose phase shifts appear strongly linked to their function of shifting the EPG bump in the EB (Green et al., 2017; Turner-Evans et al., 2017; Turner-Evans et al., 2020), we believe that the PF phase shifts offer clues as to the likely navigational function of each of these neuron types (outlined in Discussion). Next, we describe the columnar structure and connectivity of hΔ and vΔ types, which serve as the main downstream target of PF types.

<u>A constrained 2D grid for recurrent computations: the hΔ and vΔ backbone</u>
Previously referred to as 'pontine neurons' in the insect brain literature (el Jundi et al., 2009; Hanesch et al., 1989), the FB's many interneuron types create an intricately structured scaffold for intercolumnar and interlayer communication within the FB. We renamed these neurons vΔ and hΔ. vΔ refers to the predominantly "vertical" layer-skipping morphology of the constituent neurons (**Figure 31A**). hΔ refers to the predominantly "horizontal" column-skipping morphology of the constituent neurons (**Figure 31B**).

Two of these pontine neuron types are actually not FB interneurons at all, but rather are neurons that bring information into the FB from other CX structures. The vΔA_a neurons (**Figure 32Aii**) have mainly postsynaptic specializations in the asymmetrical body (AB; **Figure 31Ai**), a largely mysterious structure at the base of the FB (Pascual et al., 2004; Wolff and Rubin, 2018) that we discuss later, and send arbors up throughout the dorsal most layers of the FB. The hΔK neurons (**Figure 31Bi**), by contrast, each innervate restricted EB regions, which vary in size and coverage, and columns in intermediate FB layers before projecting to more dorsal layers of the FB. These neurons could carry head-direction information directly from the EB to the FB, bypassing the PB, but their connectivity in the FB suggests that they may also be related to behavioral state, a topic we return to in the Sleep section. With the exception of the vΔA_a, a subset of vΔA_b, and hΔK neurons, all other vΔ and hΔ neuron types are interneurons whose arbors are restricted to the FB.

There are many different types of vΔ neurons, each distinguished by the layer(s) that they innervate, by the vertical distance between their multiple (usually two) arbors, and by the spread of each arbor. These different vΔ types are shown in **Figure 31—figure supplement 1**. Note that although some neuron types, such as the vΔH, clearly respect the 9-column structural organization of the FB, most types vary in how cleanly, as a population, they tile each layer that

they innervate. Interestingly, all vΔ types also contain one or more individual neurons that project to both C1 and C9, which we refer to as 'C0' (see **Figure 31Ai**).

All hΔ neurons have two horizontally separated arbors within the FB, one of which is predominantly presynaptic (**Figure 31B**). Each hΔ type has matching left- and right-projecting neuron pairs whose two arbors largely overlap. As a population, both the right- and left-projecting populations tile all FB columns. The many hΔ types are distinguished by the layer(s) that they innervate and by the horizontal spread of their arbors. These different types are shown in detail in **Figure 31—figure supplement 2**. By contrast with the consistent 9-column tiling of the vΔ neurons, some hΔ types divide the layers they innervate into 12 columns, such as the hΔA and hΔL neurons, others into 8 columns, such as the hΔD and hΔH neurons, and one, the hΔF, into just 6 columns (**Figure 31—figure supplement 2**). In most cases, the population of neurons within each type neatly tile the layers that they innervate (see, for example, the hΔH and hΔM neurons). Overall, the structure of the hΔ and vΔ backbone provides an avenue to determine the direction of information flow through the intra-FB columnar network, which we discuss in a later section.

<u>Known and novel FB pathways to and from putative premotor and contextual centers</u>
As discussed above, the columnar LAL-projecting PFL neurons have previously been identified as the main outputs of the FB (Lin et al., 2013; Wolff et al., 2015). The projection patterns and presynaptic specializations of the PFL1, PFL2 and PFL3 neuron types in the LAL confirm that all three are indeed output neurons (**Figure 28—figure supplement 1**). In addition, the connectome revealed ~20 additional columnar types, most of which were previously unknown, that innervate either the ROB, CRE, or SNP (**Figure 32**). These types do not have arbors in the PB. Each type is composed of individual neurons that collectively tile all 9 columns of the FB (**Figure 32—figure supplement 1-2**), and the various types can be distinguished by the layer(s) of the FB they innervate, their overall columnar structure, and the extra-FB region that they project to. FR types send projections to the ROB; FS types send projections to the SNP regions; and FC types send projections to the CRE. With few exceptions, FR and FS types have primarily postsynaptic arbors in the FB and presynaptic specializations in their extra-FB neuropil (**Figure 28—figure supplement 1**), potentially forming additional columnar output pathways, as discussed in the CX outputs sections. FC types have mixed arbors both within and outside the FB (**Figure 28—figure supplement 1**), perhaps providing bidirectional communication between the FB and CRE.

<u>Does the FB have 8 functional columns?</u>
With the exception of the hΔ neuron types, most FB columnar neuron types appear to divide the FB into 9 structural columns. Interestingly, several lines of evidence suggest that these 9 'structural columns' correspond to 8 'functional columns' that encode unique allocentric directions, with C1 and C9 encoding the same direction. First, every vΔ has individual neurons with arbors in both C1 and C9, as noted above (**Figure 31Ai**). Second, PFGs neurons project from L1/L9 and R1/R9 in the PB to FB columns C1 and C9 respectively (**Figure 30Aiii**). Third, certain PFN types sample from 8 PB glomeruli in the left and right PB—each inheriting EPG

input from all EB wedges—before projecting to 8 FB columns (see, for example, the PFNp_a neuron type in **Figure 30Biii**). If the PB-FB-* columnar neurons bring head direction information into the FB, the structure's 8 functional columns would then translate to a 45° partitioning of azimuthal space.

Intra-FB connectivity of the FB columnar neurons

In the sections above, we described the major FB neuron types, with a particular focus on their morphology, columnar structure, and extra-FB innervations. We now turn our focus to connectivity within the intra-FB network and describe how columnar information may flow from PB-FB inputs to columnar outputs.

Much of the FB's columnar input comes from the PFN neurons (top few rows in **Figure 33A**), which have postsynaptic specializations in both the PB and NO. Some of the PFN types also make synapses within their type in the FB columns that they innervate—much like the PEN neurons in the EB. Though there are direct connections between PFN neurons and columnar FB output neurons in the FB (far right columns in **Figure 33A**), the majority of PFN synapses are to vΔ, hΔ, and FC neurons. Thus, much of the input to the FB passes into a multi-layer intra-FB network. To gauge the "depth" of the intra-FB network, we quantified the different 'path lengths' from PFN inputs to columnar FB output neurons (**Figure 33Bi**) (see Materials and Methods). For example, PFNa neurons have direct connections to PFL1 neurons. This is a path length of 1. PFNa neurons also have indirect connections to PFL2 neurons through first hΔJ and then hΔH neurons (**Figure 33Bi, Bii**). This is a path length of 3. We found that direct connections from PFN neurons to output neurons are relatively sparse, while many more two or three step pathways can be traced through hΔ and/or vΔ neurons (**Figure 33Bii**). While highly recurrent, the intra-FB network can therefore roughly be thought of as being 3-4 layers deep, though we note that this analysis does not include the FB tangential neurons (**See Figure 33—figure supplement 1** for a 3-layer graph of the network).

We next sought to characterize the structure of the intra-FB network connectivity. The connectivity shows clear indications of preferred pathways and subnetworks. For example, the PFNa/FC1/PFL1 types appear to form a parallel subnetwork that can be distinguished from other pathways through the FB (highlighted in **Figure 33A**). Examining this subnetwork at the individual neuron level (**Figure 33C**), we found that the connectivity patterns in the subnetwork largely matched the columnar overlap of the different constituent neurons. However, we found that direct connections from individual PFNa neurons to PFL1 neurons were not as strong or as consistent across neurons as direct connections from PFNa neurons to FC1s. FC1 neurons themselves send their outputs to a putative motor area (CRE), to PFL1s within their column, and to other FC neurons. Thus, the indirect pathways from PFNa to FC1s to PFL1s may contribute more to PFL1 activity than the direct PFNa to PFL1 connections. These indirect pathways again point to importance of depth in FB networks.

To parse additional subnetworks in the FB, we next grouped neuron types based on common connectivity patterns —specifically, their upstream and downstream partners (**Figure 33D**, see also **Figure 33—figure supplement 2**, and Materials and Methods). As expected, the FC1

neurons clustered together based on both their inputs and outputs, a clear signature of the PFNa/FC1/PFL1 subnetwork, as did some other types (see blocks around the diagonal in **Figure 6D**). However, neuron types that share an upstream cluster split into multiple downstream clusters (and vice versa) with few exceptions (**Figure 33E**, see **Figure 33—figure supplement 3** for one exception). This splitting suggests that the intra-FB network cannot, through columnar connectivity alone, be cleanly split into multiple subnetworks. Information is propagated throughout the FB, across layers and columns. However, we note that FB tangential neurons may "gate" some of these pathways, allowing for a clean functional separation of subnetworks. We return to this topic in the Discussion.

Intra-FB connectivity of the FB columnar neurons: no anatomical lateralization

As described above, PFN neuron types provide the major columnar input to the intra-FB network. How is this input structured? The PFN neurons' PB-FB phase shift predicts that activity bumps from the right and left PB will end up two columns apart in the FB (**Figure 30, Figure 34A**). This two-column phase shift corresponds to ~90°, since the FB's eight 'functional columns' sample azimuthal space in 45° increments. Importantly, these morphological phase shifts also impact PFN connectivity with downstream FB neurons (**Figure 34C**). For example, PFNa neurons that innervate glomerulus R5 synapse onto FC1B neurons in C6, and L5 neurons onto FC1B neurons in C4, consistent with their morphological phase shifts (top panel, **Figure 34C**). Postsynaptic hΔ types, which have an even number of FB columns, show a similar pattern (see PFNa-to-hΔ example in top panel, **Figure 34C**). More nuanced morphological characteristics also impact connectivity. For example, PFNp_a neurons, which arborize in spatially restricted regions in L1 (**Figure 29**), primarily connect single PB glomeruli to single FB columns, while PFNa neurons, which have slightly wider arbors, connect single glomeruli to several adjacent FB columns (**Figure 34C**). Thus, PB-FB phase shifts impact FB connectivity and have the potential to bring two spatially shifted bumps carrying the head direction signal into the FB network.

This raises the possibility of anatomical lateralization that is preserved or enhanced within the FB, a feature which could allow for each PFN type to carry two activity bumps —one from each side of the PB— to the FB, and for those two bumps to be independently propagated within the structure. One signature of lateralization of this sort would be that PFN neurons from different sides of the PB might project either to different neuron types in the same FB columns (**Figure 35A**). However, we found no systematic differences in left versus right PFN inputs to different downstream FB neurons (Figure 35B). A second signature of lateralization might be PFN neurons from different sides of the PB projecting to the same neuron types that are themselves lateralized, perhaps in demi-columns (**Figure 35C**). However, we found that the input contributions of left and right PB neurons were very similar for most downstream neurons (**Figure 35D**). Although there are some exceptions to this rule, these exceptions are almost all neurons with both weak and inconsistent connections (small dots to the top left of the plot in **Figure 35E** and an absence of large dots at the top right). Note that the PFNd neurons do preferentially and consistently make connections with other PFNd neurons. That is, within the FB, PFNd neurons from the left PB synapse onto PFNd neurons from the right PB, and those

from the right PB onto those from the left PB, but neither population makes synapses with other PFNd neurons from the same side of the bridge (neither right with right, nor left with left; see arrow in **Figure 35E**). Thus, consistent with physiological reports from some PF neuron types (Shiozaki et al., 2020), each PFN type is likely to only transmit a single bump of activity to the same downstream neurons in the FB, rather than multiple bumps that might then be processed independently within the FB. Note, however, that this does not rule out the possibility of multiple activity bumps arising from *different* PFN types, the navigational implications of which we explore in Discussion. Note also that although there appears to be no lateralization in the context of FB bump dynamics, there is considerable asymmetry in how some FB neurons from the left and right halves of the structure project to the AB.

<u>The AB: the only clearly asymmetrical structure in the fly brain</u>

The AB, which sits at the base of the FB (**Figure 36A**), is now considered a core structure of the CX (Wolff and Rubin, 2018). As suggested by its name, the structure is notable for having distinctly different sizes on either side of the midline, with the right side typically being larger than the left (Jenett et al., 2012; Pascual et al., 2004; Wolff and Rubin, 2018). The AB receives inputs from many different brain regions, including the SMP, SIP, ATL and CRE, and sends its outputs primarily to the dorsal and ventral layers of the FB (**Figure 36B**). Many of the AB neuron types that innervate the FB have been described previously (Wolff and Rubin, 2018), but we identified two new types that primarily get input in the AB, the FS4A and FS4B neurons (**Figure 36C**). FS4A and FS4B also receive input throughout the FB and output in the SNP.

The asymmetry of the two sides of the AB is clearly reflected in the connectivity of the neuron types that arborize there. The left AB primarily connects the vΔA_a neurons with the SA3 neurons, while the right AB connects many more types of AB neurons (**Figure 36D**). Another notable feature is that FB-AB columnar neurons that arborize in the right half and center of the FB send processes to the right AB, while neurons that arborize in the left FB send processes to the left AB (**Figure 36E, Figure 36—figure supplement 1A**). This is in stark contrast with the NO, whose left and right halves both receive PFN projections from all FB columns. The FB targets of AB-FB neurons are varied, including tangential, vΔ, and hΔ neurons (**Figure 36F, Figure 36—figure supplement 1B**). The role of the asymmetry introduced by the AB is not currently understood.

<u>Intra-FB connectivity: circuit motifs</u>

Unlike the EB network, whose columnar neurons form recurrent EB-PB loops, the FB network does not appear to send columnar projections back to the PB (**PB Figure 22**). This may suggest that the FB is a way station along a feedforward pathway, receiving a PB bump— modified by input from accessory structures like the NO—before initiating actions by transmitting commands to pre-motor centers. However, also in contrast to the EB, the FB is characterized by dense and highly specific inter-columnar and inter-layer recurrent connections between different neuron types. This connection matrix likely strongly influences bump dynamics and enables recurrent network dynamics *within* the structure. We now focus on describing the most prominent motifs in this recurrent network.

As described in a previous section, the backbone of the FB's recurrent network is a 2D grid formed by the hΔ and vΔ neurons (**Figure 31**). More generally, all FB columnar types can be divided into two broad categories based on the morphology of their neurons (**Figure 37A**). The processes of some "vertical" neuron types are largely confined to a single column, and include all PB-FB-*, FX, and vΔ types. "Horizontal" hΔ neuron types, by contrast, have processes in two distinct columns. As depicted in **Figure 37A**, these vertical and horizontal neuron types connect to each other in several different ways. Importantly, however, the connectivity between different vertical and horizontal types closely respects some simple rules. We found that connections were typically either localized to the same column (**Figure 37Bi**), jumped half the width of the FB (**Figure 37Bii**), or did both (**Figure 37Biii**). In this last case, a presynaptic neuron in one FB column synapses onto postsynaptic hΔ neurons, some with primarily dendritic arbors in the column and some with primarily axonal arbors in the column. Because hΔ neurons are assigned to columns based on the location of their dendritic arbor, this connectivity motif produces two diagonal bands in the column-to-column connectivity matrix (bottom panel of **Figure 37 Biii**).

It is as yet unknown whether these connections are excitatory or inhibitory, something that — much as is the case with the Δ7 neurons in the PB— would impact how activity is propagated across these connections. For example, a column-matched excitatory connection (Figure 37Bi) would preserve the phase of the bump, but if that connection were inhibitory, it would shift the bump by half the width of the FB (roughly 180° in azimuth). Similarly, a connection across half the FB's width might shift the bump by 180°, if it happens to be excitatory, or keep it in place (0°) if it were inhibitory (**Figure 37ii**). We analyzed the complete set of connectivity matrices (see, for example, the bottom row of **Figure 10Bi-iii**) between all vertical and horizontal types using principal components analysis (PCA), and found that the three broad inter-columnar motifs that we identified accounted for the most of the variance across the entire set (**Figure 37C** and **Figure 37—figure supplements 1 and 2**, see Materials and Methods). Not knowing the neurotransmitters and receptors involved in these motifs, we cannot exactly say how the activity bump from the PB is modified by the FB network. However, the connectivity strongly suggests that azimuthal comparisons and transformations of activity bumps are a key function of much of the FB's recurrent circuitry. We describe the potential significance of these specific recurrent motifs for bump-driven navigational computations in Discussion.

We now turn to the other axis of information flow in the FB, the vertical axis. As noted in a previous section, the PFN neurons provide the FB with most of its columnar input. These neurons target the ventral layers of the FB (**Figure 38A**)—in fact, most PFN neurons target Layers 1 and 2, while PFNa, PFNd, and PFNv types targets Layers 3-4 (**Figure 28—figure supplement 1**). The PFR and PFGs types are the exception to this rule, with presynaptic specializations up to Layer 6 (**Figure 28—figure supplement 1**). When we examined the 2D grid of neuron types formed by the FB's interneurons, we found a discernable vertical direction to information flow within the structure. Specifically, most of the intra-FB columnar neurons transfer information from the ventral layers of the FB to the dorsal layers, as can be seen from the distribution of their postsynaptic and presynaptic specializations (**Figure 38B,C**). Both the

hΔ and vΔ types receive most of their extra-FB columnar input in their ventral arbors and provide output in their dorsal arbors (as denoted by the dark circles being consistently higher than light circles in **Figure 38D**).

Columnar phase shifts redux: the PFL neurons

As discussed in an earlier section, the primary columnar inputs to the FB, the PFN neurons, enter the structure with different type-specific columnar phase shifts (**Figure 30**), which we believe to subserve specific vector computations required for navigation. The previously identified output neurons, three types of PFL neurons (Lin et al., 2013), also display type-specific phase shifts (**Figure 39**). These PFL phase shifts, when combined with either their asymmetric synapse counts in the PB and the LAL (described below in the Outputs section), and/or with their inputs in the FB (explored further in Discussion), position them well to modulate or control directed actions, whether towards a default goal or a goal that is specific by other neurons in the FB.

The PFL2 neurons are distinguished from all other PF columnar neurons based on their coverage of PB glomeruli. In contrast with the other columnar neurons, these neurons only receive inputs from the inner 5 PB glomeruli on each side (**Figure 39Ai-iii**). Thus, these neurons only inherit a single bump in the PB since they effectively sample from a 360° space that is split between the left and right halves of the PB (by contrast, most other PB types sample from 360° space in the left and right PB). The PFL2 neurons are also distinguished by their 4-column phase shift, giving them an ~180° phase shift in their FB innervation relative to their input PB glomerulus or glomeruli (since individual PFL2s sometimes arborize in two neighboring glomeruli) (**Figure 39Aii-iv**). Notably, these neurons send projections to both sides of the LAL, a pattern of connectivity whose potential navigational function for forward locomotion we explore in a later section on output pathways of the CX, and revisit in Discussion.

By contrast, almost all PFL1 neurons have a single column (45°) ipsilateral phase shift and project to the contralateral LAL (**Figure 39Bi-iv**). The exceptions to this rule are, first, the innermost PFL1 neurons. These neurons, which originate in PB glomeruli L1 and R1, send their outputs to the LAL on the same side. Second, the PFL1 neurons in L7 and R7 are not phase shifted as expected, but, rather, project to C1 instead of C2 and C9 instead of C8, respectively, in the FB. The function of these differences is not immediately obvious.

Finally, the PFL3 neurons display a two-column (90°) ipsilateral phase shift and also project to the contralateral LAL (**Figure 39Ci-iv**). Exceptions to this rule are described in the Figure legend. Just as the 180° phase shift of the PFL2 neurons and their projection to both sides of the LAL suggest a role in directing forward locomotion, this 90° phase shift suggests a potential role for PFL3 neurons in directing turns towards an FB-specified goal, an idea that we develop in the section on CX output pathways and in Discussion.

Overall, the PFL neurons, along with the other FB columnar outputs, may be part of dedicated circuits for a variety of bump-dependent navigational behaviors. How the FB enables the fly to set goal directions and arbitrates between these potentially different behavioral programs

based on internal state and context may be the purview of the large set of tangential inputs into the structure.

Tangential inputs to the FB network

In earlier sections we described the ring neurons and ExRs that bring tangential input to the EB. Most ring neurons bring information about directional sensory cues to the EB-PB compass network, tethering the fly's internal compass to its surroundings, while several of the ExRs likely provide modulatory input. However, in contrast to the ring neurons, the FB's many tangential inputs are less well understood. All FB tangential (FBt) neurons have presynaptic specializations in characteristic FB layers and several bring input from accessory structures of the CX, such as the CRE, SMP and LAL, as well as from the NO (**Figure 40A**). The FB4O neurons, for example, receive input in the CRE and SNP, and send their outputs solely to Layer 4 in the FB (**Figure 40Aii**). Like the ExRs, there are only a small number of neurons per FB tangential type (**Figure 40B**). Also like the ExRs, there is considerable variance across the different FB tangential neuron types. Some types, such as FB1I, target multiple FB layers (**Figure 40C**). Others, such as FB4Z, are pure FB interneurons, with no external processes (**Figure 40D**). FB4Z is also an example of an FB neuron type that does not uniformly fill its layer but instead sends out selective processes to target specific partners within the layer (**Figure 40E**). The number of different FB tangential neurons that provide output to any given FB layer and the diversity of brain regions from which they draw their inputs suggests that any accounting of the FB's functions must come with an asterisk: every navigational or other computation that the FB participates in is likely modulated by context, whether that be sensorimotor input, or the fly's internal state (see **Figure 40F**, which focuses on the inputs to the FB from the right side of the brain due to the constraints imposed by the hemibrain volume). Precisely what these modulatory influences are is largely unknown. However, some hints may come from the brain regions that target different layers through the FB tangential neurons. For example, the LAL and CRE preferentially send processes to layers 3-5, while the upper layers tend to be targeted by the SNP (**Figure 40G**, **Figure 40—figure supplement 1**).

Although little is known about most of the FB's tangential neurons, there are some exceptions. For example, the ExFl1 neurons (Homberg, 1994; Liu et al., 2006; Weir et al., 2014; Young and Armstrong, 2010b), which are likely the FB2B_a and/or FB2B_b neurons (**Figure 41A**), respond to progressive optic flow, and are strongly modulated by whether or not the fly is flying (Weir et al., 2014), providing a potential indication of the state of (forward) flight. This information is fed to other FB tangential neurons, to FB interneuron types (including some hΔ and vΔ neurons), to intermediate types (such as the FC neurons), and to output neurons (such as the FR neurons, **Figure 41B**). Such a connectivity profile is typical of most FB tangential neuron classes, as shown in **Figure 42**. A recent study focused on the lateral horn (LH) identified an FB tangential neuron type called PV5k1, which, when optogenetically stimulated under closed-loop visual conditions, leads to a reduction in the fly's wingbeat frequency. The neurons targeted by the GAL4 lines used in that study likely correspond to FB2H_a, FB2H_b, and/or FB2I_b, neuron types that target some hΔ neuron types and also the PFL2 and PFL3 neuron types, consistent with the direct influence on the fly's behavioral patterns (see later sections on PFL neurons).

The PFN neurons, the major source of columnar input to the FB, are notably sparse in the list of downstream targets of the FB tangential neurons (first block of columns, **Figure 42**). This connectivity matrix further emphasizes the importance of the FB's interneurons and recurrent network in shaping circuit dynamics in the structure: much of the contextual modulation appears to target the intra-FB network. Some FB tangential neuron types, such as the FB2B_a, have been proposed to have a major role in visual learning (Liu et al., 2006). In such a situation, the tangential neurons could be ideally placed to provide information about positive or negative reinforcers, a function typically carried out in the fly brain by dopaminergic neurons (DANs). Indeed, several FB tangential neurons are known to be DANs (discussed further below), as indicated by the gray bars in the connectivity matrix in **Figure 42**. DANs are not the only neuromodulatory neurons amongst the FB tangential neurons. It is likely that several other FB tangential neurons may be modulatory and peptidergic, but we could only confirm one additional such neuron, the octopaminergic OA-VPM3 (**Figure 42**, first row in the connectivity matrix).

As with the ring neurons of the EB, the FB's tangential neurons also synapse onto each other near their presynaptic specializations in the FB (**Figure 43**). However, in contrast to the ring neurons, there are far fewer neurons in each FB tangential type, and their subnetworks seem less tightly clustered, making it more difficult to detect hierarchies amongst the potential contextual inputs (squares marked in **Figure 44**). An additional similarity to the EB's ring neurons is that all tangential neurons of the FB, including the DANs, also receive considerable intra-FB input near their presynaptic sites in the FB (**Figure 44A**), much of it from their targets (**Figure 44B**). We also examined the connectivity matrix within and across different FB tangential neuron types at the level of individual neurons (**Figure 45A**), which revealed that a subset of FB tangential types show a strong similarity to ring neurons in their connectivity patterns. For example, FB2I neurons interact strongly among each other, and these interactions are spread uniformly across columns within their layer of innervation (**Figure 45B**). If these interactions are inhibitory, as is the case with ring neurons, such connectivity may facilitate a competition between similar contextual inputs vying to influence navigational computations based on the columnar position of the bump. We revisit this possibility in Discussion.

<u>Connections between the MB and FBs</u>

FB tangential neurons receive much of their input in central brain regions like the SNP (**Figure 28**, **Figure 40F**), which, in turn, receive inputs from many parts of the brain, consistent with the idea that the FB tangential neurons enable the contextual modulation of CX-controlled behavior. Our knowledge of the precise nature of these signals has been limited by a lack of extensive characterization of inputs to these less structured brain regions. A notable exception is the set of inputs that arrive from the MB. The MB is thought to be a center for associative learning (Cognigni et al., 2018; Modi et al., 2020). It receives inputs from nearly all sensory modalities and is innervated by multiple types of DANs (Li et al., 2020), and provides an architecture to flexibly convert sensory information from thousands of Kenyon cells (KCs) into experience-dependent (and DAN-mediated) valence signals carried by the MB's output neurons, the MBONs. MBONs broadcast these valence signals to other areas of the brain, but how these signals drive the fly's behavioral responses is not fully understood. In this section, we

analyze pathways connecting the MB and the CX. Communication between these structures may play an important role in sleep (Dubowy and Sehgal, 2017; Sitaraman et al., 2015), memory consolidation (Berry et al., 2015; Dag et al., 2019; Donlea, 2019) and perhaps also the conversion of the MB's valence signals into goal-directed actions during navigation (Collett and Collett, 2018; Sun et al., 2020).

As described in a companion paper on the MB (Li et al., 2020), about half of the mushroom body output neurons (MBONs) directly synapse onto FB tangential neurons that target the middle layers (4-6) of the FB (**Figure 46A**). Some MBONs contact only a single type of FB tangential neuron, for example, MBON09 connects exclusively to FB4R (**Figure 46Bi**), but many other MBONs contact multiple downstream targets in the FB, for example, MBON04, which targets FB tangential neurons in both Layers 1 and 6 (**Figure 46Bii**). MBONs of different neurotransmitter types, both excitatory and inhibitory, often converge onto the same FB tangential neurons (lines of different colors in **Figure 28A**; see (Li et al., 2020) for how neurotransmitters for each of the MBONs were identified). For example, the glutamatergic (and likely inhibitory) MBON04 and the cholinergic (excitatory) MBON12 both contact FB4A (**Figure 46A,Biii**), and two MBONs that express different (likely) inhibitory neurotransmitters, MBON05 (glutamate) and MBON09 (GABA), converge onto FB4R, along with a third excitatory (cholinergic) MBON21 (**Figure 46A**). FB neurons are not the only CX neurons targeted by direct projections from the MB. The glutamatergic MBON30 neuron type targets the LCNOp neurons (**Figure 46Biv**) that themselves feed the PFNp columnar neurons. The strengths of these direct connections vary widely. Some, such as connections from MBON09 and MBON21 to FB4R, represent a significant fraction of their downstream target's input (longer bars in **Figure 46—figure supplement 1B**), while others are much weaker in the influence that they exert on even their most preferred downstream partners in the CX (short bars in **Figure 46C—figure supplement 1B**). Note that, as explained in an early section of the manuscript, our summary plots and connectivity matrices exclude connected pairs whose connection weights fall below our threshold (see Materials and Methods). Thus, **Figure 46A**, for example, shows slightly fewer connections than the equivalent figure in the companion paper on the MB connectome (Li et al., 2020). This dependence of connectivity on the threshold chosen is shown in **Figure 46—figure supplement 1A,C**.

In addition to these direct connections, MBONs also strongly connect to both dorsal and ventral layers of the FB through intermediate neurons in regions like the SMP, SIP and CRE (**Figure 47**). Several of these one-hop pathways feature a mix of convergence and divergence from different MBONs onto neurons in intermediate layers (complete set of paths in **Figure 47— figure supplement 1**). Note, once again, that not all MB-to-CX pathways involve FB tangential neurons: for example, MBONs 26 and 31 reach the CX, and even the FB, through the LAL and the LCNO and LNO neurons (**Figure 29E**).

In some cases, we could use what is known about the KC inputs to different MBON types see (Li et al., 2020) to determine the type of sensory information in specific downstream FB tangential neurons. For example, several MBONs conveying valence signals associated with visual information send divergent streams of information to an intermediate layer of neurons in

diverse brain regions. These intermediate neurons, in turn, feed FB tangential neurons of different classes that go to both ventral and dorsal layers of the FB (Figure 47C). MBONs project to a largely different set of intermediate neurons, which project to their own, largely distinct set of FB tangential neurons (and LNOs) (**Figure 47D**).

In Discussion we examine the potential role of the connectivity between the MB and FB in navigation, sleep and memory consolidation.

Sleep-wake circuits in the dorsal FB and their upstream and downstream connections

A large body of previous work has established a functional role for dorsal FB (dFB) neurons in regulating sleep need and controlling sleep/wake states (reviewed in (Artiushin and Sehgal, 2017; Donlea, 2017; Dubowy and Sehgal, 2017; Helfrich-Forster, 2018)). At least a subset of neurons within the heterogeneous population of tangential neurons targeted by the R23E10 GAL4 line (**Figure 48A**; Qian et al., 2017) play a role in these processes, and show intrinsic excitability and spontaneous firing rates that track sleep need and induce sleep when activated (Donlea et al., 2014; Donlea et al., 2018; Donlea et al., 2011; Liu et al., 2016b; Ni et al., 2019; Pimentel et al., 2016; Qian et al., 2017). Consistent with this functional heterogeneity, R23E10 drives expression in multiple, distinct neuron types as defined by EM (**Figure 48B-D; Figure 48—figure supplement 1**). However, we currently lack the experimental data needed to distinguish the specific roles that these different neuron types play in sleep regulation.

We matched individual neurons in R23E10 to their corresponding EM-defined neuron type by comparing the light-level morphology of individual R23E10 neurons to the 14 candidate EM neuron types (**Figure 48—figure supplements 2-7**). Nine distinct neuron types appear to be present in the R23E10 line. Our analyses indicate that these neuron types occupy characteristic layers and sublayers of the FB, which often overlap, and send arbors to distinct regions of the superior neuropil. Such anatomical distinctions predict that there should be differences in upstream and downstream connectivity among the different neuron types. Indeed, as shown in **Figure 48C**, each type has 1 to 3 neurons per hemisphere and neurons within each type are more similar in their upstream/downstream connectivity than they are to neurons of other types, consistent with our connectivity-based definition of neuron types. While individual neuron types that show expression in the R23E10 GAL4 line have type-specific morphology and connectivity, their arbors outside the FB are mostly in superior neuropils (SMP, SIP, SLP). Their arbors both inside and outside the FB are of mixed polarity containing both pre- and postsynaptic sites.

How can the presence of multiple neuron types in R23E10 be reconciled with their functional role in sleep-wake regulation? Previous studies have shown that dopaminergic neurons of the dFB promote wakefulness (Liu et al., 2012; Ueno et al., 2012), likely through their ability to inhibit some or all of these neuron types (Pimentel et al., 2016). We reasoned that neuron types within the R23E10 expression pattern that are involved in sleep-wake control are likely those that receive direct input from wake-promoting DAN types. Using an approach similar to that described above, we identified three neuron types —FB5H, FB6H, FB7B— as likely DANs in the dFB, each of which contains one neuron per hemisphere that belongs to the PPL1 cluster

(**Figure 49**). Several lines of evidence support this identification. First, a stable split GAL4 that uses a 10kb segment of the TH genomic region as one of its hemidrivers (Aso et al., 2014a) labels three neurons per hemisphere whose cell bodies express tyrosine hydroxylase (TH). Matching morphologies of individual neuronal from this split to EM morphologies yielded high quality matches (see Materials and Methods) to FB5H, FB6H, and FB7B. Second, a driver line that specifically targets DANs, TH-GAL4, contains individual neurons whose morphology matches that of FB6H and FB7B (Fly Circuit TH collection, (Chiang et al., 2011)). Third, although the general morphology of the FB5H, FB6H, and FB7B neuron types is similar to that of R23E10 neurons, all three neuron types have arbors that lie partly outside the R23E10 pattern, demonstrating that these DAN types are not present in R23E10. These PPL1 DANs are likely the primary wake-promoting DANs of the dFB, as previously indicated by experimental studies (Liu et al., 2012; Ueno et al., 2012). Although additional DANs in the PPM3 cluster —FB2A, FB4L, FB4M— innervate more ventral FB layers, there is as yet no data to indicate that they regulate sleep.

How are the sleep-promoting cell types with the R23E10 expression patten and wake-promoting PPL1 DANs connected, and how might the network regulate sleep-wake states? To begin to address this question, we looked at the connectivity between these neuron types within the dFB, where their arbors overlap most strongly and where the majority of their presynaptic sites reside (**Figure 50**). Plotting neuron-to-neuron connectivity revealed a densely recurrent network, but with variable connection strengths (**Figure 50A**). Types that are strongly connected show all-to-all connectivity, while more weakly connected types are often only partially connected, partly because some connections drop below our threshold. Not surprisingly, given its more ventral arbors, the dopaminergic FB5H neuron type lacks any direct connections with the types present in R23E10. **Figure 50B** shows a network graph of the connectivity between all the neuron types present in R23E10 and the two remaining DAN types. Several features of this network are notable. First, the network can be roughly divided into two clusters: one containing layer 6 neurons and the other layer 7 neurons. Second, each cluster contains both putative sleep-promoting R23E10 neuron types and a putative wake-promoting DAN type. Most importantly, the two DAN types target nearly every R23E10 neuron in their layer and, in turn, receive input from the same R23E10 neurons they target. This simple motif resembles the classic “flip-flop” circuit model of brain state regulation thought to underlie sleep-wake control in mammals (reviewed in (Saper et al., 2010)). In this model, wake-promoting and sleep-promoting neuron types have reciprocal inhibitory connections, ensuring that only one population is active at a time. While dopamine is known to inhibit R23E10 neurons (Ni et al., 2019; Pimentel et al., 2016), it is currently unknown whether R23E10 neurons themselves inhibit DANs.

The ability of sleep-promoting R23E10 neurons to track sleep need and to induce sleep likely depends on their upstream and downstream connections outside of the core sleep-wake network of the dFB. Similarly, the inputs and outputs of the wake-promoting DANs remains largely unknown. Therefore, we traced pathways upstream and downstream of the neuron types to identify their major inputs and outputs. For this analysis, we used “pathway weights” to quantify the influence exerted from a given CX neuron to a different neuron (**Methods Figure**

2; also see Materials and Methods). These weights are computed by multiplying relative weights along each pathway between the two neurons and then summing across those pathways (see Materials and Methods). Pathways of increasing length tend to have a smaller pathway weight. When several distinct pathways connect the input and output types, pathway weight is summed across pathways. To identify candidate downstream pathways, neurons that receive at least 0.5% of their inputs from sleep-wake types were included. Similarly, upstream pathways that comprise greater than 0.5% of the total input to sleep-wake types were included. We focus our analysis on strong pathways, which are insensitive to the precise threshold chosen.

We found that the dFB sleep-wake neuron types have the potential to influence a large number of neuron types both inside and outside the FB (**Figure 51). Figure 51A** shows a pathway connectivity matrix that includes all neuron types up to two steps downstream of the sleep-wake neuron types; nearly all the targets reached in two steps are already present by the first step downstream (**Figure 51—figure supplement 1)**. Although the vast majority of FB tangential neuron types targeted by the sleep-wake neurons are in dorsal layers, the FB1I and FB1J tangential neuron types, with processes in both layer 1 and the dFB, provide a possible path from transmitting information on sleep state to the ventral FB. Sleep-wake neuron types also have downstream targets in the superior neuropil, consistent with the presence of mixed arbors in these regions. Several of these neurons output onto a subset of MB DANs (Li et al., 2020) and FB6A, FB6C_b and FB6I neurons all have outputs to OA-VPM3, a wake-promoting octopaminergic neuron type with processes spanning many brain regions (Ni et al., 2019; Seidner et al., 2015). FB6A is exceptional among the sleep-wake neuron types in that it makes very strong connections to several notable targets, including: PFGs, a putative neuromodulatory columnar type (as discussed in a later section); hΔk, which receives input in the EB and provides output to the dFB; and ExR3, a serotonergic neuron previously shown to be involved in sleep-wake control that innervates the EB and FB (Liu et al., 2019). FB6A also send output to 5-HTPMPD01, a serotonergic neuron, which signals to a large number of neurons in the superior neuropil.

Much like the downstream pathways, a large number of upstream neuron types have the potential to influence the sleep-wake circuit, especially through inputs in the SNP region (**Figure 52**). When considering how strong an input to a sleep-wake neuron is, it is important to take into account its polarity. In particular, while sleep-wake neurons receive inputs in the superior neuropil that have small pathway weights, these inputs may be particularly potent, since these neurons are mostly dendritic there (see early section on “Assessing the relative importance of different synaptic inputs”). With the exception of SNP inputs, it is interesting that many of neurons upstream of sleep-wake neuron types are also downstream of sleep-wake types, forming recurrent loops. For example, FB1I and FB1J both receive input from and send output to layer 7 sleep-wake neuron types, establishing direct, bidirectional communication between ventral and dorsal FB layers. Similarly, FB6A not only provides strong output to ExR3, hΔk, and PFGs, but it also receives strong input from these same neuron types as well. Related to this, ExR3 receives input primarily from FB6A but sends output to several other sleep-wake neuron types, providing a link between sleep circuits in the FB and those in the EB, as described below.

Previous studies have suggested that a recurrent loop links sleep-wake circuits in the dFB with those in the EB (Donlea et al., 2018; Liu et al., 2016a), but identifying the neurons and pathways involved has proven challenging. To address this, we constructed a network graph that contains the dFB types identified above, other previously reported CX sleep-wake neuron types — including ER5 (Liu et al., 2016a), ExR1 (i.e. 'helicon cells'; (Donlea et al., 2018)), and ExR3 (Liu et al., 2019). As shown in **Figure 53**, sleep centers in the dFB and EB are indeed reciprocally connected, and through distinct channels that bridge several clusters. ExR1 and ExR3 form a subnetwork in the EB with extensive reciprocal connections involving ER5 and ER3d types. ER5 neurons are known to be involved in homeostatic sleep control (Liu et al., 2016a; Raccuglia et al., 2019) and also receive circadian input from the anterior-projecting DN1 pathway (**Figure 6**; (Guo et al., 2018; Lamaze et al., 2018)). Circadian input may enter the circuit through other, yet-to-be identified pathways as well (Liang et al., 2019; Pirez et al., 2013). Interestingly, ER5 neurons also synapses onto EL neurons, octopaminergic cells with outputs that target nearly all other rings neuron types in the EB (see Figure 10F for network graph). This may allow ER5 neurons to impact activity throughout the EB circuit. ER3d neurons innervate the inferior-posterior portion of the BU, a region known to contain glomeruli that lack ipsilateral or contralateral visual receptive fields (Omoto et al., 2017; Shiozaki and Kazama, 2017). In addition, ER3d neuron types are targeted by a driver line that labels neurons expressing the 5HT7 serotonin receptor and have been previously implicated in the serotonergic ExR3 sleep-wake circuit of the EB (**Figure 53 S1**; (Liu et al., 2019)). hΔK neurons, in turn, have purely dendritic inputs in the EB, where they receive input predominately from PEN_b neurons. This may be one pathway by which the head direction signal, whose amplitude correlates with the fly's locomotor activity (Turner-Evans et al., 2017), can be passed directly to dFB circuits.

ExR1, ExR3, and hΔK neurons all send projections to the dFB, where they connect with distinct clusters. The first cluster consists of a recurrent FB network that includes PFGs neurons and FB6A neurons, as well as ExR3 and hΔK neurons. PFGs may provide a neuromodulatory input to the circuit, since their presynaptic sites contain dense core vesicles (as discussed in a later section). While this cluster is anchored by FB6A, it seems likely that even sleep-wake neuron types that lack direct connections with ExR1 and hΔK may be able to influence their activity, since sleep-wake neuron types are recurrently connected, as described above. Therefore, sleep-wake neuron types, especially those in layer 6, may convey information to EB circuits through their effect on ExR3 neurons. A second dFB cluster is formed by ExR1, FB8B, and hΔD. Although ExR1 has only a small number of inputs and outputs in the dFB, it receives a weak input from FB6A, consistent with previous work (Donlea et al., 2018). In addition, ExR1 indirectly communicates with the layer 7 sleep-wake circuit through FB8B and hΔD. Similarly, FB7K and FB7A, which are part of the layer 7 sleep-wake cluster, send outputs to both PFGs and hDeltaF, forming an indirect link between putative sleep-promoting layer 7 neurons with those of layer 6, as diagrammed in **Figure 53**.

These results identify a complex network that may allow for communication between sleep-wake circuits in the FB and those in the EB. However, the vast majority of the input and output to sleep-wake neuron types involve additional pathways, most of which have not been

functionally characterized, as described above (**Figure 51, Figure 52**). For example, most of the output from dFB sleep-wake neuron types is onto other FB neurons, raising the possibility that inducing sleep may involve previously unrecognized neuron types and pathways (Dag et al., 2019). Lei, Keleman et al. (in preparation) have identified the neuron that corresponds to the sleep-promoting split-GAL4 line SS57264-GAL4 that they described in (Dag et al., 2019) as FB2B_a. This neuron is not connected to the R23E10 neurons by the columnar neurons in a single step, so these sleep promoting mechanisms are not obviously coordinated.

Pathways that leave the CX: feedback, motor output and more

In the preceding sections, we focused on characterizing the connectivity patterns of different structures within the CX. We now turn our attention to potential output pathways from the CX. To define output pathways, we began by examining the projection patterns of all core CX neuron types that have a significant number of presynaptic terminals in other brain regions (**Figure 54A**, see Materials and Methods for details on significance test). These neurons target a narrow column around the CX that extends through the BU, GA, CRE, LAL, SPS, WED, RUB, ROB, SMP, SLP, IPS and SPS (**Figure 54A,B**), as partially described in previous studies (Hanesch et al., 1989; Li et al., 2020; Lin et al., 2013; Rayshubskiy et al., 2020; Wolff et al., 2015; Wolff and Rubin, 2018). The neural projections and synapse locations within these regions appear to largely segregate by type (see **Figure 54B**). Different types of neurons contribute very different numbers of synapses to their target areas, from tens of synapses for some FB tangential neurons to more than 10,000 synapses for PFL3 neurons (**Figure 54C**). Thus, information from the CX is broadcast widely across the brain, but likely through different, target-specific communication channels of various strengths.

To assess how far information spreads across all output pathways, we computed the number of neurons reached by each successive synaptic 'hop' along the individual pathways (**Figure 55A**). We found that within just two hops, that is, by the third 'layer', information from the CX reaches several thousand neuron types and over 10,000 individual neurons within the hemibrain volume (**Figure 55B**). Additionally, information from each layer feeds back into previous layers (**Figure 55A**, lower loops). There are also a large percentage of recurrent connections within each layer (self-loops in **Figure 55A,** also see **Figure 55B**).

Although many of these downstream neurons, especially those reached in later layers, arborize in regions that have been poorly characterized ("terra incognita"), we could, in some cases, classify target neurons. We labeled these targets either by their type or by their main neuropil (**Figure 55C,D**), following the same classification scheme as detailed in Table 3 in (Scheffer et al., 2020). We discovered that within 2 hops, information from the CX reached areas as far afield as the lateral horn (LH), and neuron types as diverse as MBONs, visual projection neurons (VPNs), descending neurons (DNs) and a variety of neuromodulatory and peptidergic neurons (**Figure 55D**). Different pathways out of the CX, however, show varying degrees of divergence (**Figure 55E**). Divergence here refers to the number new neuron types reached at each layer. For example, pathways that start at EL, ER6, ExR1, FS4C, FB6Q, or FB6T neurons reach very few neuron types overall, suggesting that the information in these pathways does not spread widely, reflecting a low divergence. By contrast, pathways that start at PFL3 and FS1A neurons

reach a large number of different neuron types within a few hops, suggesting that information from these types is widely shared across the brain, or highly divergent (**Figure 55E**). The number of types reached in part reflects the difference in synapse numbers (compare **Figure 55E** to **Figure 54C**). From this point on, we use pathway weights (**Methods Figure 2**; also see Materials and Methods) to quantify the influence exerted from a given CX output neuron to a different neuron.

Recurrent connectivity within lateralized neuron populations in CX accessory structures

Many of the brain regions outside but in close proximity to the CX serve as input hubs to the CX rather than as waystations to other parts of the brain. Output synapses in four structures in particular—the Gall (GA), the round body (ROB), the rubus (RUB) and the bulb (BU) —are mostly to other CX neurons, forming recurrent CX-to-CX pathways (**Figure 56A**). Recurrent pathways via the BU —which we described in an earlier section on the structure as an important input hub to the EB— are formed exclusively by ExR neurons (**Figure 56B**, see earlier section on the ExR neurons). These recurrent pathways likely serve to modulate inputs to the EB. Pathways through the GA, ROB and RUB are composed of EB and FB columnar neurons (compare CX output types in **Figure 56B**). We will focus on these three structures below.

The GA, ROB, and RUB are paired across the midline and are selectively innervated by either the left or right PB-projecting population of columnar neurons (**Figure 56C**). The Gall appears to primarily house recurrent connections between CX neurons, creating CX-to-CX recurrent loops that may allow EB columnar neurons to form connectivity patterns independent of their EB arborizations (**Figure 56D**). For example, all left EPG and PEG neurons arborize in the Gall. Depending on which EB wedge the neurons innervate, they either target the upper or lower Gall compartment (**Figure 56E**; this compartment specificity was previously described in (Wolff et al., 2015)). In their respective compartments, EPG neurons then synapse onto PEG neurons, resulting in a “checkerboard-like” connectivity pattern (**Figure 56D**). The functional significance of this connectivity motif is currently unknown. ER6, one of the few ring neuron types that does not connect to EPG neurons in the EB (**Figure 11A**, **Figure 56Fii**), provides an interesting counterexample. Both EPG and PEG neurons are recurrently connected with ER6, both in the EB and in the Gall (**Figure 56D, F**). A LAL neuron and ExR6 also output to the EPG neurons in the Gall (**Figure 56—figure supplement 1A**), and, as with ER6, ExR6 and EPG neurons are also recurrently connected in the EB (**Figure 56—figure supplement 3A, Figure 14—figure supplement 3**).

Some columnar neuron types, such as EL and PFGs, don’t target the Gall. Instead, they target a small structure next to the Gall, the Gall surround (GAs). In the GAs, EL neurons appear to primarily form all-to-all connections within either the left or the right hemisphere populations (**Figure 56D,Fi**). Curiously, neither the EL neurons nor the PFGs neurons makes many synapses in the GAs. We therefore looked in the EM images for evidence of other signaling mechanisms. We found that, both PFGs and EL neurons show elongated-bar (e-bar rather than t-bar) synapses (Shaw and Meinertzhagen, 1986; Takemura et al., 2017) and dense core vesicles (DCVs), which have been associated with neuropeptide or neuromodulator release (Burgoyne

and Morgan, 2003; Hammarlund et al., 2008; Nassel and Winther, 2010)(**Figure 56—figure supplement 1 B,C**).

The round body (ROB) and rubus (RUB) are innervated by the FB columnar PFR and FR neurons, respectively (**Figure 56Cii**). Both PFR types arborize in the ROB. There, PFR_a only receives input from PFR_b, while PFR_b neurons make all-to-all within-type connections as well as a number of output connections (**Figure 56—figure supplement 2A, B**). The most significant connection in the ROB appears to be from PFR_b to LAL002, which in turn connects to a number of other non-CX neurons in the LAL and CRE (**Figure 56—figure supplement 2B,C**). Similar to PFR neurons in the ROB, only one of the FR neurons, FR1, forms strong within-type all-to-all connections in the RUB (**Figure 56—figure supplement 3A**). However, unlike in the ROB, both FR1 and FR2 make connections to non-CX partners in the RUB (**Figure 56—figure supplement 3B**). Notably, the sets of downstream partners as well as the output pathway circuits of FR1 and FR2 are largely distinct (**Figure 56—figure supplement 3B,C**). Thus, while the ROB and RUB serve as structures for CX-to-CX recurrence like the Gall, they differ from the Gall in that they can also be considered CX output structures.

<u>CX feedback pathways through other regions</u>

CX output neurons that project to other, non-accessory regions external to the CX also participate in feedback pathways. Indeed, feedback pathways account for a large fraction of their total output pathway weights (**Figure 57A**). For example, the outputs of most ExR neurons almost entirely feed pathways that reenter the CX. Notably, however, such feedback constitutes only 25% of total pathway weights for the PFL neurons, and the ExR8 neuron type contributes virtually nothing to pathways that feedback into the CX. Indeed, most synapses made by CX output neurons in the LAL are to “true” outputs pathways that leave the CX. The situation is more mixed for regions like the CRE (including the RUB and ROB) and SMP (**Figure 57A**).

We classified the targets of external CX-neuron-to-CX-neuron pathways by grouping them into connections onto CX neurons of the same type on the same side of the brain, connections onto CX neurons of the same type on the contralateral side of the brain, connections onto the mainly presynaptic arbors of a different type of CX neurons, or connections onto mainly postsynaptic arbors of a different type of CX neurons. As mentioned above, a few columnar neuron types (for example, the EL neurons) mainly feed other neurons within their type (“self”, dark blue segments, **Figure 57—figure supplement 1A**). Neuron types whose output pathways converge onto axons of other CX output neurons outside the CX (for example, ER6, ExR1 and PFR_a neurons) are similarly sparse (“To axonal”, dark orange segments, **Figure 57—figure supplement 1A**). The most significant CX-neuron-to-CX-neuron pathways outside the CX are those that converge onto dendrites of other CX neurons (“To dendritic”, light orange segments, **Figure 57—figure supplement 1A**).

We quantified which neuron types were the main recipients, by weight, of the different CX-neuron-to-CX-neuron pathways (**Figure 57—figure supplement 1B** shows all pathways, **Figure 57B** highlights non-local, non-FB tangential pathways). We found only weak links between EB

and FB neurons. EB columnar neurons and ExR neurons mostly talk between themselves (see also **Figure 15**), whereas FB neurons mostly reach FB tangential neurons (**Figure 57—figure supplement 1B**). EB-FB and FB-EB pathways *outside* the CX are largely absent and weak when present, because EB neurons and FB neurons innervate non-overlapping areas outside of the CX (**Figure 54B**) and therefore form few direct connections between each other anywhere in these accessory regions (see **Figure 56—figure supplement 2** for a comparison of direct connections to the full pathway connections). However, there are a few notable exceptions. For example, the PFL types participate in output pathways that feed back into the EB through ER and ExR neurons (**Figure 57C**). Additionally, PFL2 and PFL3 neurons reach several LAL-NO neuron types (**Figure 57C**). The PFL2 and PFL3 neurons may carry motor commands (see Discussion), and these LAL-NO connections may therefore bring self-motion information back into the CX in the form of efference copies.

Network motifs involving CX neurons with external projections

Above we described how CX-neuron-to-CX-neuron connections outside of the CX are most commonly made between neurons originating in the same core CX structure (EB or FB). We next asked if these same neuron types also connect inside the CX (**Figure 58**). We followed a similar line of analysis as we used for analyzing ExR connectivity (**Figure 15**). We distinguished 3 possible motifs (**Figure 58A**): “canonical feedback”, “parallel connections”, and “linked targets”. The “canonical feedback” motif corresponds to cases in which CX output neurons make extra-CX synapses onto other CX neurons, which, in turn, project back to a core CX structure in which they contact the original output neurons. For example, PFL1 neurons provide convergent input to FB2B_b neurons, which then feed back onto PFL1 neurons in the FB (**Figure 58B**). Similar feedback motifs have frequently been found to be involved in inhibitory gain control and (Womelsdorf et al., 2014). However, particularly in a structure associated with motor control, such a motif could also enable a broad class of neurons, including all the columnar neurons, to be notified of an upcoming CX-initiated action (see Discussion). The second motif involves “parallel connections” from one type onto another in multiple structures. For example, neurons of the tangential type FB6T synapse onto neurons from the ipsilateral FB6E type in both the SMP/SIP and the FB (**Figure 58C**). The function of such a motif is not clear, but it is likely to depend on the extent to which electrical activity in these neurons is compartmentalized. The third and final motif involves neurons projecting to “linked targets”. These recurrent subnetworks are composed of multiple neuron types that connect to each other. A prominent example of such a case is the FB8F_a neuron type that projects to a set of FB6 neurons interconnected in the FB (**Figure 58D**).

Neurons that participate in any one of three motifs are usually also part of a larger network (**Figure 58B,C,D** right panel; note, however, that in **Figure 58D**, the FB8F-fed subnetwork is the main output of that neuron type). **Figure 58E** summarizes the prevalence of the three different motifs for all CX output neurons. EPG, PEG and ExR3 neuron types, for example, participate in all three of these different motifs, while others, such as the PFL2, FR1 and FB6A neuron types, participate in only one of these motifs. Finally, some types, such as the FS4C neurons, do not participate in any (**Figure 58E**). Overall, although the connectivity of CX neurons outside of the CX is not nearly as structured as the connectivity within its core structures, the reoccurrence of

a small set of motifs, together with the segregation of EB and FB pathways, may point to as yet unknown common functional roles. For example, the EB's involvement in a ring attractor may explain its high degree of recurrence and the presence of a large number of these motifs.

CX projections to brain areas outside the CX

We now turned our attention to pathways that leave the CX and send their information on to other brain regions. These pathways likely enable the CX to influence the fly's behavior. For example, during sleep, the CX is thought to trigger the consolidation of courtship memories by driving dopaminergic neurons that project to the MB (Dag et al., 2019). The CX also influences the fly's wakefulness and activity levels based on internal states, such as sleep (Liang et al., 2019) and the need for certain nutrients (Sareen et al., 2020). This influence may be achieved not only by directly activating motor pathways, but also by modulating the gain and tuning of early sensory pathways. To this end, the CX is well known to play an important role in the initiation and direction of movement (Bender et al., 2010; Guo and Ritzmann, 2013; Harley and Ritzmann, 2010; Krause et al., 2019; Martin et al., 2015; Poeck et al., 2008; Rayshubskiy et al., 2020; Strauss, 2002; Strauss and Heisenberg, 1993; Triphan et al., 2010; Triphan et al., 2012).

Different CX neurons make vastly different contributions to downstream networks (**Figure 59A**), reflecting their unequal number of synapses (**Figure 54C**), spread (**Figure 55E**) and contribution to CX-to-CX loops (**Figure 57A, Figure 57—figure supplement 1A**). The PFL, PFR_b, and FS4 neurons are the strongest contributors to pathways external to the CX, followed by ExR8, FR and FS1-3 neurons. FC neurons, ExR7, ExR3 and a handful of FB tangential neurons make weak contributions. All other CX output neurons that we considered are purely involved in CX-to-CX loops. Our efforts to infer the role of networks that are downstream of the CX were limited by our inability to identify neurons with projections outside the hemibrain volume, and also by the limited characterization of neurons in less structured brain regions labeled 'terra incognita' (Panser et al., 2016). Indeed, for all CX output types, most of the downstream targets are labeled as 'unidentified', meaning that they had no assigned type or had a terra incognita neuropil type attached to them (**Figure 59A**).

The majority of neurons in networks that are external to the CX receive only a very small contribution from the CX (**Figure 59B**, note log scale, without which the handful of neuron types receiving strong CX contributions would be invisible; see Materials and Methods for details). We therefore focused on analyzing only those neuron types that receive more than 0.5% of their inputs from pathways originating in the CX. Simply plotting the weights of pathways from CX neurons onto strong downstream targets (see Materials and Methods) and clustering them by their input revealed that these targets are largely segregated (**Figure 59C**). That is, the majority of external targets receive significant input from only one CX type. Surprisingly, this segregation of target networks is even maintained several synapses downstream (**Figure 59—figure supplement 1 and 2**). Thus, as was suggested in our earlier analysis of all CX neuron types that leave the region (**Figure 55**), it is likely that CX output channels differ in the information that they carry, with each channel serving the distinct functional needs of its downstream circuits. For columnar neuron outputs, this segregation raises the intriguing

possibility that different columnar output neuron types may control distinct sets of behaviors mediated by different actuators (see Discussion).

This segregation is less evident when mapping brain regions reached by different CX neurons (**Figure 59D**), even if each type targets a unique set of regions. Many different FS neuron types, for example, contact distinct downstream circuits in the SMP and SIP, and different PFL neuron types largely project to type-specific targets in the LAL. The finer-scale segregation by downstream target type is only partially visible as sub-clusters within brain regions (**Figure 59E** provides an illustration of such clustering by CX neuron type of origin at a hemibrain-wide scale, see **Figure 59—figure supplement 3-5** for a breakdown by type of origin). The picture that emerges is of every CX output neuron type targeting a relatively strongly interconnected subnetwork only weakly linked to the target subnetworks of other CX output neurons (**Figure 59—figure supplement 2**). Note that because of the weak links between subnetworks, virtually any neuron in the downstream pathways is reachable by most CX neurons, albeit through very weak pathways in most cases.

Known neuron types reached by CX output pathways

Although the majority of pathways that exit the CX lead to neurons whose function is as yet unknown, we were able to identify some known types that receive input from these pathways (**Figure 60A,B**). These types include neuromodulatory or peptidergic neurons, MBONs, visual projection neurons (vPNs), and descending neurons (DNs), among others. Direct connections between CX outputs and known types tended to be weak, and indirect connections were often channeled through larger networks, leading to low total pathway weights. Of note, MBONs receive input from FR, FS, PFR_b, and FB8F_a pathways; vPNs receive input from ExR8 and PFL3 pathways; and DNs receive input from PFL2 or PFL3 pathways (**Figure 60C**). We discuss these pathways in more detail below.

CRE and SMP connections to MBONs and DANs

As discussed in an earlier section, the MB is a highly conserved center for associative learning and memory. These functions are, in part, mediated by interactions between MBONs and DANs. In the previous section, we focused on direct and indirect inputs from MBONs to the CX. We now turn our attention to information flow in the reverse direction. Many of these connections are also discussed in (Li et al., 2020). The functional role for these interactions is still unclear, although some of them may enable the CX to trigger the consolidation of courtship memories by driving dopaminergic neurons that project to the MB (Dag et al., 2019). Other connections likely also play a role in the learned behaviors that the MBONs are thought to drive (Aso et al., 2014b).

The FR, FS1, FS2 and PFR_b neuron types, and one type of FB tangential neuron (FB8F_a), all send outputs to a weakly connected subnetwork of PPL DAN and MBON neuron types (**Figure 61A**). The central part of this subnetwork is constituted by the outputs of FR1, FR2, FS1A and FS1B neurons. These CX output neurons reach two “atypical MBONs”, which have part of their dendritic arbors outside of the MB (Li et al., 2020): MBON30 —a direct FR1 target— and MBON27 —a direct target of FS1B. Interestingly, the MBONs in this subnetwork are not among

the prominent MBON inputs to the CX (**Figure 46**, **Figure 47**). It is unclear why MBON27, which is known to receive inputs from visual KCs in the MB and projects to DNs in the LAL (Li et al., 2020), is specifically targeted by the CX. The CX output neurons also reach 3 PPL DANs: PPL102, PPL103 and PPL107. These PPLs DANS are also linked by a set of CRE and SMP interneurons (**Figure 61B-E**).

We consider three additional subnetworks to also be a part of the central CRE/SNP network. The PPL104 DANs, which connect to each other, are a prominent direct target of FS2 neurons. The FS2 neurons also connect to the rest of the circuit via SMP interneurons. The PPL105 DANs receive input from the FB8F_a neurons and drive MBONs that, in turn, drive the FB8F_a neurons in a closed loop, which we discuss further in the sleep section. Finally, PFR_b neurons reach MBON20 neurons through the LAL002 neuron (**Figure 61B,** see figure legend for a more detailed description of the network). MBON20 neurons are themselves strongly linked to two DNs, DNp42 and DNb05. DNb05 is required for innate aversive olfactory behavior (Huoviala et al., 2020). The large inhibitory oviposition interneuron oviIN is also adjacent to this MBON subnetwork (**Figure 61B** and **Figure 61—figure supplement 1**) (Wang et al., 2020). oviIN is targeted by FC2B and FS1A neuron types, the latter bilaterally. oviIN, in turn, feeds back onto FC2B neurons. It also provides input to MBON27 (which is also targeted by FS1B and, indirectly, FS1A neurons). The CX thus directly and indirectly connects to a number of MBONs.

<u>Projections to, and shared targets with, vPNs</u>

Few of the outputs from visual areas to the central brain—many of which are visual projection neurons (vPNs) (Mu et al., 2012; Panser et al., 2016; Wu et al., 2016)— reach the CX. Instead, the vPNs likely reach direct pathways to the motor system (Namiki et al., 2018). However, we found intriguing links from CX outputs to a few vPNs, suggesting that the indirect CX pathway may exert some influence on more direct visuomotor pathways.

Four types of CX output neurons (PFL1, PFL3, PFR_b and ExR8 neurons) interact with visual pathways (**Figure 62A**, although the MBON20 and mALD1 neurons, both of which receive inputs from the PFR_b pathway (see earlier section), do contact a set of vPNs, see **Figure 62—figure supplement 1**). The PFL3 neurons contact two lobula columnar (LC) neurons, LC33 and LC10 in what is thought to be their output region. LC33, a type newly described in the hemibrain dataset (Scheffer et al., 2020), is an atypical LC neuron that send outputs to the LAL in a region overlapping with PFL3 outputs (**Figure 62B,C**). Out of the 15 neurons present in the dataset, only 4 receive synapses from PFL3, and only 3 do so consistently (**Figure 62—figure supplement 2**). This might suggest the existence of subtypes within LC33. As a population, the LC33 neuron type innervates the most medial part of the lobula, and the arbors of the neurons contacted by PFL3 are situated in the center along the dorso-ventral axis. LC33 neurons also share a number of targets with PFL3, but not reliably. Even when we restricted our analysis to the LC33 neurons targeted by PFL3 neurons, each LC33 neuron appears to have a distinct output profile that only partially overlaps with the PFL3 output profile. Their function is as yet unknown (**Figure 62—figure supplement 2**).

PFL3 neurons also contact LC10 neurons in a similarly selective fashion, although more indirectly. These LC10 neurons interact with two AOTU-LAL neurons (AOTU042 and AOTU019), which themselves are major target of PFL3 neurons (**Figure 62D,E**). The interaction of the LC10 neurons with AOTU042, whose LAL innervation is on the contralateral side, is bidirectional. Consistent with the AOTU042 innervation pattern in the AOTU, the connections from these neurons onto LC10 neurons excludes 35 (out of 449) LC10 neurons that innervate the most dorsal part of the AOTU. More generally, the connection strength seems to follow a gradient along the dorso-ventral axis in the AOTU, with LC10 neurons that have the most ventral innervation receiving the strongest input from AOTU042. In contrast, AOTU019 innervates a more dorsal part of the AOTU (see anatomy in **Figure 63C**). For LC10 neurons, the dorso-ventral axis in the AOTU corresponds to the antero-posterior axis in the lobula, which is likely to correspond to functional subdivisions (Wu et al., 2016). LC10s are thought to be involved in small object tracking. The interplay between the LC10 and PFL3 downstream networks could allow small object tracking responses to be modulated by more general orientation behaviors. By contrast with LC33, however, the only targets that LC10 and PFL3 neurons share are the above-mentioned two AOTU neurons.

PFL1 neurons link to a network with LC27 neurons, another newly discovered LC neuron type (Scheffer et al., 2020). The connection is made through LAL-PLP neurons and PLP interneurons that selectively innervate the glomerular region in the PLP targeted by LC27 (**Figure 62F and G**). The function of LC27 is unknown. Given the predominance of the PLP and PVLP in PFL1 outputs (**Figure 59 Di**), and the fact that the optic glomeruli are positioned at the PVLP and PVLP/PLP boundary, it is possible that this motif is repeated for other LC neurons that are not presently identified or are only partially traced in the volume. This is also the only strong link we could discern between PFL1 neurons and an identified neuron type outside the CX. In short, the PFL1 and PFL3 neuron types each seem to regulate the output of a distinct set of LC neurons, potentially regulating more hardwired visual to motor responses (see Discussion).

Until now, we have focused on output neurons with their primary inputs in the PB and FB. However, a single output neuron type from the EB also contacts visual pathways, and, specifically, “centrifugal” visual neurons: the dorsal centrifugal horizontal (DCH) and ventral centrifugal horizontal (VCH) neurons. CH neurons, whose response properties have been characterized in the blowfly (Hausen, 1976), are part of the horizontal-motion-sensing network of the lobula, where they are both pre- and postsynaptic. However, these neurons receive the majority of their inputs in the central brain, and this is also where they get their CX input. In blowflies, the VCH and DCH neurons are both non-spiking and inhibitory, and they confer their motion sensitivity to at least one of their downstream targets, the small-object-motion-sensitive FD1 neuron (Egelhaaf et al., 1993). All these neurons respond to motion both in the ipsilateral (front-to-back) and contralateral (back-to-front) direction. The FD1 neuron, in particular, has been hypothesized to suppress motion responses during saccades (Hennig et al., 2011). Its central input has not been characterized.

The ExR8 neurons link the EB to these CH neurons. Specifically, the ExR8 neurons make direct and indirect connections (through PS047 neurons) to the DCH and VCH neurons (**Figure 62H,I**).

Unusually for an ExR neuron type, ExR8 neurons, which have not yet been functionally characterized, contribute virtually all of their outputs to "true" outputs pathways that leave the CX (**Figure 57A**). They likely constitute the only strong and near-direct connection to motor pathways from the EB. The ExR8 neuron type receives input in a compartment of the NO (NO1) from PEN_b neurons on one side (**Figure 25B**), suggesting that it could relay information about angular velocity from the fly's movements to circuits that detect visual motion.

CX outputs to the motor system

As mentioned above, the CX is known to play a role in determining the fly's movements. Movements are controlled through DNs, which carry motor commands from the central brain to the ventral nerve cord (VNC) (Hsu and Bhandawat, 2016; Namiki et al., 2018). Light microscopy has enabled the identification of a few LAL-projecting CX neuron types that may link the CX to DNs (Hanesch et al., 1989; Heinze and Homberg, 2009; Lin et al., 2013; Wolff et al., 2015). A complete connectome should, in principle, allow us to identify all CX to DN neural pathways. However, the limited volume of the hemibrain has permitted only a small fraction of these neurons to be identified (Scheffer et al., 2020). We used the identified DNs to focus our analysis of CX output neurons, while noting that a larger number of CX output neurons that project to terra incognita and to as-yet-unassigned neural segments might well make contributions to DNs that were difficult to identify in the hemibrain volume.

The main source of CX input to DNs comes from PFL2 and PFL3 neurons, with additional smaller contributions from ExR7, ExR8 and FR2 neurons (**Figure 63A**). Multiple DN types receive strong PFL2/PFL3 inputs. Most prominent among these are DNa02, DNa03, DNa04 and DNb01 neuron types, as well as a putative DNg neuron (DN?_5813078378). DNa02 is known to be involved in turning in walking flies (Rayshubskiy et al., 2020). DNa02, DNa03, DNa04 and DNb01 share inputs to varying degrees, suggesting that they could be part of an interacting premotor network. This network is symmetrically influenced by PFL2 neurons and asymmetrically influenced by PFL3 neurons. These connectivity patterns are consistent with the possibility that PFL2 neurons control forward walking, and that PFL3 neurons control turns by differentially influencing a similar network. In such a scheme, turns in walking flies would be controlled by asymmetric modifications of the fly's gait (DeAngelis et al., 2019; Strauss and Heisenberg, 1990). No other CX output neurons appear to be in a position to execute such a function (also see Discussion). It is interesting to note that the DN neurons in question innervate a variety of leg and wing neuropils in the VNC (Namiki et al., 2018), suggesting that control of different actuators is somewhat integrated or coordinated in the LAL. DNg and DNa01 are in circuits targeted only by the PFL3 neurons and not PFL2 neurons. PFL2 and PFL3 neurons also reach the moonwalker neuron MDN, which has a bilateral innervation pattern. This connection is almost exclusively contralateral (**Figure 63—figure supplement 1A,B**).

PFL2 and PFL3 connections to their downstream DN targets are both direct and indirect, through a LAL interneuron network (**Figure 63B-E**). PFL2 neurons project to both sides of the LAL, where they connect to DNa03 neurons on either side of the brain. Interestingly, PFL3 neurons tend to reach contralateral DNs through strong connections to LAL-LAL interneurons like the LAL212 and AOTU019 (the same neurons that interact with LC10 in the AOTU), whereas

PFL2 contribute mostly to ipsilateral networks. Whether these intermediate midline crossing LAL neurons could be part of a flip-flop circuit through inhibition (Iwano et al., 2010) is, as yet, unknown.

ExR7 and ExR8 also indirectly reach some DNs in this network (**Figure 63B**). Additionally, we observed that ExR8 neurons target, through posterior slope neurons (**Figure 63—figure supplement 1 C-E**), a different set of DNp descending neurons. These DNp neurons are thought to innervate neck and haltere neuropils. Finally, FR2 reaches DNp32 through a SMP interneuron (**Figure 63—figure supplement 1 F**). It is likely that a lot of similar connections in the dorso-posterior part of the brain are lost as a result of the limitations of the hemibrain dataset.

Asymmetries in the distribution of columnar outputs

Differences in the number of synapse counts from PFL2 and PFL3 neurons that arborize in different PB glomeruli (and, therefore, different FB columns) are likely to contribute to asymmetries in their activity profiles. Specifically, EPG bumps in PB glomeruli L3 or R3 would likely induce stronger activation of their downstream targets (**Figure 64** shows that the outputs of the left PFL3 neurons are strongest for the individuals that get input in PB glomerulus L3. By symmetry, R3 would similarly be the site of strongest output on the right side). Since L3 and R3 are 180 degrees apart (when the bump is in L3, it is also in R7 and vice versa), if the EPG bump of activity was in either glomerulus and passed to the PFL3 neurons, the resulting activity would create asymmetries in PFL3 output, likely generating a turn (see Discussion section on goal-directed navigation). A similar interpretation of somewhat different asymmetries in PFL3 neuron synapse counts observed across the hemibrain and FAFB volumes has been independently proposed by (Rayshubskiy et al., 2020). The FS4A and B neuron types also show an interesting asymmetry (**Figure 65A**). FS4A prominently targets neurons projecting to the FLA (**Figure 65B**), suggesting that they may be involved in directing feeding behaviors.

Known unknowns downstream of CX output pathways

As discussed earlier, many of the neurons and circuits that receive projections from CX output neurons have never been investigated experimentally. Beyond brain regions that are directly downstream of CX output neurons (for example, the LAL for PFL neuron types, the CRE for FC, FR and PFR neuron types, the SMP/SIP for the FS neuron types), a few other, more distant neuropils reappear regularly in these downstream circuits. The IB and ATL are targeted by prominent neurons in the PFL3, FS3, FC2C, and PFR_b downstream networks. The lateral part of the PLP is part of the PFL1, FC1E, and FR1 networks. As mentioned before, LC neuron output circuits likely also innervate some parts of the lateral PLP (Scheffer et al., 2020). The ExR8 and a FS1B networks reach ventral brain regions (SPS/IPS down to the GNG). Deciphering the organization and function of those areas will be necessary to obtain a complete picture of the many ways in which the CX likely shapes the fly's behavior.

Overall, our analysis of the 'true' outputs of the CX reveals that EPG-bump-fed and FB-modulated PFL2 and PFL3 neuron types directly and indirectly contact DNs, likely enabling flexible, context- and goal-dependent control of the fly's orientation and locomotion. The more dorsal output networks of the FB, which act through FC, FS, FR, PFR_b neurons, are likely

involved in modulating and directing innate behaviors that depend on the animal being correctly oriented in its surroundings, like oviposition or feeding. A prominent feature of nearly every layer of these output circuits is strong feedback to the CX, suggesting that all information related to the fly's intended actions is relayed back to the core CX structures. All these output circuits are also densely recurrent, allowing for exquisite fine-tuning and coordination during action selection. Finally, there are indications of interactions between CX-related output circuits and more direct visual output pathways, suggesting that these different sensorimotor pathways may differentially modulate each other during specific behavioral contexts. More broadly, while it is important to keep in mind that many of the CX's output pathways cannot be resolved in the hemibrain's volume, it is already clear that information from the CX likely influences a wide variety of targets across the brain, from neurons that arborize in sensory systems to DANs and DNs.

DISCUSSION

Recent physiological and anatomical studies have highlighted strong links between circuit structure —even at the level of topography— and function in the adult fly central brain (Green and Maimon, 2018; Turner-Evans et al., 2020), something that has proven to be valuable both for generating hypotheses and for experimentally testing them (Green et al., 2017; Kim et al., 2017b; Turner-Evans et al., 2017; Turner-Evans et al., 2020). This recent history gives us reason to expect that connectomic studies (Eichler et al., 2017; Ohyama et al., 2015; White et al., 1986; Zheng et al., 2018) will significantly accelerate studies of circuit function (Bentley et al., 2016; Deutsch et al., 2020; Eschbach and Zlatic, 2020; Gordus et al., 2015; Jovanic et al., 2016; Ohyama et al., 2015; Schretter et al., 2020; Tastekin et al., 2018; Turner-Evans et al., 2020). CX circuits, in particular, are thought to be involved a wide variety of flexible, context-dependent behaviors (Honkanen et al., 2019; Turner-Evans and Jayaraman, 2016). In the Results sections of this manuscript, we have tried to provide a detailed description of CX neuron types and circuits, with a particular focus on extracting and analyzing network motifs from the perspective of what we currently know about CX circuit function and CX-mediated behavior. We found many repeating motifs, suggesting that an understanding of the computational roles of some of these may generalize to others. Some of these recurrent motifs match those that have been proposed previously to implement ring attractors for head direction (Hulse and Jayaraman, 2019; Turner-Evans et al., 2020). Others seem suitable for gain control in multiple structures. And still others seem to be ideal for vector computations that would be required for robust navigational behaviors. We found that information from the CX's output neurons is broadcast widely, not just to premotor centers but to sensory regions and, importantly, back into the CX itself. In the sections that follow, we discuss some functional implications of these motifs and of other results of our analyses. We derived these functional implications not just from our connectomic analyses and the historical precedent of structure predicting function in many different neural circuits, but also on published physiological and behavioral studies. Testing the hypotheses that we outline below will require a long series of functional experiments, but the connectome provides an invaluable guide for the design and prioritization of such experiments.

EM circuit reconstruction: how complete is complete enough?

The value of EM-level connectomes in understanding the function of neural circuits in small and large brains is widely appreciated (Abbott et al., 2020; Litwin-Kumar and Turaga, 2019; Schlegel et al., 2017). Although recent technical advances have made it possible to acquire increasingly large EM volumes (Scheffer et al., 2020; Zheng et al., 2018) and improvements in machine learning have enabled high-throughput reconstruction of increasingly large neural circuits (Dorkenwald et al., 2020; Januszewski et al., 2018), the step from acquiring a volume to obtaining a complete connectome still requires considerable human proofreading and tracing effort (Scheffer et al., 2020). As part of our analysis of the CX connectome, we found that although increased proofreading led to an expected increase in the number of synaptic connections between neurons, it did not necessarily lead to significant changes in the relative weight of connections between different neuron types (**Figures 3-5**). While it is important to note that we made comparisons between the hemibrain connectome at fairly advanced stages of proofreading in the CX, our results do suggest that it may be possible to obtain an accurate picture of neural circuit connectivity from incomplete reconstructions. It may be useful for future large scale connectomics efforts to incorporate similar validation steps of smaller sample volumes into reconstruction pipelines to determine appropriate trade-offs between accuracy and cost of proofreading.

Connectivity and neural processing beyond the typical synapse

Although we provide a detailed description of the CX's hundreds of neuron types, recurrent networks and pathways, there is still more information that could be extracted from the CX connectome. The CX is innervated by a large number of modulatory and peptidergic neurons (Kahsai et al., 2012; Kahsai et al., 2010; Kahsai and Winther, 2011), many unidentified and almost all of unknown function. These neurons likely significantly modulate the function of recurrent networks in ways that few studies address (Bargmann and Marder, 2013). Knowing their identities would help guide circuit studies into context- and internal-state-dependent processing in the CX.

A large number of CX neuron types that make t-bar and e-bar synapses (Shaw and Meinertzhagen, 1986; Takemura et al., 2017) in CX structures also send projections to other structures in which they make no such synaptic connections. We investigated these projections in more detail and consistently found dense core vesicles (Burgoyne and Morgan, 2003; Hammarlund et al., 2008; Nassel and Winther, 2010) in these otherwise nearly synapse-free processes (**Figure 56—figure supplement 1B,C**). Although the involvement of some of these neuron types, such as the PFGs, in sleep-wake circuits suggests a plausible scenario for state-dependent modulation of CX circuits, such explanations are not easily available in all cases.

As previously discussed, the hemibrain connectome also does not capture glial networks or gap junctions. Despite all these limitations, the identification of chemical synapses between CX neurons allowed us to extract network motifs that make strong predictions about function. We discuss these insights in the following sections.

What the CX's network motifs tell us about its navigational computations

Many flexible, goal-driven behaviors unfold over longer durations than fast reflexive responses and are robust to the temporary loss of sensory cues directly associated with the goal. Desert ants, for example, use path integration to return to their nests after long foraging trips in relatively featureless landscapes (Wehner, 2020), and mammals use working memory to perform delayed match-to-sample tasks (Romo et al., 1999). For such behaviors, brains are believed to rely on intermediate representations and neural dynamics that persist or update even in the absence of direct sensory inputs. Such persistent representations have long been believed to be generated and maintained by recurrent attractor networks (Brody et al., 2003; Durstewitz et al., 2000). These more abstract intermediate representations also enable disparate sensory and self-motion cues of different modalities —for example, polarized light, visual optic flow and proprioceptive cues in the path integrating ant (but see (Pfeffer and Wittlinger, 2016))— to be registered to a shared reference frame. To enable an animal to learn from experience, any past associations of the current context with good or bad outcomes must be recalled and used to modify neural dynamics in these intermediate reference frames, raising computational questions that have been explored in the field of reinforcement learning (Sutton and Barto, 2018). Ultimately, information in this reference frame must still be dynamically converted to a body-centered reference frame for situation-appropriate action. Decades of experimental work in a variety of species have led theorists to propose gain field networks for the implementation of such coordinate transformations (Andersen et al., 1993; Pouget and Sejnowski, 1997; Pouget and Snyder, 2000; Salinas and Abbott, 2001; Zipser and Andersen, 1988), but the predicted neural circuit connectivity has not been directly identified. The repertoire of flexible navigational behaviors that flies display suggest that their small brains successfully solve many of these computational challenges, and, further, that fly circuits may have evolved solutions to these problems that resemble those proposed by theorists to account for neural response properties in mammalian circuits (Hulse and Jayaraman, 2019).

Flies use short-term memory to orient towards the last-known positions of attractive visual beacons that have disappeared (Neuser et al., 2008). They learn about their body size and use that information when attempting to cross gaps (Krause et al., 2019). They learn to avoid heat punishment by using visual patterns around them to orient to safety (Liu et al., 2006). Although they are not central place foragers like bees and ants, they are capable of returning to a spot of food even when exploring their surroundings in darkness (Brockmann et al., 2018; Corfas et al., 2019; Kim and Dickinson, 2017), and of remembering visual landmarks to navigate to safe spots in an otherwise hostile open space (Ofstad et al., 2011). The CX is thought to be essential for many of these behaviors. In the sections that follow, we will discuss how the patterns of connectivity revealed by the CX connectome may enable the neural dynamics, coordinate transformations, and learning-induced changes in action selection associated with meeting the computational challenges of some of these behaviors.

<u>Sensory cues to obtain a stable allothetic representation of head direction</u>

Head direction representations enable an animal to flexibly rely on a variety of different cues, including self-motion, to navigate. Work in *Drosophila* and other insect species has established

that the CX generates a stable head direction representation from directional sensory cues carried by ring neurons, such as polarized light, visual landmarks, and wind direction (**Figure 66A,B, Table 4**) (Heinze and Homberg, 2007; Homberg et al., 2011; Okubo et al., 2020; Seelig and Jayaraman, 2015; Varga and Ritzmann, 2016). In *Drosophila* visual head direction information reaches the CX via the anterior visual pathway, which appears to convey different visual information in separate, parallel "channels" (**Figure 6-8**) (Omoto et al., 2017; Seelig and Jayaraman, 2013; Shiozaki and Kazama, 2017; Sun et al., 2017; Timaeus et al., 2020). Some "channels" of this pathway have been characterized functionally, while the function and sensory tuning of other groups of neurons remains elusive. For example, most of the ring neurons (and their inputs) in the superior BU are spatiotemporally tuned to bright visual features with some degree of orientation preference (Seelig and Jayaraman, 2013; Sun et al., 2017) and the pathway through the anterior BU appears to be dedicated to polarization signals (Hardcastle et al., 2020) (see below). In contrast, little is known about the role of ring neurons in the inferior BU neurons in informing the head direction representation (Omoto et al., 2017; Shiozaki and Kazama, 2017). Wind stimuli reach the head direction circuitry through separate input pathway via the LAL (Okubo et al., 2020) and it unknown whether other sensory modalities are conveyed through this route.

Our connectome-based analysis suggests that there are 22 ring neuron types, 18 of which receive inputs via the anterior visual pathway. In contrast, an anatomical and developmental characterization of ring neurons found only 11 distinct types (Omoto et al., 2018). Given that past neurophysiological studies have only tested tuning to a relatively small number of sensory stimuli, it remains to be seen how many functionally distinct input types exist.

The connectome reveals mechanisms by which these sensory pathways are integrated and suggests that different cues exert differing levels of influence on the EPG neurons that carry the head direction representation. An implicit prioritization of certain sensory cues is reflected in the relative locations of synaptic input from different sensory streams onto the EPG dendrites in the EB (**Figure 12**), in the relative weight of those inputs (**Figure 11A**), in the feedback that some ring neuron pathways receive from the EPG neurons, and in the relative weight of across-type inhibition from some ring neuron types onto others (**Figure 13A,C**). The implicit hierarchy of ring neuron inputs to the fly compass indicates that the EPG head direction representation preferentially tethers to environmental references that are likely to indicate a global direction. Bright visual landmarks, for example, may originate from celestial bodies such as the sun, but they could also be generated by local terrestrial objects (for example, open spaces in a forest canopy). In contrast, the polarization pattern, if available, represents a reliable global reference, which might explain the observed circuit motifs that suggest preferential use of polarization cues to update the fly's head direction representation (**Figure 66B**).

Particularly when navigating over long distances, skylight cues allow the head direction representation to be tethered to global landmarks such as the sun, and to the polarized light patterns of the sky (Heinze and Reppert, 2011). Indeed, polarized light e-vector information has long been thought to be important for the determination of sky-compass-based head direction in many insects, and that is the case in the fly as well (Hardcastle et al., 2020; Mathejczyk and

Wernet, 2019; Warren et al., 2018; Weir and Dickinson, 2012; Wernet et al., 2012), despite their comparatively small dorsal rim area (Fortini and Rubin, 1991; Wada, 1974; Wernet et al., 2003)—a dorsal band of the insect eye that is structurally specialized for the detection of polarized light e-vectors in the sky (Labhart, 1999). Sensory information about the celestial polarization pattern reaches the Drosophila CX via a dedicated pathway ((Hardcastle et al., 2020; Weir et al., 2016), **Figures 6-8**); although only 5 ER4m neurons from each hemisphere showing strong tuning to e-vector orientation, this tuning collectively covers a large part of the 180 degree range of possible e-vector orientations (Hardcastle et al., 2020; Weir et al., 2016). However, in contrast to the position of the sun, the 180-degree symmetric polarized light patterns do not immediately provide the ability to distinguish a specific direction from one directly opposite to it. The CX connectome suggests that the fly's compass may have evolved a solution to this problem. For the polarization-tuned ER4m neurons, we observed that synapse numbers to EPG neurons varied smoothly along the circumference of the EB, but with mirror symmetric profiles for ER4m neurons from the left and right hemisphere, respectively, resulting in stronger connections from the left hemisphere for EB wedges on the right half of the EB and stronger connections from the right hemisphere to the left half of the EB (**Figure 11D, Figure 67A**). This structure was even more clearly revealed when we analyzed the pairwise correlation of EPG neurons according to their ER4m inputs (**Figure 11—figure supplement1, Figure67B**): all EPG neurons on the right side of the EB were positively correlated with each other, while being anticorrelated with those on the left side, and the inverse pattern was observed for the left EPG population. Given the mirror symmetry of the represented direction of polarization(Hardcastle et al., 2020; Weir et al., 2016), this connectivity pattern may allow the fly to generate a complete, 360-degree head direction representation from polarized light input (**Figure 67B**). One possible mechanism by which this could be achieved hinges on the geometry of the fly's polarization sensors in the dorsal rim area and how it interacts with the natural polarization pattern of the sky. The receptive fields of the fly's polarization sensors in the left and right eye face the contralateral celestial hemisphere and tile a small strip along the rostral-caudal axis of the fly (**Figure 67C,D)**(Heinze, 2014). Along this strip tuning to e-vectors varies continuously and covers nearly the full 180-degree range of possible e-vector orientations. Given the naturalistic celestial polarization pattern schematized in (**Figure 67Ci, Di**), the geometry of the slightly curved receptive field "strip" might act as a rough 'matched filter', such that neurons in the dorsal rim area on the side of the sun (facing the contralateral sky) are systematically more strongly activated than those on the side facing away from the sun. The all-to-all inhibition between left and right ring neurons in the EB (**Figure 14A**) may then systematically select either the left or the right ring neurons to tether the head direction depending on which direction the fly is facing relative to the current position of the sun, thus disambiguating the 180-degree mirror symmetry in the polarization signal. In locusts, TL-neurons, homologs of the fly's ring neurons, and PB neurons have been shown to exhibit matched-filter like tuning to the full-sky polarization patterns generated by the sun (Bech et al., 2014; Zittrell et al., 2020). A corollary of these studies is that individual TL neurons in the locust have receptive fields that span large parts of the sky. Indeed, the area of the sky that is sampled by photoreceptors in the dorsal rim area is significantly larger in locusts compared to flies (Heinze, 2014), and it is plausible that further sensory processing along the anterior visual pathway toward the CX differs between

species as well, in which case different insects might employ different strategies for disambiguating polarized light stimuli.

The mechanism described above would not require that the sun be directly visible, but it might still be beneficial to have ring neurons that have multimodal tuning to both polarized light and sun-like stimuli. Such cells have been described in other insects (el Jundi et al., 2015; Pegel et al., 2018; Pfeiffer et al., 2005). While this has yet to be demonstrated experimentally, multimodal ring neurons tuned to both visual features and polarized light e-vector orientation may also exist in flies. Hardcastle and colleagues report polarization-tuned neurons in the superior bulb (Hardcastle et al., 2020), where tuning to bright features has also been observed (Omoto et al., 2017; Seelig and Jayaraman, 2013; Sun et al., 2017).

Besides visual cues, mechanosensory wind stimuli can drive the fly's head direction system in the EB (Okubo et al., 2020) (**Figure 9**). Information about wind direction reaches the EB via ring neurons that arborize in the LAL. Wind tuning has been demonstrated in both ER3a and ER1 neurons, although only ER1 neurons were able to update the head direction estimate (Okubo et al., 2020). Analysis of the connectome suggests that both the ER1 and ER3a neuron populations consist of multiple types with distinct inputs. We found that only ER1_b and ER3a_b neurons got strong inputs from cells that we believe to be the wind-sensitive WLL and WPN neurons (**Figure 9**) (Okubo et al., 2020; Suver et al., 2019). The connectivity of these two ring neuron types onto EPG neurons, with strong connections from ER1_b but no connections from ER3a_b neurons, is consistent with the observation that ER1 but not ER3a neurons can drive the head direction representation (**Figure 11A**). It is also noteworthy that in the EB, ER1_b neurons deviate from the within-type all-to-all inhibition motif that all other ring neurons show in the EB (**Figure 13A, Figure 66B**). A possible reason is that an accurate mapping from ER1 neuron activity to a head direction representation requires pooling information from multiple ring neurons at once (Okubo et al., 2020). Although our analysis also suggests that ER1_b input to the EPG neurons is suppressed by ER1_a neurons, it is presently unknown whether ER1_a neurons also encode wind direction or whether these neurons are tuned to a different stimulus (**Figure 66B**). However, ER1_a and ER1_b inputs in the LAL are distinct and unfortunately little is known about the inputs ER1_a receives.

Whether over short or long distances, olfactory cues are strong indicators of good food sources. Flies are known to fly upwind when they encounter an appetitive odor (Budick and Dickinson, 2006). Based on the proximity of different ring neuron inputs to the likely spike initiation sites of EPG neurons, wind direction carried by ER1_b neurons (and perhaps also ER1_a neurons, although their function is currently unknown) may be another cue that strongly tethers the head direction representation (Okubo et al., 2020), and likely guides their behavior. The relative importance of synapse location in determining the cues to which the EPG compass tethers will only be clear with in-depth investigations of EPG neuron biophysics.

More broadly, future studies of ring neuron and EPG interactions insights should provide an implementation-level understanding of a variety of computations related to dynamic

multisensory integration (Pouget et al., 2002) and the resolution of conflicts between cues of different reliability (Deneve et al., 2001; Hoinville and Wehner, 2018; Wystrach et al., 2015).

All-to-all inhibition for noise reduction in sensory inputs to the compass network

Recent studies have established an important role for fast-timescale, short-term plasticity in the synaptic weights between ring and EPG neurons in enabling the EPG compass to quickly adapt to different sensory settings (Fisher et al., 2019; Kim et al., 2019). But the ring neuron network may preselect more salient cues for the compass through all-to-all inhibitory connectivity within each type. The precise impact of all-to-all inhibition on the ring neuron network's preprocessing of localizing cues that are used to generate the head direction representation will depend on the timescale of the inhibitory conductance (Ermentrout, 1992), which is as yet unknown. If the inhibitory conductance is fast, all-to-all inhibition would create winner-take-all dynamics in which a few ring neurons receiving the strongest inputs effectively shut down all other ring neurons. In sensory settings characterized by a single dominant sensory cue, such as the sun or polarized light e-vector orientation in a desert landscape during the day (Coyne et al., 1982; Weir and Dickinson, 2012), only a handful of ring neurons with appropriately tuned receptive fields (Fisher et al., 2019; Omoto et al., 2017; Seelig and Jayaraman, 2013; Shiozaki and Kazama, 2017; Sun et al., 2017) would be active within each type for any particular head direction. Fast all-to-all inhibition in this setting would enhance the activity of the most dominant ring neuron within each type and would minimize the impact of noise from the others, which might otherwise disrupt the stability of the EPG compass. However, stable and unique heading representations are also generated within scenes with multiple strong cues, such as within a forest or when walking on the branches of a tree, as long as the two-dimensional arrangement of cues allows for a unique determination of heading (Kim et al., 2019). In the presence of multiple salient cues, we would expect multiple ring neurons to respond with comparable strength for any given heading of the fly and several to respond weakly to clutter in the scene. Although fast all-to-all inhibition in this scene would still filter out the weaker responses, it could allow multiple, strongly responsive ring neurons to remain active for each heading. A slower inhibitory conductance would, in this situation, induce oscillatory spiking dynamics between these multiple 'winners' (Ermentrout, 1992), a situation that been referred to as 'winnerless competition' (Rabinovich et al., 2001), and that has suggested to be useful for sequential memory (Seliger et al., 2003).

Modulation of sensory inputs to the EPG neurons

It is important to note that ring neuron responses are not determined purely by sensory cues. These neurons appear to be modulated by state, maintain a baseline level of activity, and may be biophysically configured to support oscillatory population activity that is revealed during sleep (Raccuglia et al., 2019). The many additional inputs that many ring neuron types receive in the BU (**Figure 8**) provide clues as to how the activity of these neurons might be modulated by the fly's behavior and its internal state. The visually-tuned superior BU ring neurons primarily receive input from a large interhemispheric AOTU neuron (AOTU046), which is likely mediating the dynamic stimulus selection through delayed contralateral inhibition (Sun et al., 2017). The same group of ring neurons also shows changes in activity with the fly's behavioral state (flight vs. walking) (Seelig and Jayaraman, 2013) and indeed many of these neurons

receive input from a dopaminergic ExR neuron that has been linked to changes in the fly's movement pattern (Kong et al., 2010). A different set of ring neurons that receive their inputs in the inferior BU receives strong inputs from two ExR neurons —ExR1 and ExR3— that have been linked to the control of sleep (**Figure 8, Figure 15**) (Donlea et al., 2018; Liu et al., 2019).

A ring attractor network with all the trimmings

The fly's head direction representation tethers to directional sensory cues conveyed by ring neurons, but is also updated by self-motion cues (Green et al., 2017; Green and Maimon, 2018; Hulse and Jayaraman, 2019; Seelig and Jayaraman, 2015; Turner-Evans et al., 2017; Turner-Evans et al., 2020) and is maintained across periods of immobility (Seelig and Jayaraman, 2015). Strong experimental and theoretical evidence suggests that the representation is maintained by a ring attractor network (Kim et al., 2017b), which includes at least some of the recurrently connected columnar neurons that link the EB and the PB —the EPG, PEN_a, PEN_b and PEG neuron types (Green et al., 2017; Turner-Evans et al., 2017; Turner-Evans et al., 2020). The patterns of connectivity between individuals of these types are consistent around the entire EB and across the length of the PB. Similarly, the broad connectivity patterns of individuals within these types to tangential neurons —Δ7 neurons in the PB and different types of ring neurons in the EB— are similar across these structures. Notably, however, two distinct classes of neurons (EPGts and the P6-8P9 neurons, **Figure 18** and **Figure 21**, respectively) only innervate the edges of the network —the outer glomeruli of the PB and the corresponding wedges in the EB. These types may help to stitch together what might otherwise be a discontinuity in the ring attractor network at these positions.

The hemibrain connectome further allowed us to identify several neuron types that are likely involved in the network's function, but whose role awaits experimental investigation. Many of these additional types are tangential neurons. Some of them appear to provide additional sources of inhibition, potentially regulating the overall network activity (Franconville et al., 2018; Turner-Evans et al., 2020). The ER6 neurons, for example, receive input from the EPG and PEG neurons in the Gall and send outputs to the EPG and PEN_b neurons in the EB (**Figure 10, 2, 2—figure supplement 1**), potentially modulating the EPG-to-PEG-to-PEN_b-to-EPG feedback loop. The dopaminergic LPsP neurons (**Figure 23**) may enable changes in synaptic strength in the PB. Such plasticity in the PB has been suggested to allow flies to calibrate their directed movements to their body size (Krause et al., 2019). The PB also receives neuromodulatory input in the form of the octopaminergic P1-9 neurons, which contact the EB-PB columnar neurons (**Figure 23**). In sensory brain regions, octopaminergic neurons are known to modulate neuronal conductances based on the fly's behavioral state (Strother et al., 2018; Suver et al., 2012), and it is an open question whether the P1-9 neurons play a similar role in the PB.

Two additional classes of columnar neurons also contact the ring attractor network. The connectivity pattern of EL neurons in the EB is remarkably similar to that of the EPG neurons (**Figure 11**), but their function is unknown. In the PB, the IbSpsP neurons bring input into specific glomeruli from regions associated with premotor functions, potentially allowing them to exert an influence on the movements of the bump outside the EB.

The fly's head direction system uses 8 computational units

The existence of 8 computational units in the PB and EB network suggests that the fly's head direction system samples angular space at 45-degree intervals (**Figure 68A)** (Hanesch et al., 1989; Lin et al., 2013; Seelig and Jayaraman, 2015; Wolff et al., 2015), consistent with anatomical and physiological work in other insects (Heinze and Homberg, 2007, 2008; Honkanen et al., 2019; Pfeiffer and Homberg, 2014; Strausfeld, 2012; Williams, 1975). Importantly, this does not mean that the system cannot resolve head directions at resolutions below 45 degrees, because the differential activation of different columnar neurons can effectively represent any arbitrary angle within the 360 degrees around the fly. Within the PB, FB columnar neurons inherit a head direction bump directly from EPG neurons and indirectly through Δ7 neurons (**Figure 68B**) (Franconville et al., 2018; Green et al., 2017; Turner-Evans et al., 2017; Turner-Evans et al., 2020). The EPG-to-Δ7 transformation ensures that the bump is cosine shaped (**Figure 20**), a particularly suitable representation for vector-based computations (**Figure 68C**) (Touretzky et al., 1993). PB-FB columnar neurons convey activity bumps from the left and right PB to the FB (Shiozaki et al., 2020). Thus, the major columnar input to the FB network is a cosine-formatted head direction signal sampled at 45-degree intervals using 8 functional and 9 structural columns. Viewing the directional representations in this way has important implications for how the PB-FB phase-shifts of different FB columnar neurons may relate to the circuit's navigational function.

Path integration: an example of a canonical vector-based computation

Path integration is a canonical vector-based navigation strategy used by a diverse array of both flying and walking insects (reviewed in (Collett, 2019; Heinze et al., 2018; Wehner, 2020)), potentially including *Drosophila* (Brockmann et al., 2018; Corfas et al., 2019; Dethier, 1957; Kim and Dickinson, 2017; Murata et al., 2017). In its most basic form, 2D path integration requires that an animal keep track of its direction and distance relative to a stored goal location, such as a food source or nest, often without the use of external landmarks. The direction and distance to the goal location is thought to be computed through the integration of self-motion signals and stored as a 'home vector'. To return to the goal location, animals are thought to read out the home vector by comparing their current heading to the stored home vector in order to generate appropriate motor commands. While many insects are thought to generate and use visual snapshots of their surroundings to guide return trips (Collett et al., 2013; Collett and Zeil, 2018; Freas et al., 2019), and while this may involve the MB (Buehlmann et al., 2020; Collett and Collett, 2018; Kamhi et al., 2020; Sun et al., 2020), we will focus here on how the home vector might be constructed and read out in the CX using only a stable head direction signal. Although there is as yet no definitive evidence that the CX is used for path integration, in the next few subsections, we show how a network built from FB-inspired circuit motifs could compute an allocentric translational velocity vector whose integration would yield a home vector. We note that the framework for vector computations that we describe below is likely to be useful for a much broader array of behaviors involving oriented action selection.

The potential for vector computations in the FB

The one-column contralateral phase shift that is characteristic of all PFN neuron types (**Figure 30A**), which serve as the major columnar input to the FB network, implies that activity bumps from the left and right PB would produce bumps in 90° phase-shifted (2-column-separated) locations in the FB (**Figure 69A**). The amplitude of these activity bumps is likely to be strongly influenced by the different inputs that they receive through their lateralized projections in the NO (**Figure 25**), setting up the possibility of bump-based vector computations in the FB. As discussed above, in similar computations in the EB, the PEN_a neurons, which are conjunctively tuned to head direction and angular velocity, provide contralaterally phase-shifted input to the EPG neurons, thereby updating the position of the EPG bump when the fly turns (Green et al., 2017; Turner-Evans et al., 2017; Turner-Evans et al., 2020). Inside the FB, vector computations fed by phase-shifted PFN bumps whose amplitudes are controlled by different conjunctive signals could ultimately drive PFL neuron types to generate appropriate motor commands (**Figure 62**) (Hartmann and Wehner, 1995; Stone et al., 2017; Wittmann and Schwegler, 1995). Although the intra-FB columnar network is highly recurrent, it is built from a limited number of circuit motifs. These motifs serve as the backbone of a 2D grid in which activity bumps are constrained to either maintain their phase (that is, maintain their column) while moving across layers via the vΔ neuron types, or shift phase by 180° (that is, shift by half the columns of the FB) via the hΔ neuron types. While some pathways directly connect PFN neurons to output pathways, such as those involving PFL neurons, many more pathways run through this 2D grid. Thus, the network has depth, providing multiple layers with which to process activity bumps. In addition, a large number of tangential neuron types that selectively innervate different layers of the FB from integrative regions, suggesting that the FB's vector computations are influenced by context and internal state.

In the sections that follow, we draw from published experimental and theoretical work to explore the navigational implications of PF-neuron phase shifts given the type of information that these neurons receive from their NO inputs, the FB's 2D grid, the tangential inputs to the layers of the FB, and the connections from the region's many columnar neuron types to FB neurons that project to premotor areas.

<u>Potential function of PFN phase shifts: forward models and coordinate transformations</u>

Despite the PFN phase shifts, the two 90°-separated activity bumps arising from a single PFN type cannot propagate independently through the FB network, because nearly all single neurons and neuron types that are postsynaptic to PFN neurons sample from left and right populations equally (**Figure 35**). Instead, just like EB neurons postsynaptic to the PEN neurons, each postsynaptic neuron type in the FB likely sums the bumps from both the left and right PFN populations, indicating that each PFN type provides a single bump as input to the FB columnar network.

Further, much like the PEN neurons, the PFN neurons innervating the left PB project to the right NO and neurons innervating the right PB project to the left NO (Lin et al., 2013; Wolff et al., 2015). One potential function that this differential NO input to the left and right PFN populations could serve is to produce a new, transformed directional representation that could

take on angles +/- 45 degrees around the fly's instantaneous head direction. For example, as shown in **Figure 69B**, a strong excitatory input to the right nodulus would increase the bump amplitude of the left PFN population relative to the right PFN population. In turn, the summation of these two bumps by a postsynaptic neuron type in the FB would result in a new bump that lies closer to that of the left PFN population.

Figure 69C shows a phasor diagrams of this process. Viewed in this way, the cosine-formatted activity bumps from the left and right PFN populations become vectors whose magnitude reflects bump amplitude and whose angular position indicates bump phase in 'functional column' space, with each phase mapping to an allocentric direction. Critically, because these vectors can only take on positive values (firing rates above 0), such differential input could only shift the resulting vector's phase by +/- 45 degrees around the fly's instantaneous head direction. What might this transformed directional representation encode? The answer likely depends on the nature of the input that PFN neurons receive in the NO (Wolff and Rubin, 2018). Recent work in *Drosophila* has shown that some PFN neurons show differential activity in the NO that reflects the fly's turning behavior during flight (Shiozaki et al., 2020), but the nature of the rotational velocity signal remains to be determined. We briefly outline two hypothetical scenarios that differ in the specific information carried by PFN neurons.

First, PFN phase shifts should enable coordinate transformations, such as converting the allocentric head direction representation into an allocentric body direction representation (**Figure 69D**) (Andersen and Cui, 2009; Andersen et al., 1993; Batista, 2002; Bicanski and Burgess, 2020). Flies make head movements that change their head-body angles by as much as 30° during both flight (Duistermars et al., 2012) and walking (Fujiwara et al., 2017; Geurten et al., 2014). In this scenario, inputs to PFN neurons arborizing in the left and right NO would encode how much the head is rotated to one or the other azimuthal direction of the body's axis, perhaps derived from proprioceptive information (Paulk and Gilbert, 2006; Preuss and Hengstenberg, 1992). When properly calibrated, such differential input to the NO could allow the PFN phase shift (which, at 45°, is sufficient to encode the entire range of head-body angles) to rotate the head direction vector by an angle equal to the head-body angle. This would effectively transform the fly's head direction vector into an allocentric body direction vector. To do so, the network could use gain fields (Andersen et al., 1993; Pouget and Sejnowski, 1997; Pouget and Snyder, 2000; Salinas and Abbott, 2001; Zipser and Andersen, 1988), with an intermediate layer composed of PFN neurons whose head-direction tuning curves are gain-modulated by shared input related to the head-body angle. The neuron types downstream of PFN neurons would complete the coordinate transformation through their structured sampling of PFN neurons with different tuning properties. Coordinate transformations such as these may be useful when combining allocentric directional representations with body-centered velocity estimates to estimate direction and distance. We return to this idea in more detail in subsequent sections focused on vector computations related to path integration.

Second, as shown in **Figure 69E**, if we assume that NO inputs carry a motor efference copy of the fly's rotational velocity, as has been shown to exist in the fly visual system (Fujiwara et al.,

2017; Kim et al., 2017a; Kim et al., 2015), then bump shifts driven by differential input to the NO could function as a forward model (Webb, 2004) that encodes a prediction of the fly's future head direction or body direction. Why might a forward model of head or body direction be useful? Intracellular recordings from neurons in the ring attractor network have revealed that PEN activity is tuned to the fly's rotational velocity, but that this activity lags behavior by ~100 ms (Turner-Evans et al., 2017). Similar lags may result from propagation delays in neural processing, either along sensory pathways into the EB, or in passing the bump from EB to FB. In situations where flies might rely on the CX to direct their movements —especially in time-critical scenarios— such delays in updating the compass could be costly. One way to overcome this is for the PFN network to compute the fly's approximate future head or body direction so that navigational decisions can effectively be made in real-time, a strategy that dragonflies have been shown to use during rapid prey capture maneuvers (Mischiati et al., 2015). A forward model could also allow the fly to distinguish changes in its head or body direction associated with voluntary movements from those induced by external disturbances, such as changes in wind direction (Currier et al., 2020). More generally, matching the predicted head and body direction with the actual direction could enable the fly to fine tune its movements to produce intended motor outputs (Krause et al., 2019).

The PFN neuron types that the computations hypothesized above might involve is as yet unclear. However, our analysis of PFN inputs in the NO does allow us to draw some inferences about the sort of self-motion information that different PFN neuron types might carry. For example, considering that PFL2 neurons provide selective feedback to the LNO3 neuron type and considering that the PFL3 neurons feed the LCNOp neurons (**Figure 57C**), we would hypothesize that the former may provide its downstream PFN neurons —the PFNv neurons (**Figure 27**)— with efference information related to translational (and potentially forward) movement, and that the latter my provide its PFN targets —several PFNp sub-classes (**Figure 27**)— with efference information related to rotational movements. Physiological recordings in behaving flies would be needed to test such hypotheses.

<u>Intra-FB circuit motifs for vector computation</u>

Phase shifts of a single PFN neuron type could enable the generation of vectors that are within ±45° of the fly's instantaneous head direction. Intriguingly, the FB network appears to be wired to expand this range to allow for computations with vectors of arbitrary angles.

As shown in **Figure 70**, the vΔ and hΔ neuron types could, in principle, allow for vector computations across arbitrary azimuthal angles. In this example, we consider two hypothetical PFN populations (PFN1 and PFN2), both with the characteristic 1-column contralateral phase shift, as shown in the phasor diagrams in **Figure 70B**. On their own, these PFN populations are limited to directional representations spanning 45° around the head direction signal that they inherit. However, an excitatory hΔ (or an inhibitory vΔ) would invert the PFN2 vectors, shifting them by 180°. Thus, if a postsynaptic neuron type were to sum the input from the non-inverted PFN1 neuron population and an inverted PFN2 neuron population, it could form a representation of any arbitrary vector over the full 360° range, even though the PFN1 and PFN2

populations are individually range-limited (**Figure 70C/D**). A similar inversion could happen at the level of the PB, if one PFN population were to receive excitatory Δ7 input while the other received inhibitory Δ7 input, which is likely how the 180° separation of PEN_a and PEN_b population bumps is generated and maintained in the PB (Green et al., 2017; Turner-Evans et al., 2020). Together, the non-inverted PFN1 and inverted PFN2 neuron populations form a basis set of four orthogonal basis vectors, all separated by 90° (**Figure 70C**). As mentioned previously, the requirement for PFN neurons to have a positive firing rate prevents any single PFN population from forming a basis set on its own; instead, forming a basis set requires four independent bumps located at 90° intervals. When this situation is achieved, independent NO input could alter the relative amplitudes of bumps carried by each of the four PFN populations (inverted and non-inverted PFN1 and PFN2 populations), enabling their sum to encode a vector with any angle (**Figure 70D**). Many PFN neuron types receive non-overlapping inputs in the NO (**Figure 25**) and could thus represent such independent vectors. Importantly, during navigation, the orientation of this set of four vectors would be dynamically updated with the head direction representation, such that any computations derived from these vectors would be independent of the fly's current head direction (**Figure 70—figure supplement 1**).

Could the intra-FB network support the construction of arbitrary vectors, which requires two layers beyond PFN input? The type-to-type network graph in **Figure FB 6—figure supplement 1** suggests that there are many pathways within the FB's 2D grid that could potentially implement a four-vector basis set. We chose two arbitrary PFN types —PFNd and PFNp_c— and used their connectivity with two downstream vΔ and hΔ neuron types —vΔK and hΔA— to illustrate how this might work (**Figure 70—figure supplement 2**). We also show how a downstream neuron type —PFL3 in this case— could sum the input from the vΔ and hΔ to represent arbitrary vectors determined by independent NO inputs to the left and right PFNd and PFNp_c populations (**Figure 70—figure supplement 2C**). Physiological investigations will be required to establish which of the FB's many pathways implement such computations, and whether or not the large number of these pathways is an indication of vector computations in different behavioral contexts.

<u>Connectome-driven assessment of models of path integration</u>

Having established that the FB network of *Drosophila* could, in principle, compute arbitrary vectors, we now explore the potential utility of PB-FB offsets and intra-FB connectivity motifs for path integration. A variety of models have been proposed for path integration (Benhamou, 1997; Benhamou et al., 1990; Benhamou and Séguinot, 1995; Bernardet et al., 2008; Cheung, 2014; Gallistel, 1990; Goldschmidt et al., 2017; Haferlach et al., 2007; Hartmann and Wehner, 1995; Issa and Zhang, 2012; Jander, 1957; Kim and Hallam, 2000; Kim and Lee, 2011; Maurer, 1998; Merkle et al., 2006; Mittelstaedt, 1983; Mittelstaedt and Mittelstaedt, 1972; Muller and Wehner, 1988; Stone et al., 2017; Vickerstaff and Di Paolo, 2005; Wittmann and Schwegler, 1995). These models have several differences, including whether the home vector is stored in an allocentric reference frame or an egocentric reference frame, and whether it is stored using a 'static vectorial basis' or a 'dynamic vectorial basis' (Heinze et al., 2018; Vickerstaff and Cheung, 2010). Here we focus on models that store the home vector in an allocentric reference

frame using a static vectorial basis (Hartmann and Wehner, 1995; Stone et al., 2017), which has been shown to have several theoretical advantages (Cheung and Vickerstaff, 2010; Vickerstaff and Cheung, 2010) and whose implementation is directly suggested by the FB's network architecture. Path integration models can be further divided into two groups according to whether the home vector is stored and read out as independent components or as a single vector.

An example of the first type of path integration model, which stores the home vector as two independent components, was recently put forward by Stone et al. (Stone et al., 2017). This work combined anatomical and functional data from bees, including physiological recordings of optic flow-sensitive NO inputs and EM data, to build an anatomically inspired model of path integration based on the projection and innervation patterns of CX neurons, but without access to their synaptic connectivity. The model utilized PB-FB offsets to read out a home vector and, importantly, could also account for holonomic motion, which occurs when animals move in directions that are not aligned with their head/body axis, an issue we return to below. At its core, this model and those derived from it (Le Moel et al., 2019) function by modulating the amplitude of left and right PFN bumps according to the insect's motion in the leftward or rightward direction, respectively. Integration of the left and right PFN activities can then store a home vector as two independent components. During readout, a population of PFL neurons are assumed to compare the insect's current head direction to that of directions 45° to the left and to the right to decide which direction is closer to the implicitly stored home vector. This 45° 'functional offset' (phase-shift) was derived from physiological recordings demonstrating that some LAL-NO neurons function as optic flow sensors whose optimal expansion points are 45° to the left and right of the bee (Stone et al., 2017), a feature we return to below. While conceptually elegant, one major feature of this model is inconsistent with the anatomy and connectivity of the homologous neurons in the *Drosophila* connectome. In particular, the model requires that the left and right PFN bumps independently propagate to right and left PFL populations, respectively. This operation is unlikely to be supported by the FB columnar network, since every neuron and neuron type postsynaptic to PFN neurons receives input from both the left and right populations (**Figure 35**).

In the next two sections, we use the additional anatomical and connectivity information provided by the CX connectome to propose two conceptual models for computing an allocentric translational velocity (TV) vector whose integration could be stored as a single home vector. The first model builds on the work of Stone et al. and uses PFN offsets to simplify home vector computation. The second model is more relevant to walking insects and incorporates a head-to-body coordinate transformation to compute the fly's translational velocity vector. In both cases, the key computation performed by the FB network is a coordinate transformation that ensures that egocentric velocity signals and allocentric directional representations are directionally aligned.

<u>Computing an allocentric translational velocity vector using head-centered optic flow sensors during flight</u>

Flying insects are thought to perform visual odometry by relying on optic flow sensors to estimate their velocity relative to the ground (Leitch et al., 2020; Srinivasan, 2014, 2015), consistent with leg-based cues being of little use and motor signals being unreliable in the face external perturbations like gusts of wind. In addition, unlike walking conditions, during flight many insects make banked turns involving body rolls that are accompanied by gaze-stabilizing head rotations that keep the head near the horizontal plane (Kim et al., 2017a; Muijres et al., 2015). Importantly, flight trajectories often contain a significant sideslip component as well, during which the insect's translational velocity is in a direction that is different from that of its head-body axis (Braun et al., 2012).

The FB's recurrent circuitry described above could use such cues to compute a flying insect's allocentric TV vector. One potential model is shown in **Figure 71**. This model is composed of two PFN neuron types that receive independent input from two LAL-NO neuron types (**Figure 71B**), for which there are multiple candidates (**Figure 25**). It exploits the FB network's ability to form a set of four basis vectors to compute a single TV vector. To do so, it employs the optic flow sensors described by Stone et al. —with their preferred expansions points spaced at 45° intervals around the fly's head— to modulate the amplitudes of the four basis vectors such that their sum encodes an instantaneous allocentric TV vector (**Figure 71C-E)**. Importantly, this model relies on the fact that the basis vectors and optic flow sensors are directionally aligned (**Figure 71C-D**). That is, at every moment in time, each bump in the basis set has its amplitude modulated by a velocity input that senses movement in the same direction as encoded by the bump. Much like the model of Stone et al., this model can account for holonomic motion. Another interesting feature of this model is that it should be insensitive to head movements in the yaw plane, since the optic flow sensors and FB basis vectors are both in head-centered coordinates. The next conceptual model explores how this might work in walking insects, when the velocity sensors may be in a body-centered egocentric reference frame while the directional representation is in a head-centered allocentric reference frame.

<u>Computing an allocentric translational velocity vector using body-centered velocity estimates during walking</u>

Could the FB network compute an instantaneous TV vector in cases where its velocity estimates and directional estimate are in different reference frames? The model shown in **Figure 72** explores such a scenario using a hypothetical example of a walking fly whose velocity estimates are computed using cues that operate in an egocentric, body-centered reference frame. These velocity estimates could be derived from motor efference copies or proprioceptive cues, and we assume the existence of estimates for both forward (parallel to the body axis) and sideslip (perpendicular to the body axis) velocity. Computing a TV vector in this scenario is more complicated than in the previous model because the direction of the head and that of the body are not necessarily aligned, which requires a head-to-body coordinate transformation. As shown in **Figure 72B-D** this model uses the head-body angle to compute the total TV vector as the sum of two components, which represent the distance traveled parallel and perpendicular to the fly's body axis. To compute the parallel and perpendicular components of the TV vector,

the model uses two basis sets that receive NO input related to the head-body angle as well as either a forward or sideslip velocity signal.

A circuit for computing the component of the TV vector parallel to the fly's body axis is shown in **Figure 72B**, which involves two calculations that occur in parallel. The circuit recruits two independent PFN populations, one to encode movement in the forward direction, and the other for movement in the backward direction. A velocity signal increases the amplitude of the two PFN vectors that point in the direction the fly is moving (for the example situation in **Figure 72B**, in the forward direction), resulting in a vector whose amplitude encodes velocity and whose direction is either the fly's head direction (**Figure 72B**), or its reverse head direction, which would be captured by the PFN2 population in **Figure 72B**. At the same time, an input related to the head-body angle transforms the head-centered vector into a body-centered vector, as described above (**Figure 69**). The result is a single vector encoding the component of the fly's movement parallel to the body's axis in either the forward or backward direction. As mentioned above, this sort of computation could employ gain fields (Andersen et al., 1993; Pouget and Sejnowski, 1997; Salinas and Abbott, 2001), but with the transformed representation (that is, body direction) being scaled by the fly's velocity in the process.

The component of the TV vector that is perpendicular to the fly's body axis could be computed using the same circuitry as above, but with right/left sideslip velocity signals (**Figure 72C**) instead of forward/reverse velocity signals. As shown in **Figure 72—figure supplement 1**, such a circuit would work regardless of whether the fly is sideslipping right or left or whether its head is to the right or left of the body axis. The output of these two circuits could then be summed to compute a single vector that encodes the fly's instantaneous translational velocity in an allocentric reference frame (**Figure 72D**).

<u>Summary: translational velocity computation</u>

The conceptual models described above —one for flight and the other for walking— could, in principle, compute an allocentric translational velocity vector whose integration would yield an exact home vector. To accomplish this, the models use coordinate transformations to ensure that allocentric vectors are directionally aligned with the egocentric velocity estimates that control their amplitudes. While these particular models highlight the general utility of such transformations, the FB circuitry could, in principle, accommodate many similar models. In addition, it is possible that animals structure their movements during outbound paths to simplify the computation of the translational velocity vector. For example, if an animal were to only move forward during outbound paths, then circuit components dedicated to encoding backward motions would not be needed by the path integration circuit, a feature explicitly used by the model of Stone et al (Stone et al., 2017). Similarly, it is possible that an exact solution is not always required to perform path integration. For example, if a model generates errors that tend to cancel out during the integration process, the home vector can still effectively guide behavior. The local search behavior of foraging *Drosophila*, for example, involves relatively short loops that may not require a precise accounting of the goal location (Brockmann et al., 2018; Brockmann et al., 2017; Kim and Dickinson, 2017). In most situations, the fly should also

be able to use local sensory cues in addition to path integration during such search behaviors. Finally, it is possible that egocentric velocity signals could come pre-aligned to FB bumps, assuming that the LAL could implement the trigonometric functions needed to scale velocity signals according to, for example, head-body angle. Taken together, these models highlight the connectome's ability to inspire novel, implementation-level hypothesis about network computation. They also provide a framework for generating many similar models, with specific implementations that likely depend on cell type, species, and behavioral need. Ultimately, evaluating models like these necessarily requires physiological recordings from animals in specific behavioral contexts.

Potential mechanisms for translational velocity integration and home vector storage

Theoretical work has suggested several potential ways to integrate translational velocity vectors. First, translational velocity could be integrated and stored using two separate circuits: a ring attractor that encodes the angle of the home vector and a line attractor that encodes the length of the home vector (Hartmann and Wehner, 1995). Rather than keeping track of the distance traveled in each allocentric direction, this network would shift the columnar location of an activity bump to encode the phase of the home vector. Solutions like these seem unlikely to be implemented by the FB, since they require FB-centered attractors with circuit motifs for shifting the bump similar to those found in the EB-PB attractor, which we see no evidence for in the FB network. Second, the model of Stone et al. employed 18 neurons per FB column and used structured recurrent connections between them to integrate and store a two-component home vector (Stone et al., 2017). The hemibrain connectome provides little evidence for such structured recurrent PFN connections, especially in the NO, where some PFN types show all-to-all connectivity. Finally, the conceptual models described here allow for the computation of a single TV vector, suggesting that the FB network could simply integrate the corresponding activity bump and store the resulting home vector directly. This model would function by keeping track of the distance traveled in each FB column's allocentric direction. How might the network integrate and store a single TV vector?

Integration and storage could occur through several complementary mechanisms operating at different scales, from changes in synaptic strength or the excitability of individual neurons to persistent activity (Major and Tank, 2004) at the single neuron (Yoshida and Hasselmo, 2009) or network level (Aksay et al., 2007). In addition, integration and storage mechanisms may vary across species and environmental context depending on the animal's needs. For example, desert ants can maintain multi-day memories of multiple goal vectors to food sites and remember home vectors over 1-2 days of captivity (Wehner et al., 2004). This sort of long-term maintenance would favor a stable storage mechanism, such as changes in synaptic strength. In contrast, *Drosophila* performing relatively brief local searches close to a food source may rely on short-term mechanisms that could involve persistent neural activity. The connectome alone does little to constrain the space of possible storage mechanisms, but it can provide information regarding the likely site of storage and inspire a few conceptual models for how the vector could be stored.

Several considerations narrow the potential site of home vector storage. Assuming the conceptual models described above are utilized in ways similar to that imagined, the home vector would be stored downstream of the four vector basis sets used to compute the TV vector. The PFL2 and PFL3 neuron types are well positioned to read out the home vector by comparing it to the fly's instantaneous head direction (see section below), suggesting that the home vector is perhaps stored by neuron types that provide inputs to the PFL neurons. The PFL neuron types could also store the home vector themselves. Some insects are likely to maintain more than one goal vector (Dacke and Srinivasan, 2008; Mangan and Webb, 2012), but the PFL neurons could store these different goal vectors through input-synapse-specific presynaptic (Goldschmidt et al., 2017) or postsynaptic plasticity. In addition to direct PFN input, PFL2/3 neurons receive shared input from a handful of hΔ types, several FC2 types, one or two vΔ types, and many FB tangential neuron types, each of which could also potentially store a home or goal vector.

Several potential storage mechanisms seem plausible. Many hΔ neuron types have within-type recurrent connections, forming small loops that connect pairs of hΔ neurons that encode directions 180-degrees apart. If the biophysical properties of these neurons allowed for graded, persistent activity, and hΔ neurons have inhibitory connections, each column-pair could encode the direction traveled along one dimension. Alternatively, while the FC neurons providing input to PFL2/3 neurons largely lack within-type recurrent connections, they could maintain a vector in working memory through graded changes in their excitability or activity. Finally, the FB's tangential neurons could potentially store home vectors through column-specific plasticity, as is known to occur between ring neurons and EPG neurons. In general, some recurrent architectures may allow for the storage of home vectors (Wittmann and Schwegler, 1995), but an FB ring attractor, if it were to exist, would likely not allow for home vector storage, since these networks have the undesirable property of forming a single activity peak at the expense of the activity in distance columns that would be needed to fully encode the home vector.

Overall, although the connectome can do no more than rule out some circuit implementations of how the home vector might be stored, it should prove useful in prioritizing a search for the likely neural targets for such a function. It is important to note that the entire circuitry described above must function in different modes depending on the animal's behavioral needs —integrating direction and distance traveled to update the home vector when the fly is searching, but switching to reading out the home vector when the fly is attempting to return to a previously visited spot. The likeliest candidates for such behavioral mode switching are the FB's tangential neurons.

<u>Reading out the home vector</u>

Once formed, how might an insect 'read out' the home vector to return to its goal location? In our formulation, the home vector points *from* the nest *to* the insect's current location. Returning home, then, requires that an insect move in a direction *opposite* to the home vector. To accommodate the other behaviors and computations that these circuits are likely to be involved in, we refer to the home vector as the 'stored vector', which is read out to orient the

insect towards a 'goal vector' (**Figure 73**, bottom panel). However, unlike an ant or bee, the fly is not a central place forager. Thus, 'goal' in this context refers only to a spot that the fly is likely to return to during a local search, such as a food source (Brockmann et al., 2018; Kim and Dickinson, 2017). PFL neurons are generally regarded as the major columnar output of the FB network (el Jundi et al., 2015; Hanesch et al., 1989; Heinze et al., 2013; Heinze and Homberg, 2008; Homberg, 1985; Lin et al., 2013; Skutt-Kakaria et al., 2019; Wolff et al., 2015), and their PB-FB offsets strongly implicate them in reading out stored vectors in ways first proposed by theoretical work (Hartmann and Wehner, 1995; Wittmann and Schwegler, 1995). In particular, PFL neurons may use their PB-FB phase shifts to compare the fly's instantaneous head direction, which they receive in the PB, to that of the stored vector, which they may receive in the FB, to generate appropriate motor commands to guide the fly to its goal. In doing so, they effectively generate egocentric motor commands based on allocentric directional variables. Interestingly, each of the three PFL types have characteristic phase shifts that strongly predict their involvement in generating distinct motor commands (**Figure 73**).

PFL2 neurons may use their 180° offset and bilateral LAL projections to increase the fly's forward velocity when its heading is directly away from the stored vector, which in our formulation is towards the goal location (**Figure 73A**, bottom panel). Unlike the other PFL types, PFL2 neurons receive only a single bump as input in the PB (**Figure 39**). This suggests that the population cannot make left versus right activity comparisons. In agreement with this, individual PFL2 neurons make bilateral projections to the left and right LAL. Because of their 180° phase shifts, the PFL2 population activity will be largest when the fly is heading directly towards its goal location. The above characteristic suggests that PFL2 neurons are ideally suited to generate a motor command related to forward velocity.

PFL3 neurons may use their 90° phase shifts and lateralized LAL projections to orient the fly towards the goal. As shown in **Figure 73B**, their 90° offset predicts that the left and right PFL3 populations will have their maximum activity when the fly is 90° to the right or left of the goal direction, respectively. If the left PFL3 population generates left turns and the right PFL3 populations generated right turns, then the orienting behavior of fly will have two equilibrium points: a stable equilibrium that occurs when the fly is oriented towards the goal direction and an unstable equilibrium when the fly is oriented in the opposite direction. This sort of read out would ensure that flies orient directly towards the goal location. It is additionally possible that across-column inhomogeneities in the EPG->PFL synaptic profile (**Figure 73—figure supplement 1**) and also in the PFL->LAL network (**Figure 64**) may provide the fly with a 'default goal' even in the absence of FB input and be modulated by such input when available, similar to a hypothesis recently advanced in an independent study (Rayshubskiy et al., 2020). The 45° offset of PFL1 neurons may serve a related function, although they target distinct downstream neurons compared to PFL2/3 (**Figure 57C**). One possibility is that the PFL2/3 neurons affect body orientation while the PFL1 population controls a separate variable, such as sideslip or head-body angle. Ultimately, it is also important to remember that brain regions like the LAL and CRE house complex recurrent networks with inter-hemispheric pathways that are likely to be inhibitory (see Output sections) (Iwano et al., 2010). Thus, it is highly likely that our hypotheses

about directed movement arising from PFL neurons are vastly under-estimating how much and in what ways the processing of these regions impacts any activity from PFL neurons.

Summary: vector computations in the FB

The discussion above supports the notion that the FB network has the computational capacity to compute, store, and read out vectors in support of goal-directed navigational behaviors. While we have focused on path integration as a canonical vector-based computation, *Drosophila* are known to perform several other behaviors that may rely on the formation of goal vectors, including: local search, a path-integration-based foraging strategy (Corrales-Carvajal et al., 2016; Dethier, 1957; Kim and Dickinson, 2017); menotaxis, where a constant heading is maintained relative to an arbitrary goal direction to generate straight trajectories that support long-distance dispersal (Giraldo et al., 2018; Green et al., 2019; Leitch et al., 2020); place learning, which requires associating visual cues with the presence of a cool spot in an otherwise hot 2D environment (Melnattur et al., 2020; Ofstad et al., 2011); and the detour paradigm, where flies orient towards directions associated with attractive landmarks even after they have disappeared (Neuser et al., 2008). In addition, ethologically-based studies in behaving insects have established a range of vector-based behaviors, from long distance migrations that require a time-compensated sun compass (Heinze and Reppert, 2011; Perez et al., 1997) to the waggle dance that bees use to communicate the distance and direction of a food source (Frisch, 1967). The ability of some insects to store multiple goal vectors and the fact that different insect species may use vector computations to support distinct behaviors has important implications for FB circuits. The FB may have evolved as a general vector calculator that can be co-opted, whether by evolution or in support of distinct behaviors, to support vector-based navigation strategies generally, which could explain the highly conserved nature of FB circuit and neuron types. Additionally, the ability of some insects to store multiple goal vectors requires mechanisms for switching between them, a function perhaps mediated by the large class of FB tangential neurons that could convey context and state information to the columnar networks involved in vector operations.

The role of the MB in memory-guided action-selection and navigation

The connection between the MB —as a potential source of snapshot memory— and the CX —as a steering center— has been the subject of considerable conceptual interest in the insect navigation field (Collett and Collett, 2018; Sun et al., 2020). However, the connections between these regions may also play critical roles in sleep and memory consolidation (Dag et al., 2019). Some of the neurons connecting the MB and the CX, which have been mentioned in the companion MB manuscript (Li et al., 2020) as well as in the MB section in this manuscript, are likely to bring valence and novelty signals extracted from past associations that the fly has made with its current sensory surroundings.

Some outputs from the CX contact a limited number of MBON-associated networks (**Figure 61**). These connections may allow the CX to modulate some behavioral responses to specific sensory contexts that have been associated with negative or positive valence through the MB. Although we do not yet know which behaviors these are, we would expect these behaviors to benefit

from information about the fly's orientation in its surroundings. The fact that an oviposition neuron (oviIN) is associated with those networks (**Figure 61**) is thus not entirely surprising. There have been suggestions that oviposition decisions are informed by several external factors (Yang et al., 2008; Zhang et al., 2020). What is less clear is how and why CX signals, particularly, for example, from the columnar FR1 neurons, might drive MB neurons (in the case of the FR1 neurons, the MBON30 neurons). Such interactions between the MB and CX suggest that memory-guided navigation may benefit from a study of both regions acting in concert. Consistent with such an idea, the atypical MBON, MBON27, targets the DNa03 neuron type (Li et al., 2020), which is also targeted by PFL3 neurons.

Beyond navigation: sleep and internal state in the CX

In addition to its role in generating flexible, oriented locomotion, the CX is known to regulate the fly's overall activity level based on circadian cues (Liang et al., 2019) and the animal's sleep needs (Donlea et al., 2014; Donlea et al., 2018; Liu et al., 2016a; Pimentel et al., 2016) and nutritive state (Dus et al., 2013; Sareen et al., 2020). Thus, the CX likely tracks and incorporates internal state variables in selecting and generating appropriate actions.

While its function(s) remain largely unknown, sleep is associated with a variety of processes in *Drosophila*, including synaptic homeostasis (Bushey et al., 2011), memory formation and consolidation (Berry et al., 2015; Dag et al., 2019; Donlea et al., 2011), changes in gene expression (Cirelli et al., 2005; Zimmerman et al., 2006), and several metabolic processes (Kempf et al., 2019; Vaccaro et al., 2020). Sleep in flies is behaviorally defined as a reversible state of immobility that is homeostatically regulated and associated with an increased arousal threshold (Hendricks et al., 2000; Shaw et al., 2000). It is marked by drastic changes in brain-wide activity patterns (Nitz et al., 2002; Tainton-Heap et al., 2020; Yap et al., 2017). The neural circuits involved in tracking sleep need and inducing sleep are thought to partially reside in the CX. In particular, a population of FB tangential neurons labeled by the R23E10 GAL4 line induces sleep when activated and tracks sleep need through changes in baseline firing rate and intrinsic excitability (Donlea et al., 2011; Pimentel et al., 2016) (but see also (Tainton-Heap et al., 2020)). Importantly, our analysis revealed that this line contains ~9 distinct neuron types (**Figure 48**), consistent with previous work (Qian et al., 2017). Future experiments using highly specific driver lines are needed to assess which of these types is mostly strongly tied to sleep homeostasis. By contrast, dopaminergic neurons in the dorsal FB are thought to promote wakefulness by inhibiting R23E10 neurons (Ni et al., 2019; Pimentel et al., 2016) (**Figure 49**). Our connectomic analysis revealed extensive, reciprocal connections between these sleep-promoting and wake-promoting populations (**Figure 50**), a circuit that could function as a 'flip-flop' switch to ensure that only one population is active at a time (Saper et al., 2010). Pathways upstream and downstream of this putative flip-flop circuit are likely to influence the fly's sleep-wake state. Our pathway tracing analysis identified a large number of previously undescribed pathways leading to and from dorsal FB sleep-wake neuron types whose potential involvement in sleep-wake control requires future investigation (**Figure 51-55**). Our analysis also identified reciprocal pathways connecting sleep-wake neurons in the EB with those in the dorsal FB (**Figure 53**), as proposed by prior work (Donlea et al., 2018; Liu et al., 2016a). In particular, ER5

neurons, whose excitability correlates with sleep need (Liu et al., 2016a), may signal to dorsal FB circuits through two types of extrinsic ring neuron types: ExR1 and ExR3. ExR3 neurons are serotonergic and were recently shown to be involved in sleep-wake control, along with a population of ER3d neurons that they are reciprocally connected to (Liu et al., 2019). Importantly, ExR1 and ExR3 neurons allow for bidirectional communication between EB and dorsal FB circuits, consistent with previous work suggesting that ExR1 neurons can convey information from the dorsal FB to the EB (Donlea et al., 2018). In addition to these bidirectional pathways, a previously undescribed columnar type, hΔK, conveys information from PEN_b neurons in the EB directly to dorsal FB circuits.

Links between the CX's role in sleep and navigation have begun to be explored both experimentally (Donlea et al., 2018; Liang et al., 2019) and computationally (Valle et al., 2020), but the CX connectome suggests that the number of pathways and neuron types that connect circuit elements known to be involved in these functions may have been underestimated. Further, the dorsal layers of the FB, which have been associated with sleep-wake functions in the fly may well be involved in internal states beyond sleep. Specifically, tangential neuron types in the dorsal FB have been implicated in feeding decisions based on the nutritive value of foods, and they may incorporate past experience into these computations (Sareen et al., 2020).

Regardless of the range of internal-state-related computations that the dorsal FB may be involved in, these computations likely involve the FB's columnar neurons. These columnar neurons are part of the sleep-wake networks of the EB and FB. Although we believe this columnar structure —and the FB's 2D grid more generally— to be convenient for vector computations, why this columnar structure may be needed for sleep-wake control or for feeding and satiety related computations remains mysterious. One possibility is that the head direction signal may be used as a proxy for tracking the quality or quantity of the fly's waking experience, perhaps as a means to estimate sleep and/or nutritional need. Alternatively, the head direction signal may be inherently activity-promoting, since it likely drives premotor neurons in the FB. Therefore, the head direction signal may require suppression to establish a sleep state or to enable a hungry fly to stop on a patch of nutritive food.

Both sleep and feeding are known to be under circadian control (Dubowy and Sehgal, 2017; Murphy et al., 2016; Xu et al., 2008). Flies are most active around dawn and dusk, and show consolidated sleep throughout the night and during a daytime siesta (Dubowy and Sehgal, 2017). This daily rhythm is imposed by outputs from the circadian network and functions to restrict behavior to appropriate times of day. Previous studies have identified a population of anterior-projection DN1 clock neurons that convey circadian information through TuBu neurons to EB ring neurons, including ER5 (**Figures 6C, 7D**) (Guo et al., 2018; Lamaze et al., 2018). Thus, in addition to keeping track of sleep debt, CX circuits are likely to receive circadian information that could be used to select behaviors according to time of day.

The need for high-level behavior selection may explain the potential interactions of circuits related to navigation, feeding and sleep. Hungry flies, for example, are known to forgo sleep in

favor of foraging (Keene et al., 2010). Many of the FB's tangential neurons in Layer 6 that have been implicated in sleep-wake control are highly recurrently connected. It is possible that some of these neurons are involved in decision-making related to feeding (Sareen et al., 2020). If so, inhibitory interactions between these different tangential neurons may —in a manner similar to the interactions of ring neurons for sensory control of the fly's compass— enable the fly to select appropriate actions based on internal need. Testing such ideas will require a finer-resolution analysis of the role that these neurons play in sleep and feeding.

Other neuron types in the sleep-wake network whose function remains unknown are the ExR1 and ExR3 neurons, which innervate regions outside of the EB and FB. ExR3 neurons, for example, provide input to several neuron types in the BU, including ExR1 neurons and several types of ring neurons. One possibility suggested previously (Donlea et al., 2018), is that sleep-promoting neurons may gate incoming sensory information through their effect on ExR neurons. A similar gating of sensory information may also be important for a hungry fly to be able to continue a feeding bout.

A different puzzle concerns the potential role for neural oscillations in sleep-wake control (Raccuglia et al., 2019; Yap et al., 2017). How the highly recurrent networks in the EB and FB might support such oscillations remains to be determined. One possibility is that all-to-all inhibition between ring neurons in the EB could, with the appropriate inhibitory conductances, induce such patterns of activity. A different issue raised by the highly recurrent architecture of the sleep-wake networks concerns the manner in which activity may propagate in these networks. Artificial stimulation of neurons within such potentially self-regulating networks may trigger downstream activity that is never seen in more naturalistic situations, something that may be important for experimentalists working in the circuit to be aware of.

Several limitations of the hemibrain dataset are notable in the context of sleep: neurons that that show structural changes as a function of the fly's sleep-wake history (Bushey et al., 2011), such as ER5 (Liu et al., 2016a), could have sleep-state-dependent connections different from those described here; similarly, at present, the hemibrain connectome does not include reconstructed glia, which are also known to be involved in sleep-wake control; lastly, the hemibrain dataset cannot resolve the presence of gap junctions, which may also be important for sleep-wake control (Troup et al., 2018).

The CX as a multifunctional network: context-based action selection

While we have focused much of our discussion on column specific, vector-computations, the FB has a second organizational dimension with nine layers stacked along its dorso-ventral axis.

The 9 layers of the FB contain ~150 tangential cell types, most of which arborize throughout a single layer. Most of these tangential neurons provide inputs to the CX, though a handful of types appear to be CX output neurons. Inputs from the NO only enter the FB through the ventral layers (1-4) and the most dorsal layers (7-9) only receive inputs from the SMP/SIP/SLP. Tangential neurons in layers across the FB receive direct or indirect input from MBONs and

many tangential neuron types receive recurrent signals from CX output pathways. Further, dopaminergic FB tangential neurons can be found in many layers. Although only a handful of these neurons have been investigated experimentally, their physiology and influence on behavior strongly suggest that FB tangential inputs provide contextual information to the FB. Dorsal tangential neurons arborizing in layers 6 and 7 have been associated with sleep and wakefulness (Artiushin and Sehgal, 2017; Donlea, 2017; Dubowy and Sehgal, 2017; Helfrich-Forster, 2018). Layer 6 neurons have also been linked to modulating food choice based on internal metabolic state (Sareen et al., 2020). Finally, either the FB2B_a or FB2B_b neurons indicate the state of flight (Weir et al., 2014). The diversity of contextual information coming into these layer-specific FB tangential neurons could be used to set (or reset) context-specific goals or to switch which goals will influence the FB outputs. Switching could occur by releasing or restricting specific pathways in the FB network or by more broadly altering the network properties through neuromodulator release.

Considering that the highest layers of the FB are associated with modulating the fly's activity depending on sleep state and satiety levels, this connectivity pattern suggests that information about the fly's current navigational state may enter the FB ventrally, that additional processing may happen in the middle layers, and that this processing may then determine the fly's next actions (or inaction) in the dorsal layers. An additional possibility suggested by the flow of bump information from ventral to dorsal layers of the FB, and by the diffusion of columns in the dorsal layers, is that the specificity of actions is organized along the vertical axis of the FB, with oriented actions modulated and signaled by output neurons originating in the middle layers (see next section) and the fly's overall state of activity modulated in directionally non-specific ways by the highest layers.

Directing movement based on the fly's current state

The structure of the FB suggests that it modifies the head direction signal, possibly in a context specific manner, to orient the fly with respect to specific "goal" directions. These directions are likely meaningful to the fly's survival, such as a source of food or safety or, for female flies, a good site for oviposition.

The outputs of the CX likely direct the fly to these goal locations. Depending on the type of goal, output signals could fall into one of two (or more) categories. First, the CX outputs could control the animal's movements and/or orientation relative to its global environment, directing the fly to reach a desired location or to avoid one associated with danger. Examples of computations that fall into this category include localizing the source of an attractive odor and local search during foraging. Second, the CX outputs could release motor programs when the animal is oriented in a specific goal direction. Examples of behaviors that may involve such computations include feeding, oviposition, fighting, and negotiating uncertain terrain.

Output neurons with bilateral innervation patterns in premotor regions such as the LAL and CRE are likely to modulate symmetric actions (for example, forward walking), while those with unilateral innervations in such regions likely control asymmetric actions (for example, turning).

Examples of the former include the PFL2, FS1-3 neurons, while PFL1, PFL3, PFR, FR, FS4 and FC neurons all show unilateral innervations of premotor regions. Note that the actions that are controlled by the CX may not be limited to walking and flight. Based on the range of the downstream targets of these different CX output neuron types, which include not just the DNs, but neurons and regions involved in feeding and oviposition, we speculate that the CX may also be involved in directing voluntary take-offs, proboscis extension during feeding, or abdomen bending for egg laying in a manner that incorporates the fly's internal sense of its body size, posture and orientation relative to its surroundings.

We found that output channels, including the columnar output systems (PFL, PFR, FR, FC and FS neurons), feed relatively independent output subnetworks. This suggests that each subnetwork controls an independent set of behaviors (**Figure 74**, which is partly based on **Figure 74—figure supplement 1**, proposes a speculative set of modules for this). If true, each subnetwork could allow for the maintenance of independent goal locations, each specific for a given behavior and carried by a specific FB columnar type (or set of types). For example, some subnetworks could control behaviors related to goals in front of the animal, such as feeding or gap crossing (Poeck et al., 2008; Triphan et al., 2016; Triphan et al., 2010), while others could control behaviors related to goals behind the animal, such as egg-laying (Yang et al., 2008). Another axis along which behaviors can be subdivided is the scale of orientation control. From the body to the head and legs, proboscis, abdomen or antenna, all body parts have an orientation relative to the environment. Each of these body parts could benefit from coordinated but independent control and could be individually targeted by CX outputs. Alternatively, these independent subnetworks could also support, through unknown mechanisms, the maintenance of independent goal locations associated with independent behaviors.

The PFL2 and PFL3 neurons provide the most direct link from the CX to the motor center, known as the ventral nerve cord (VNC). These two neuron types are also the major output channel of the CX. They are therefore a prime candidate to guide orientation and/or movements to a fixed goal. However, these actions would need to be coordinated with movements of body parts that alter the sensed orientation, most notably head movement. The ExR8 neuron could carry out these corrections. Of note, both PFL3 and ExR8 neurons are associated with visual pathways.

The other FB columnar output types (PFR, FR, FC and FS neurons) are well positioned to control behaviors that only depend on orientation. We found connections from these neurons to the oviposition network, to regions controlling feeding, and, more generally, to parts of the MBON/dopaminergic system. The MBON/dopaminergic connection pathway suggests that some of the behaviors controlled by the CX could also be learned behaviors.

We remain in the dark concerning a number of CX output neurons (PFL1, ExR7 and most of the FC neurons) and a vast majority of the downstream circuitry. Further characterization of the terra incognita regions, and a more complete connectome, would help to identify the function of these pathways.

Navigation with small networks and with numerical variation in columnar neurons

The remarkable behavioral repertoire of insects is still more remarkable when considering their small brains. The CX connectome suggests that part of the secret behind this wide-ranging repertoire lies in having evolved architectures that are precisely configured for sophisticated behavior, but with weights that are plastic to allow these behaviors to flexibly adapt to context and situational demand. It is likely that the impressive computational power of their brains may also derive from an under-explored aspect of their neurons: their capacity for arbor-specific local computations, possibly even subthreshold computations in which synaptic release does not require the spiking of neurons that can otherwise spike and/or molecular computations through signal transduction cascades. These issues will require further experiments, but the connectivity we observe in the EB, for example, hints at a rich potential for insights into subcellular computation in the CX.

Regardless of the true computational capacity of single neurons, the fact that the fly can navigate with a head direction system of 8 computational units and just a few thousand neurons performing vector computation is remarkable. In principle, such small networks should be exquisitely sensitive to any variations in the number of neurons in each computational unit. However, the CX connectome revealed a striking mirror-symmetry in the different number of columnar neurons that innervate each of the 18 PB glomeruli, and the 9 structural columns of the FB. Although several studies have investigated the developmental origins of columnar CX neurons (Pereanu et al., 2011; Sullivan et al., 2019; Walsh and Doe, 2017; Yang et al., 2013; Young and Armstrong, 2010a), we do not know of any that have noted or focused on this systematic asymmetry. There are indications of numerical variations in some columnar neurons, such as the EPG, PEN and PFL neurons, in the FAFB volume as well (Rayshubskiy et al., 2020; Turner-Evans et al., 2020), and more complete EM reconstructions of that volume (Dorkenwald et al., 2020; Li et al., 2019) should be able to clarify whether these variations exactly match what is seen in the hemibrain volume.

We do not know if these asymmetries in columnar neuron numbers are also present in the CX of other insects. Some diptera, including Drosophila, have a closed EB, in contrast with most other insects, whose Central Body Lowers have an open, FB-like structure (Strausfeld, 2012). The non-uniform distribution of the EPG neurons at the base of the EB (four coming from glomeruli L8 and R8 of the PB and two coming from glomeruli L1, L2, R1, and R2), EPGt innervation at that location, and systematic modifications to neuron number across the columnar neuron types may represent an evolutionary adjustment to the closing of this structure.

The functional consequences of the systematic variation of neuron numbers across columns are entirely unknown. It is possible that this variation builds redundancy into a critical navigational system, or that the increased numbers of neurons in specific glomeruli ensure a preferred "default" location for the bump to occupy within the more central columns of CX structures, and perhaps also a "default" heading for the fly to adopt (**Figure 64**, **Figure 73**), an idea that is similar to a suggestion advanced independently in (Rayshubskiy et al., 2020). A different possibility is that such variation is not stereotyped across flies, but is specific to individuals, and

that this may account for locomotor biases across the population (Ayroles et al., 2015; Buchanan et al., 2015; Skutt-Kakaria et al., 2019). Functional experiments with specific perturbations of neuron numbers in different columns may be necessary to further investigate this issue. Regardless of their functional role, how such mirror symmetric numerical variation is achieved may be an intriguing question for future studies of CX development.

The CX as a tractable deep recurrent neural network

Technical advances over the past several decades have enabled increasingly large-scale recordings of neural activity from the central brains of a wide range of animals (Jun et al., 2017; Lu et al., 2020; Stringer et al., 2019; Vanwalleghem et al., 2018). These recordings have, in turn, enabled high-throughput studies of neural response properties that have focused on relating patterns of neuronal activity to sensory, behavioral and internal state variables. However, the biophysical and circuit mechanisms underlying these response properties have been more challenging to access. Similarly, dramatic progress in the field of machine learning has enabled the creation of sophisticated artificial agents that can solve a variety of different cognitive tasks, including flexible navigation (Banino et al., 2018; Cueva and Wei, 2018). Some units in these deep networks develop response properties broadly similar to those observed in real brains. However, insights into how such artificial neural networks generate the representations observed in their units —something that could, in principle, guide mechanistic hypotheses for the function of natural neural networks— have been slower to come (but see (Cueva et al., 2019; Uria et al., 2020) for progress in uncovering the architectural basis of navigational responses in these networks). In this era of deep learning, a broader question concerns the level of understanding that is appropriate or even possible for the function of large and complex neural networks (Gao and Ganguli, 2015; Hasson et al., 2020; Lillicrap and Kording, 2019; Richards et al., 2019; Saxe et al., 2020; Yamins and DiCarlo, 2016). What seems achievable is an understanding of learning rules and objective functions that can, in principle, generate networks with realistic population responses for specific cognitive tasks. The conservation of the CX's structure across arthropods (Honkanen et al., 2019; Strausfeld, 2012; Turner-Evans and Jayaraman, 2016) perhaps highlights the extent to which the region has, in practice, been shaped by such rules over evolutionary timescales in the service of flexible behavior. But what of an understanding of the actual network implementation itself? Some have argued against the necessity or desirability of such a level of understanding (Hasson et al., 2020; Richards et al., 2019). The fly's relatively brief history in systems neuroscience provides an increasingly compelling counterargument and may eventually offer a roadmap for implementation-level understanding that could scale to much larger brains and more complex cognitive functions.

The fly displays an interesting repertoire of flexible behaviors, and some of its recurrent neural circuits show short and long timescale dynamics that have been linked to associative learning and navigation across animals. Its 100,000-neuron brain circuits may appear complex, but they also feature modularity and type-specific connectivity that is genetically prespecified and has been refined over its evolutionary history. Both these features apply to much larger brains as well (Hodge et al., 2019; Maruoka et al., 2017; Saunders et al., 2018; Strange et al., 2014; Tasic

et al., 2018), although there is likely greater flexibility in the wiring of mammalian circuits and greater heterogeneity within cell types in the mammalian brain (Cembrowski and Menon, 2018; Cembrowski and Spruston, 2019). Such developmentally-driven organizational features of natural brains may actually make them more tractable than artificial neural networks for an understanding of their function (Zador, 2019). **Figure 75A**, for example, shows the connectivity of a small fraction of the fly CX's many neuron types arranged by layers. Taking a single-neuron-resolution view of this subnetwork shows just how densely recurrent it is, even at a small scale (**Figure 75B**). Indeed, if the types and connectivity of these neurons were unknown, extracting network structure from population responses would be a challenge. However, sorting the neurons into types —in this case, inhibitory types— makes the logic of the network clearer (**Figure 75C**; also see **Figure 75—figure supplement 1** for the part of the CX that we believe to be structured for navigational computations). Combining this circuit connectivity with physiological studies has enabled not only the generation of hypotheses for the computations that may be carried out by subnetworks at each layer, but, increasingly, tests of these hypotheses (Fisher et al., 2019; Green et al., 2017; Kim et al., 2019; Kim et al., 2017b; Turner-Evans et al., 2017). As a result, it is possible to establish circuit-level mechanisms underlying the generation of different response properties. Importantly, fly circuit connectivity is not always structured (Caron et al., 2013), many synaptic connections are plastic, and information from one part of the network often flows to all other parts of it (as we detail in sections of this manuscript). Nevertheless, the developmentally pre-specified organization of these networks makes them experimentally tractable. Although the computational capacity of the morphologically and biophysically complex neurons in these networks has likely been vastly underestimated, the connectome thus raises the prospects for at least a circuit-level understanding of how the fly's CX generates many of this small animal's flexible behaviors.

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MATERIALS & METHODS

Nomenclature

Briefly, many neurons exhibit a unique morphology that could be matched to previous light-based descriptions. Neurons with practically indistinguishable shapes but with different connectivity patterns were split into connectivity subtypes within a morphology type (for example the sub-classes of PFN neuron types). For this connectivity-driven sub-classification, we used a cell type clustering tool called CBLAST (Scheffer et al., 2020) in an iterative process, using neuron morphology as a template, and regrouping neurons after more careful examination of neuron projection patterns and their connections. CBLAST usually generated clusters that were consistent with the morphological groupings of the neurons, but often suggested new sub-groupings. This clustering served as a naming guide after manual inspection (see **Table 1** and **2** for all neuron types, with numbers for each type; see Materials and Methods for notable changes in names).

All CX neurons have been given two names, a short name that we use throughout the manuscript, and a longer name that captures the overall morphology of the neuron. Both long and short names for the vast majority of PB, NO and AB neurons are published (Wolff and Rubin, 2018) and remain largely unchanged, even though slight changes were made to certain formats in the FB nomenclature (Scheffer et al., 2020). For example, abbreviated names published in (Wolff and Rubin, 2018), used a one-letter rather than a two-letter code for CX

neuropils and non-CX neuropils included lower case letters (for example,Sps, Ib). There are several cases in which these published names are identical to either abbreviated names in other brain regions or in other systems. These redundant names have consequently been changed, as follows: LN was changed to LNO, LGN was changed to LGNO, and EB ring neurons were changed from R1-R5 to ER1-ER5 to distinguish them from the names of fly photoreceptors. Details for the PB/NO/AB nomenclature scheme can be found in (Wolff and Rubin, 2018). Below, we describe in broad terms the approach that was taken to name previously undescribed CX neurons, a more detailed account of which is published in (Scheffer et al., 2020).

The same general rules used to name PB, NO and AB neurons were followed for FB nomenclature, as follows:

1. A maximum of three neuropils, the two with the greatest input (except for tangential neurons , which are named for the neuropils with second and third highest input, see Scheffer et al. for details), followed by one output, constitute the backbone for/of? the name. The input neuropil with a larger arbor (and greater number of synapses) is listed first. Connectivity data was used to confirm the relative proportions of synapses in input neuropils. Neurons that project to only two neuropils are named by the input followed by output neuropil.

2. The standard two-letter abbreviations are used for CX neuropils (PB, FB, EB, NO, AB) whereas the three-letter abbreviations described in (Ito et al., 2014) are used for non-CX neuropils.

3. The highest synaptic input for many FB neurons is restricted to a relatively small group of neuropils, leading to a great deal of redundancy in anatomical names. Neurons that innervate the same set of neuropils are therefore distinguished from one another by the underscore symbol followed by a number (_1, _2, etc.).

4. Numbers that follow “FB” indicate the FB layer arborized by the neuron. Confinement to the dorsal or ventral portion of an FB layer is indicated by a “d” or “v.” A hyphen between FB layers indicates the arbor is more or less continuous across the noted layers. Numbers and letters that follow “NO” identify either the nodulus or nodulus subcompartment. In an effort to keep the anatomical names as short as possible, only the subcompartments are included for neurons that arborize in NO3, abbreviated NOa (anterior), NOm (medial) and NOp (posterior).

Whereas the FB tangential neurons **target a single FB layer**, the FB columnar neuron arbors often span more than one layer, either as continuous arbors or as distinct arbors in different layers and brain hemispheres. The width of these arbors varies with neuron type, and therefore, column number is fluid and neuron type-dependent. In other words, there is no fixed number of columns in the FB as is shown for PB glomeruli. The placement of arbors, both within specific layers and across columns, is a defining morphological feature and is therefore included in columnar neuron names, as described in the following rule.

5. There are two basic blueprints for columnar neuron architecture: one in which input arbors are vertically arranged within a single column and is indicated by the prefix “v” in the short

name and D0 in the anatomical name, and a second in which the arbors are horizontally distributed across the width of the FB and indicated by the prefix "h" in the short name and D# in the anatomical name (see below; further details in (Scheffer et al., 2020)). The input arbor(s) for the horizontal class of neurons are vertically arranged within a column, as with the vertical class, and is/are separated by a neuron type-specific number of columns. The number of columns between these two arbors is referred to as "delta" and is indicated in the neuron name by "D" followed by the number of columns that separate the input from the output arbors (details described in (Scheffer et al., 2020)). The following sample neuron illustrates the nomenclature scheme described above: FB2,3,4,5D5FB4,5v (hDeltaI) has an input arbor in layers 2,3,4 and 5 that extends through one column, its output arbor "skips" 5 columns (D5) and extends vertically, within a column, through layers 4 and the ventral half of layer 5.

A detailed account of the naming scheme for the FB neurons has already been described (Scheffer et al., 2020). We briefly use the FB "tangential" neurons as examples to illustrate the scheme. We found a total of 574 FB tangential neurons. Each neuron has widely extended processes spreading over a thin layer of the FB. The tangential processes typically extend to a single FB layer, but a few neuron types have multi-layered arborizations. Their morphological classification was achieved based on the FB layers they innervated as well as their arborization patterns outside the CX and locations of their cell bodies. **Table 2** shows the number of neuron types for each FB layer and their cell counts. The numbers in the type name indicate the layer in which they arborize (that is, FB1, FB2, …), followed by upper case letters classifying their morphological types. When connectivity subtypes were identified, lower case letters are used after underscores, for example, FB1E_a and FB1E_b. **Methods Figure 1** shows an example of identified connectivity types within a morphology type. We judged that these neurons were nearly indistinguishable based on morphology, so they were all given the same type name, FB2F. However, since CBLAST suggested these neurons should be clustered into three groups, FB2F was split into three connectivity types, FB2F_a, FB2F_b and FB2F_c, with manual confirmation of their actual connections. For each morphological or connectivity type, there are always at least two neurons because we found pairs of neurons in the right and left side of the brain (even though the left side is only partially captured by the reconstructed hemibrain volume). We also identified several intrinsic columnar neurons in the FB, including hDelta and vDelta types, and columnar projection neurons, for example, FR (FB-RUB), FC (FB-CRE) and FS (FB-SMP). In addition, in the FB, we identified the EL (EB-LAL) neuron type.

Software and code availability

We accessed the neuPrint database through its R API, neuprintr (http://natverse.org/neuprintr/). Analysis was done in R. All analysis code will be made available upon peer review

Validation Methods

We compared the version of the dataset used in this manuscript (v1.1) to an older version (v0.9) to test how much the connectivity motifs depend on tracing completeness. We selected neuron instances containing at least 200 upstream (respectively, downstream) synapses in the EB in the v1.1 dataset and quantified how their inputs changed (resp. outputs) from the older version. We only considered connections between bodyids present in both datasets and compared neuron to neuron connection weights.

We also compared different PB glomeruli that were traced to different levels of completeness. To compare inputs (respectively, outputs) of neurons of the same type across glomeruli, we chose types with at least one instance that had at least 20 upstream (resp. downstream) synapses in each of the glomeruli that were considered. Further, at least 80% of that neuron instance's total synapses in those glomeruli were required to be upstream (resp. downstream). The comparisons were made at the type-to-type level, between mirror symmetric glomeruli. When compiling type to type connection weights for a single glomerulus, we only considered instances innervating the glomerulus in question (for example, only the EPG_L3 instances in glomerulus L3) even though the strict "type" definition covers all glomeruli.

To compare the third column of the FB to the rest of the FB, we selected neurons with at least 200 upstream (respectively, downstream) synapses in the FB and at least 80% of those in FB column 3. Note that this restricts the analysis to columnar types. We compared type to type connections between these selected neurons and all other instances of neurons of the same type. To do so, we modified type definitions by dividing types between instances in column 3 and other instances.

<u>Quantifying and normalizing connectivity between individual neurons</u>

Throughout, as per neuPrint convention, the number of synapses between two neurons is the number of postsynaptic sites on the postsynaptic neurons (Scheffer et al., 2020). Note that if polyadic synapses are present, as is common, the number of presynaptic sites will not equal the number of postsynaptic sites. In all analyses in which we quantify connections, we filter out all cases with strictly less than 3 synapses between two neurons. This significantly lightens processing load and gets rid of spurious connections.

Depending on the analysis, we used several different types of measures to quantify connection strength. As a default, we used **relative weight**, which is the relative input weight from some neuron *a* to some neuron *b* within a region of interest (ROI). Thus, to compute the relative weight of a connection from neuron *a* to neuron *b* in a region X, we counted the synapses from *a* to *b* in region X and divided this number by the total number of inputs that *b* received in X. We feel that this metric is the best approximation of a functionally relevant measure. We also occasionally used a second connectivity measure, **output contribution**. Output contribution is the relative output weight from neuron *a* to neuron *b*, computed by dividing the number of synapses from neuron *a* to neuron *b* in region X by the total number of outputs that *a* sends to *b* in X. While this measure is unlikely to be functionally meaningful, it is useful for comparing the output composition of neurons (for example, **Figure 26B** bar graphs and validation).

Quantification of connectivity between neuron types

Each neuron type can consist of many neurons. If these neurons are connected to neurons of another type, the individual weights between partners may vary considerably. We therefore devised two criteria to judge if the connections between two types of neurons were significant in a given ROI. For our first criteria, we calculated the normalized total connection strength from neuron type A to neuron type B in the chosen ROI: $\frac{W}{D_A}$. $W = \sum_1^n \sum_1^m w_{ij}$ is the total weight from type A, with n individual neurons, a_1 to a_n, to type B, with m neurons, b_1 to b_m, in the chosen ROI. w_{ij} is the weight of the connection from a_i to b_j in the chosen ROI. D_A is the total number of synapses downstream of type A neurons (to all neuron types) in the chosen ROI. If the normalized total connection strength was greater than 0.8, we considered the connection significant. This criterion is meant to retain cases in which a neuron type sends a small process to a neuropil where it makes exclusive contact with another type.

For our second criterion, we calculated the relative weight contributed by neurons of type A to each type B neuron in a given ROI: $Wrel_j = \frac{(\sum_1^n w_{ij})}{U_{bj}}$. U_{bj} is the total number of upstream synapses of neuron b_j in the ROI. We then considered the sample $(Wrel_1 \ldots Wrel_m)$. In the case that $m = 1$ (i.e. type B consisted of a single instance) the connection was considered significant if $Wrel_1$ was above a predetermined threshold (in practice 0.01). Otherwise, we used R's t.test with p=0.05 to determine if the connection was significant. For significant connections, we used the mean relative weight $\sum_1^m \left(\frac{W_{relj}}{m}\right)$ to quantify the connection from type A → B.

Deciding which neurons innervate a given ROI

A given type is considered to innervate an ROI if it satisfies two criteria:.

1. Half of the instances of the type make synapses in the ROI
2. The type makes at least one significant type to type connection in the ROI.

For example, we use this method to select neuron types to include in the connectivity graph in **Figure 10F**.

In neuropil innervation plots, only those regions where significant type to type connections exist are shown.

Neuron renders

The 3D renderings of neurons presented in the Figures were generated using the visualization tools of NeuTu (Zhao et al., 2018).

Connectivity graphs

Graphs were laid out manually (for example, **Figure 6C**, **Figure 62**), by manually specifying the layer of each type and using the Sugiyama layout in igraph (for example, **Figure 60 - figure supplement 1**), or by using the stress majorization layout of the graphlayouts package (**Figure 17, Figure 58 - figure supplement 2, Figure 60, 9 to 22**).

Community detection

Community detection in **Figure 58 - figure supplement 2** is done using the label propagation algorithm implemented in the igraph package.

Morphology analysis

The electrotonic distance of a synapse from the putative spike initiation zone was computed utilizing tools from the R packages nat and igraph. We started by generating a graph representation of a neuron. Using nat, we read in a neuron's skeleton and transformed this object into a directed graph for which the weights of the edges are the Euclidean distance between the vertices and the edges were directed away from the soma. Assuming the skeleton nodes are placed in such a way to capture the curvature of the neuron's arbors, the Euclidean distance between two nodes is a decent approximation of the arbor distance between these two points. Hence, the neuron graph contains sufficient information to calculate the distance along a neuron's arbor between any two points. These neuron graphs also contained information about the width of the arbor at each vertex. Each synapse location was projected to the closest (in Euclidean distance) graph node location. For more info see http://natverse.org/nat/articles/neurons-as-graph.html.

Once we had the graph representation, we identified a root point that approximates the putative spike initiation zone. Extrapolating from studies in Drosophila projection neurons, we assumed the spike initiation zone to be near the roots of the neuron's arbors in the EB (Gouwens and Wilson, 2009). To determine the root point, we intersected the EPG skeleton with the EB ROI. This process often "fractured" the skeleton, generating several disconnected subgraphs. This was a problem because we rely on the graph representation of the neuron to calculate distances along the arbors. To "heal" these fractures, we took each connected subgraph and repeatedly added the respective parent node from the original full graph back to the subgraph until all subgraphs were connected to each other again. We then pruned down any nodes that were added during the "healing" but were unnecessary to keep this graph connected. This ensures that the parent node of the graph, which will be the root point, is the point closest to the EB for which there exists a path between this point and each of the neuron's synapse locations in the EB. Note that, for our purposes, any point between this putative spike initiation zone and where the synapses of interest occur will be a sufficient point for the root point as the ordering of the synapses will be consistent relative to any of these points. This analysis is aimed at comparing the electrotonic distance between different synapses and the spike initiation zone for a single neuron. Hence, any stretch of arbor that occurs between the spike initiation zone and all the synapses of interest will necessarily be included in the calculation of electrotonic distance for all synapses and thus will not affect our comparison. The synapses of interest are determined to be those that occur within the EB ROI.

With the root point identified, we calculated the electrotonic distance between each synapse and this point using functions from the igraph package. Since the width of the arbor changes, we calculated the length constant λ for each edge separately (prescribing the width of the edge to be the width at the terminating vertex of the edge). The electrotonic distance of that edge then is the length of the edge normalized by the edge's length constant. To determine the electrotonic distance between a given synapse and the root point, we summed the electrotonic distances of all edges between the root point and that synapse. For example, suppose the length constants of the edges in the path between a particular synapse and the root point are given by $\lambda_1, \lambda_2, \dots, \lambda_n$ and the lengths (or weights) of these edges are $w_1, w_2, \dots, w_n$, then the electrotonic distance between this synapse and the rootpoint is given by $\frac{w_1}{\lambda_1} + \frac{w_2}{\lambda_2} + \cdots + \frac{w_n}{\lambda_n}$. We assumed that the specific intracellular resistivity R_i and the specific membrane resistivity R_m are constant across the neuron. Hence, we did not need to include these values in our computation of the length constants as they become a constant factor on each electrotonic distance. Since we are comparing the electrotonic distances of synapses on the same neuron, this factor will not affect their relation.

<u>Analysis of synapse locations: 2D histograms, synapse densities and mean synapse locations.</u> The hemibrain database contains information regarding the spatial location of every synapse, along with the identity of the presynaptic and postsynaptic neuron. In addition, each neuropil has an associated mesh that defines its boundary in three dimensions, which can be used to restrict a neuron's synapses to the subset contained within a given ROI. We used these two sources of information to quantify and visualize the spatial extent of neuronal innervation patterns in various ROIs. Our plots showing synapse locations or distributions are viewed from an ROI-specific perspective, which is in most cases different from the front, top, and side views defined by the x, y, and z axes used by the hemibrain database. For example, to visualize the concentric innervation pattern of EB ring neurons, it helps to view the EB from directly above, looking through its central canal (for example, **Figure 10—figure supplement 1A**). Similarly, an FB neuron's columnar location is most easily seen by choosing a perspective that places the FB's layer in the image plane (for example, **Figure 29C**). To define these perspectives, we first performed PCA on the x, y, and z locations of all synapses within the ROI. In most cases, the individual principal components defined the major axes of each structure but in some cases, small rotations were performed to manually adjust PCA-derived axes. Finally, a coordinate transformation was used to convert each synapse location from the hemibrain reference frame to the PCA-derived reference frame. For some ROIs, synapse distributions are shown from several orthogonal perspectives (for example, **Figure 6D**). The approximate direction of the anterior-posterior, dorsal-ventral, or medial-lateral directions are indicted for each plot. For anterior-radial projections along the EB circumference, the distance of each synapse from the center of mass of the EB ROI mesh was used as an additional coordinate. The outline of each ROI was computed by finding a convex hull that traced the border around the vertex locations of the ROI mesh using the 'ahull' function from the R package 'alphahull'. To visualize projections of synapse distributions we used two-dimensional histograms of synapse counts (for example, **Figure 10—figure supplement 1A**) and normalized synapse densities (for

example, **Figure 6D**) generated with R's ggplot2 package. In some analyses, such as for defining the columnar structure of FB neuron types (for example, **Figure 29C**), we approximated the spatial location of a neuron's arbor in a given ROI as the average location of all of its synapses.

EB modularity analysis

We used Pearson's correlation to compute the similarity between the ring neuron inputs to two different EPG neurons: $\rho_{ab} = (\overline{w}_a \cdot \overline{w}_b)/(\|\overline{w}_a\|\|\overline{w}_b\|)$, where $\overline{w}_a = w_a - \frac{1}{n}\sum_{i=1}^{n} w_{ai}$ is the mean-subtracted vector of inputs onto neuron a, and $\overline{w}_b$ is the mean-subtracted vector of inputs onto neuron b for the same set of inputs. We chose this similarity measure (as opposed to cosine similarity, used elsewhere) because of the high density of connections between ring neurons and EPG neurons. We then normalized the correlation values between 0 and 1, and used this as a measure of adjacency between neurons a and b: $A_{ab} = (\rho_{ab} + 1)/2$. We ordered the resulting adjacency matrix according to the EB wedges that the EPG neurons innervate. We used these wedges to define clusters c, and then we computed the modularity of this matrix with respect to these clusters. We interpret the elements of the adjacency matrix as a measure of connection weight between the network of EPG neurons. For weighted networks, the modularity Q is given by: $Q = \frac{1}{2m}\sum_{ab}\left(A_{ab} - \frac{k_a k_b}{2m}\right)\delta(c_a, c_b)$, where $m = \frac{1}{2}\sum_{ab} A_{ab}$ is the total weight of the edges in the network, $k_a = \sum_b A_{ab}$ is the degree of neuron a, and $\delta(c_a, c_b)$ is a function that equals 1 if neurons a and b are in the same cluster (i.e., neurons a and b innervate the same EB wedge, and $c_a = c_b$), and 0 otherwise. Q can take values between 0 and 1; in our case, a value of 1 indicates perfect correlation within clusters and perfect anti-correlation between clusters. Conversely, a value of 0 indicates that correlations within each cluster are no stronger than would be expected by chance, give the average correlation of each EPG with all other EPG neurons. To measure the statistical significance of modularity values, we computed the distribution of modularity values for shuffled version of the connectivity matrix from ring neurons onto EPG neurons. For each shuffling, we randomly permuted the connections from each ring neuron onto its set of EPG outputs, and repeated this independently for each ring neuron. We then recomputed the adjacency matrix and the corresponding modularity. We reported P-values as the fraction of 1000 permutations for which the shuffled connectivity matrix had a higher modularity value than the unshuffled matrix. We repeated this analysis using both the relative weight and the synapse count as a measure of connectivity, and using connections from rings neurons to EPG neurons and vice versa.

Analyzing the Δ7 connectivity profile

Each Δ7 neuron outputs in two glomeruli that are spaced 7 glomeruli apart and receives varying input from the EPG neurons in the glomeruli in between. This creates a double peaked EPG to Δ7 connectivity profile. To find the mean profile shape, the EPG input profile to each Δ7 was fit with two Gaussians. The profile was then shifted so that the Gaussians with the higher peak

were aligned across all Δ7 neurons. The mean and standard deviation of the aligned profiles was then calculated. Note that while the EPG neurons were arranged by the order of their PB glomerular arborizations (from R8 to L8), each PB glomerulus has multiple EPG neurons. A random order of EPG neurons was chosen for plotting and fitting within each individual glomerulus.

Propagating fictive EPG activity through the Δ7 neurons

The EPG activity was assumed to have one of two shapes: a von Mises or an impulse profile. In the former case, the 16 wedges of the EPG were each assigned a value from 0 to 2π, and a von Mises function with $\kappa = 2.5$ was assumed. The profile was normalized so that the fictive activity ranged from 0 to 1. For the impulse function, all EPG neurons that arborize within a given PB glomerulus were assumed to have an activity of 1 while all others were assumed to have an activity of 0. When generating the summary statistic, the mean of the von Mises function or the location of the impulse were each shifted to cover all 16 possible permutations (one for each glomerulus).

The fictive EPG activity was then converted into a vector. This vector was first multiplied by the EPG to Δ7 connectivity matrix and then by the Δ7 to X connectivity matrix, where X is a columnar PB-EB or PB-FB neuron. The activity was then averaged across all neurons within each glomerulus. This generated a 16 x Y matrix, where Y is the number of glomeruli that each columnar type X covers in the PB. The 16 comes from the 16 different permutations. The 16 activity profiles were then aligned by circularly permuting them by their order in the permutation (for example, the first profile wasn't changed, the second was shifted by one glomerulus, the third by two, etc.). Additional alignment corrections were made to those neuron types that arborize in all PB glomeruli (the PFG and PEG neurons) or those that arborize in fewer than 16 glomeruli (the PFL1, PFL3, and PFR_b neurons) as appropriate. The mean of the aligned profiles was then calculated. A cosine was fit to the mean using the 'nls' function in R, and the residual was taken as the summary statistic.

Assigning neurons to FB columns:

FB columnar neurons were assigned to FB columns using several complementary methods that depended on cell type. First, neurons belonging to an FX type (FS, FR, or FC) were manually assigned to one of nine columns (C1 to C9) by viewing population renderings in 3D. Several types, such as FR1 and FC2A, are composed of 18 neurons total with 2 neurons per column, which made columnar assignments unambiguous. For types with a less clear columnar structure, direct comparisons to strongly columnar types could be used to aid in column assignment. FX types were assigned to columns first, since they collectively innervate all FB layers and could therefore be used as a backbone for defining the average position of the 9 FB columns. Second, PB-FB-X and vΔ neurons were assigned to FB columns using an automated approach. For each type, we began by finding the FB layer that contained the most synapses (presynaptic and postsynaptic) and then calculated each neuron's average synapse location using all three dimensions and assigned it an FB column corresponding to the closest FX-

defined column from that layer. Automated column assignments were manually checked by viewing 3D population renderings and plots of average neuron locations (for example, **Figure 29**). vΔ types contain individual neurons that innervate both C1 and C9. These neurons were manually identified and assigned to the "C0" column as a way to separate them from neurons that innervate single columns (C1 to C9). Third, hΔ types form a variable number of FB columns that depends on cell type (**Figure 31—figure supplement 2**), ranging from 6 to 12 columns. Many types, such as hΔA and hΔG, have as many neurons as they have columns, and each column shows minimal overlap with neighboring columns, which made columnar assignments unambiguous. Other types show a less clear columnar structure, such as hΔC. These types were manually assigned to a 12 column scheme by finding leftward and rightward projecting pairs that were approximately mirror symmetric.

Every FB columnar neuron in the database contains its FB column assignment as part of the "instance field", but each type's total column number needs to be taken into account, since "C2" for an FX type is not the same as "C2" for an hΔ type. In addition, it is important to recognize that for many types, column assignments are discrete, despite spatial variation in neuron locations that are rather continuous.

<u>FB motif analysis:</u>

Figure 37 employs PCA on column-to-column FB connectivity matrices, establishing the existence of three connectivity motifs. This analysis starts with column-to-column connectivity matrices, three examples of which are shown in the bottom panels of **Figure 37B**. These matrices were constructed by averaging relative weights across presynaptic and postsynaptic neuron pairs, grouped by their columnar locations. Because of this, the analysis does not take into account PB-FB offsets, only how neurons in each FB column connect to one another. Next, since FB neurons have type-specific column numbers, all column-to-column connectivity matrices were coerced to a 9 column scheme by rounding to nearest column. For example, hΔB has a total of 12 columns, and neurons in C11 (in the 12 column scheme) would get mapped to C8 (in the 9 column scheme), since $9\left(\frac{11}{12}\right) = 8.25$, which rounds to C8 (in the 9 column scheme). To these 9x9 column-to-column connectivity matrices were added a C0 column to include those vΔ neurons that innervate C1 and C9. Together, this produced 903 column-to-column connectivity matrices which all had the same dimensions: 10x10. Next, every column-to-column connectivity matrix was transformed into a vector by concatenating rows, and the resulting vectors were grouped into a new matrix whose dimensions were 903x100. We refer to this grouped matrix as the "connectivity feature matrix", since each row in the matrix contains a vectorized column-to-column connectivity matrix between two FB neuron types. Because strong connections will account for more variance than weak connections, even though their column-to-column structure could be the same, we performed PCA on the binarized connectivity feature matrix. As discussed in the results section (**Figure 37**), the first two PCs accounted for much more variance than any of the subsequent PCs. To visualize the column-to-column connectivity space, each row in the connectivity feature matrix was projected onto the first two PCs to generate the scatter plot shown in **Figure 37C**.

FB columnar steps from inputs to outputs

To count the steps between FB columnar inputs and outputs (shown in **Figure 33B**), the connectivity matrix shown in **Figure 33B** was converted to a network. The shortest path between the input node and the output node was then used to determine the number of steps that connected inputs to outputs.

ROI groups

For the analysis in the Output section, all ROIs on the right side of the brain (with the exception of the CX) were grouped as a super-ROI. All statistics were then recomputed within that super-ROI. This approach avoids ROI specific artifacts that result from large neurons that innervate small, sometimes ill-defined regions with processes that are part of a much larger arbor that spans multiple ROIs.

Similarity plots/clustering

To compare the similarity of the inputs to two different types of neurons, we computed their cosine similarity: $(w_a \cdot w_b)/(||w_a||||w_b||)$, where w_a is the vector of all input weights to a neuron a and w_b is the vector of all input weights to a neuron b for the same set of inputs. We used the same method to compare outputs, though, in that case, w_a and w_b contained all output weights. We chose this metric as it is suitable for very sparse vectors while still conveying information about the proportions in which a neuron contacts its targets. Similarity matrices were ordered (and in some cases clustered) by using hierarchical clustering based on the complete linkage algorithm.

Lateralizing neuron types

For some analyses, we subdivided types into left and right populations (respectively post-fixed with _L or _R). Right or left lateralization was determined by the position of the cell bodies, as taken from the "name" field in neuprint. The only exception to this rule was made for PFL1, PFL3, PFR_a and PFR_b. For these types, their assigned side corresponds to the side opposite to the hemisphere that the neuron innervates outside of the central complex (the LAL or the round body). In a few instances this side assignment differs from the cell body side.

Pathways

Pathways between neuron types were determined by walking downstream (or upstream) of a set of types of interest. All significant downstream (upstream) types were determined, then all significant downstream (upstream) types of those types were found, and so on, for a defined number of steps. For the analysis in the Output section, for example, 5 steps were considered. Further, for this output analysis, type-to-type significance was determined using a t-test as described above (see section: Quantification of connectivity between neuron types). For sleep pathway analysis, significance was defined as any type-to-type connection whose relative weight exceeded 0.01. We added this alternative criterion because some FB tangential types

are composed of only two neurons that can have strong but variable connections with neurons of another type, and these connections would occasionally be filtered out by our first criterion. This procedure leads to a connectivity graph, which we processed further as described below.

In the output analysis, we extended the connectivity graph by simulating contralateral connections at every step. These simulations mitigate the incompleteness of the EM volume on the left side. For example, if A_R contacts B_R with weight w in a lateralized region (in this case the full right side of the brain excluding the central complex), we added to the graph an equivalent connection from A_L to B_L on the other side. This was particularly useful when neurons crossed over the midline, but did not propagate if the symmetric neuron was not identified in the dataset.

Pathway weight

Given two types A and B in a connectivity graph, we defined the **pathway weight** from A $\longrightarrow$ B as the summed weight over all pathways, where the weights of individual pathways were obtained by multiplying relative weights along the steps of a given pathway, i.e. $W_{pathway} = \sum_{p}^{Pathways} \prod_{s}^{Steps} w_{ps}$. w_{ps} is the weight between partners *p* at step *s*. This is equivalent to multiplying the adjacency matrix A_g with itself. In practice, because of recurrence, there are infinitely many pathways, and we therefore cannot loop over all of them. For the sleep pathway analysis, we used pathways that are no longer than the number of steps used to build the graph in the first place. For the output pathway analysis, given the high recurrence in the circuit, we multiplied $A_g$until its norm converged to zero (in practice when it becomes smaller than 10^{-8}). This multiplication always converges because the metric used (the relative weight) is smaller than 1.

To determine the relative contribution a neuron makes to a set of pathways, we ran the same computation but instead of using w_{ps}, we used the weight relative normalized by the sum of weight relative contributed by the presynaptic type. This yielded a metric that sums to 1 if all the pathways emanating from a given type are summed. The CX-to-CX fraction of individual synapses in Figure 56C is the relative contribution made to CX neurons by the neuron targeted by the synapse considered.

When considering pathways from the CX, we ended pathways as soon as they looped back to the CX. When considering pathways to a set of known types, we ended pathways as soon as a known neuron was reached. This was done by removing the corresponding line (column) in the resulting matrix to be recursively multiplied by the adjacency matrix.

Immunohistochemistry:

To determine whether neurons in the SS56699 split GAL4 line expressed tyrosine hydroxylase (TH), immunohistochemical processes was performed as described in (Aso et al., 2014). Briefly, ten GFP expressing brains were fixed (2% paraformaldehyde in Schneider's medium), permeabilized in PBT (0.5% Triton X-100 in PBS), and blocked (5% normal goat serum for 90

minutes). Subsequently, brains were incubated in primary antibodies (diluted in 5% serum in PBT at 4°C for 2–4 days): Chicken anti-GFP (Abcam ab13970; 1:1000); anti-TH mouse monoclonal (SIGMA MAB318; 1:200); anti-TH rabbit polyclonal (SIGMA AB152; 1:200). After several washes (PBT for 30 min), brains were then incubated in secondary antibodies (diluted in 5% serum in PBT at 4°C for 2–4 days): Alexa 488 anti-Chicken IgY (Invitrogen A11039; 1:400); Atto 647N anti-mouse IgG (Rockland 610-156-121; 1:400); Alexa 568 anti-rabbit IgG (Invitrogen A11036; 1:400). Finally, brains were washed thoroughly (PBT four times for 30 min or longer) and mounted on glass slides for confocal imaging.

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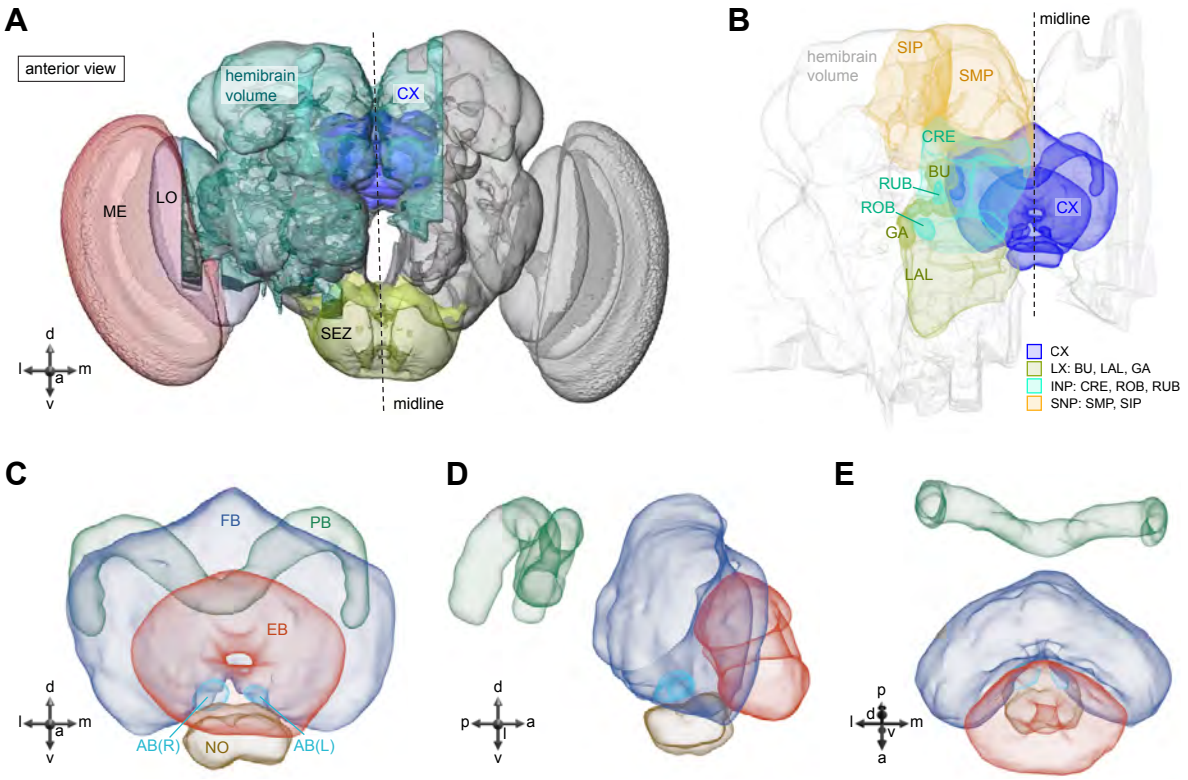
Figure 1: The central complex and accessory brain regions



CX	central complex
ME	medulla
LO	lobula
SEZ	subesophageal ganglion zone
SIP	superior intermediate protocerebrum
SMP	superior medial protocerebrum
LAL	lateral accessory lobe

CRE	crepine
BU	bulb
RUB	rubus
ROB	round body
GA	gall
INP	inferior neuropil
SNP	superior neuropil

EB	ellipsoid body
BU	fan-shaped body
PB	protocerebral bridge
NO	nodulus
AB	asymmetric body

Figure 1: The central complex and accessory brain regions

A) The portion of the central brain (aquamarine) that was imaged and reconstructed to generate the hemibrain volume (Scheffer et al., 2020) is superimposed on a frontal view of a greyscale representation of the entire Drosophila brain (JRC 2018 unisex template; Bogovic et al., 2019). The central complex (CX) is shown in dark blue. The midline is indicated by the dotted black line. The brain areas LO, ME and SEZ, which largely lie outside the hemibrain, are labeled.

B) A zoomed-in view of the hemibrain volume, highlighting the CX and accessory brain regions.

C) A zoomed-in view of the structures that make up the CX: the EB, PB, FB, AB and paired NO.

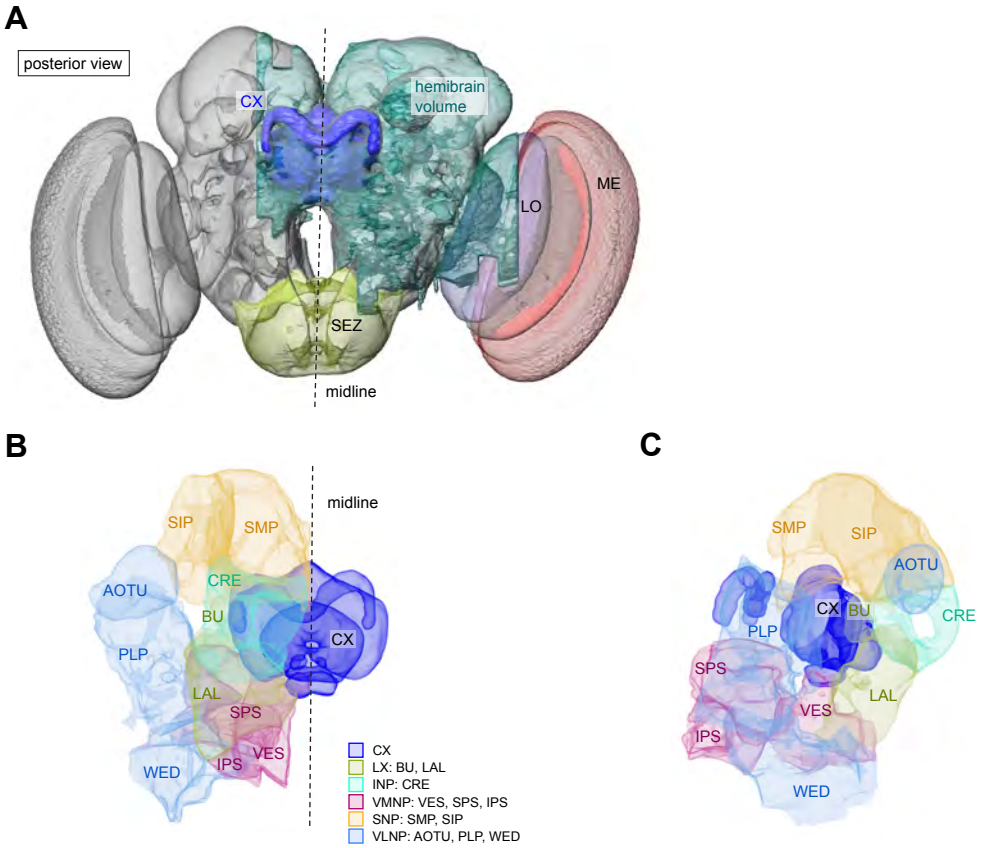
D) The same structures viewed from the lateral side of the brain.

E) The same structures viewed from the dorsal side of the brain.

The table below shows the abbreviations and full names for most of the brain regions discussed in this paper. See Scheffer et al., (2020) for details.

Anatomical axis labels: d: dorsal; v: ventral; l: lateral; m: medial; p: posterior; a: anterior.

Figure 1—figure supplement 1: The central complex and additional accessory brain regions



AOTU	anterior optic tubercle
PLP	posterior lateral protocerebrum
WED	wedge
SPS	superior posterior slope
IPS	inferior posterior slope
VES	vest

Figure 1—figure supplement 1: The central complex and additional accessory brain regions

A) Posterior view of the hemibrain volume shown in **Figure 1A**.
B) A zoomed-in view of the CX and additional accessory brain regions not shown in **Figure 1**.
C) Lateral view of **B**.

The table below shows the abbreviations and full names for the brain regions shown here that were not shown in **Figure 1**. See Scheffer et al., (2020) for details.

Figure 2: High-level schematic and an example sensorimotor pathway through the CX

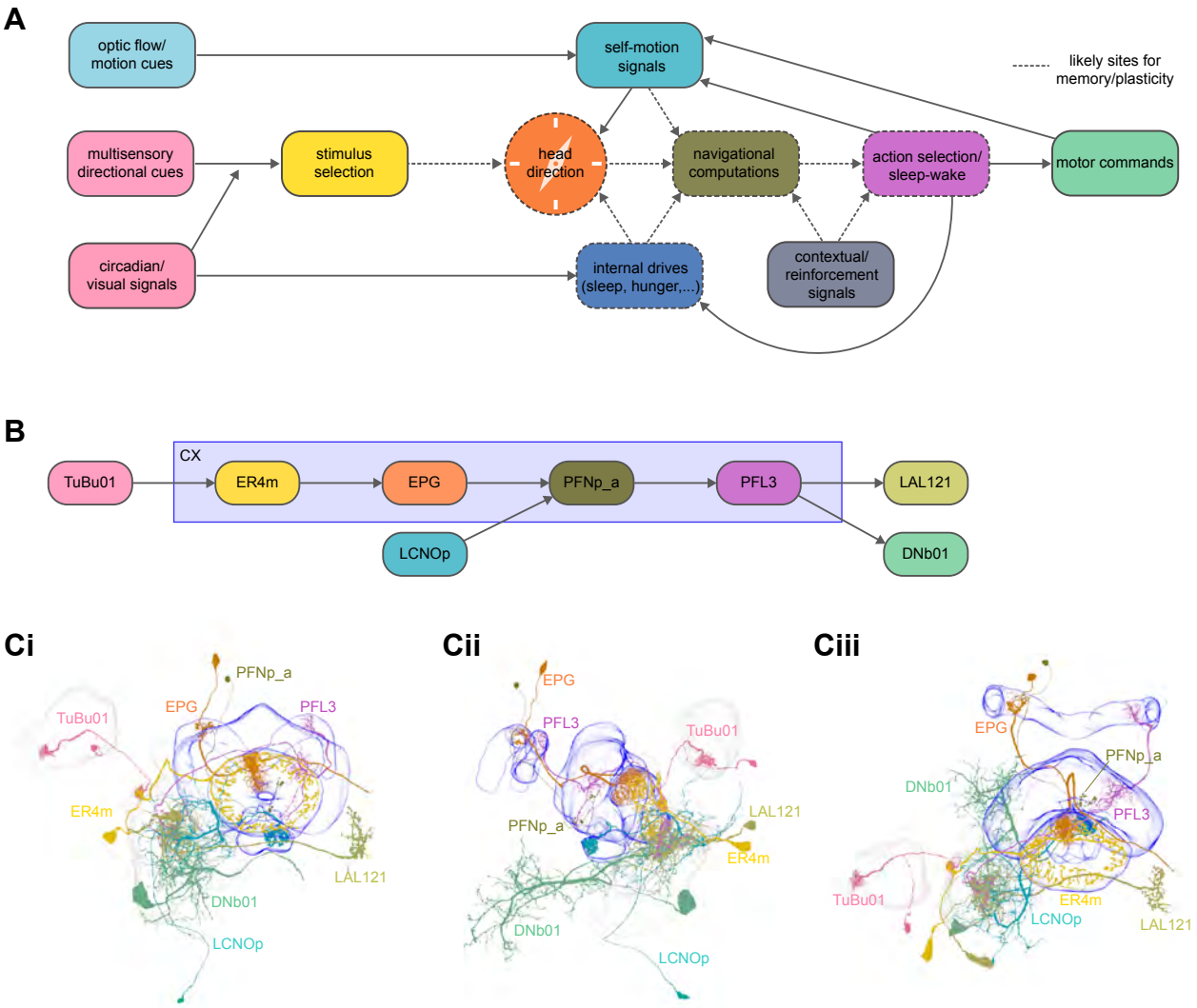


Figure 2: High-level schematic and an example sensorimotor pathway through the CX

A) The CX integrates information from multiple sensory modalities to track the fly's internal drives and its orientation in its surroundings, enabling the fly to generate flexible, directed behavior, while also modulating its internal state. This high-level schematic provides an overview of computations that the CX has been associated with, loosely organized by known modules and interactions.

B) A sample neuron-type-based pathway going from neuron types that provide information about sensory (here, visual) cues to neuron types within the core CX that generate head-direction to self-motion-based modulation of the head-direction input and ultimately to action selection through the activation of descending neurons (DNs). The neurons shown here will be fully introduced later in the manuscript. Note that the schematic highlights a small subset of neurons that are connected to each other in a feedforward manner, but the pathway also features dense recurrence and feedback.

C) **Ci-iii** show three different views (anterior, lateral, dorsal, respectively) of individual, connected neurons of the types schematized in **B**.

Figure 2—figure supplement 1: Examples of different inter-connected CX neurons

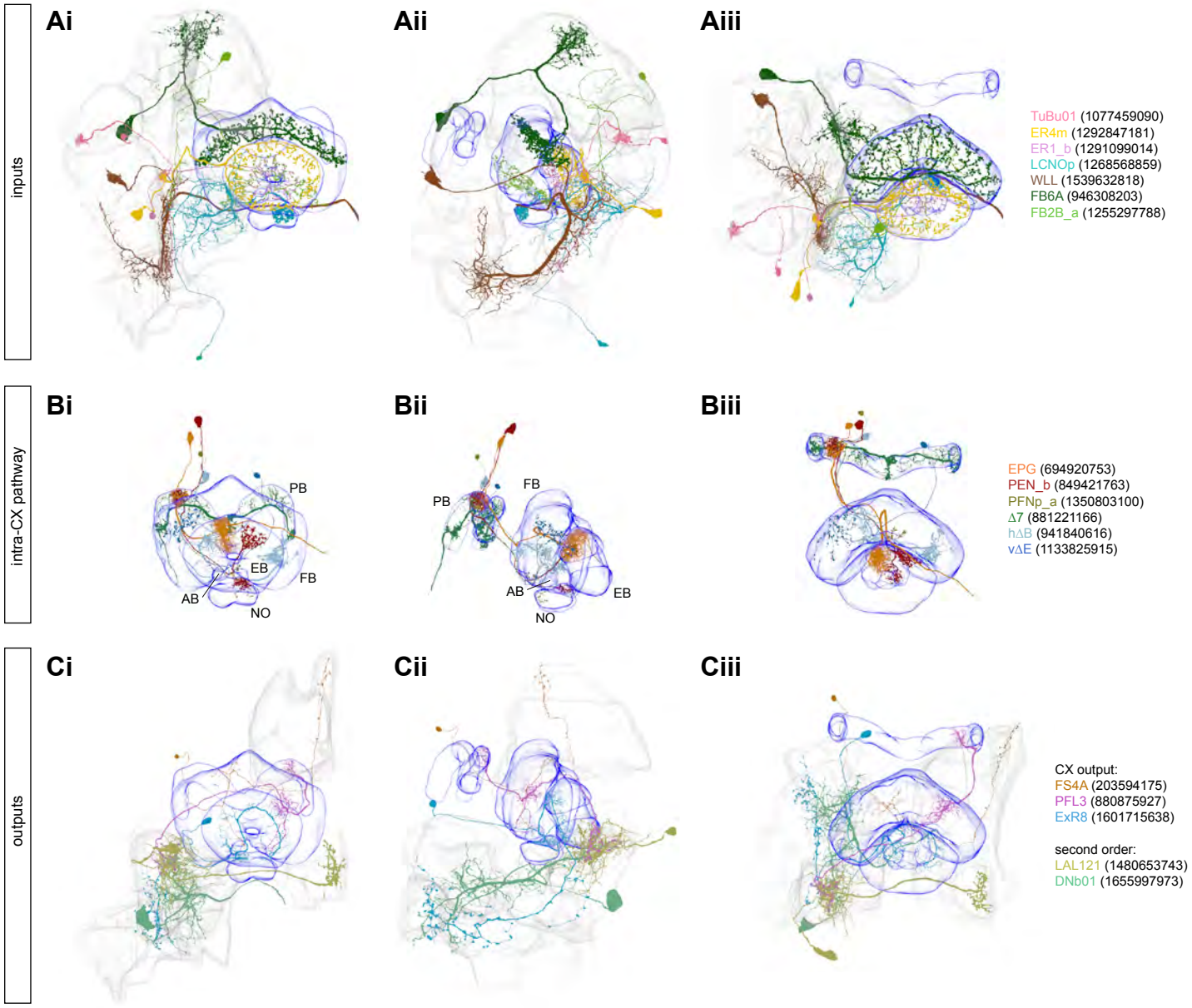


Figure 2—figure supplement 1: Selected CX input, intra and output neurons

A) Three different views (**Ai**: anterior, **Aii**: lateral, **Aiii**: dorsal, respectively) of selected individual neurons that provide input to the CX.

B) Same as **A**, but for intra-CX connections.

C) Same as **A**, but for CX output pathways.

Figure 3: Comparing EB connections before and after dense tracing

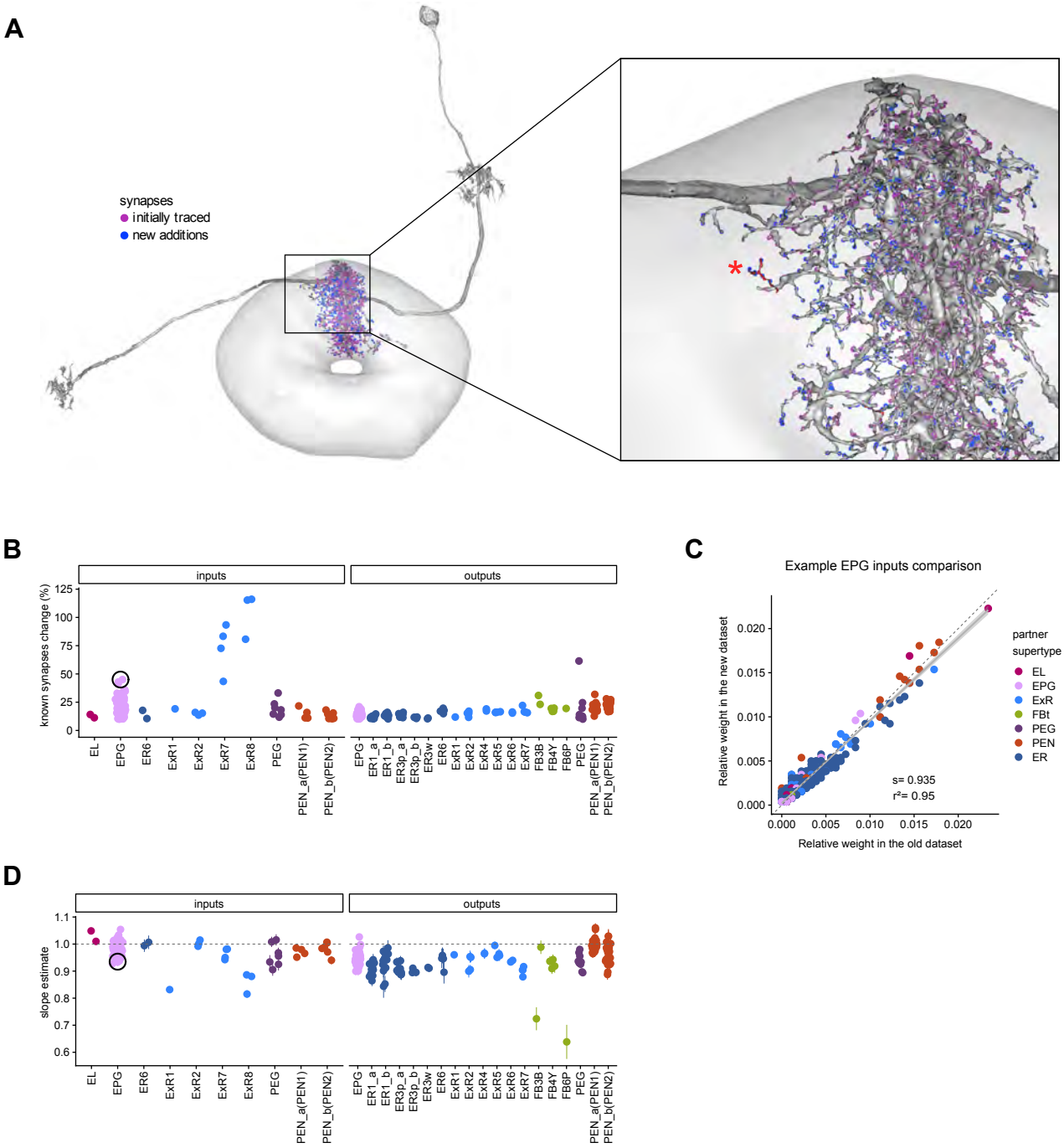


Figure 3: Quantitative impact of different levels of proofreading on neuronal connectivity in the EB

A) Morphological rendering of an example EPG neuron before and after dense tracing in the EB. Inset, zoomed in view of part of the EPG arbors highlighting changes as a result of dense tracing. The neuron segmentation is in grey. One newly added fragment is colored in red and marked with a red star. Synapses to neurons that were initially identified are in pink. Synapses to neurons that were identified after dense tracing are in blue. These new additions often resulted from joining previously unidentified fragments to their parent neuron.

B) Change in the number of input synapses from known neurons (left panel) and output synapses to known neurons made with selected EB neurons after dense tracing (selection criteria: at least 200 synapses in the EB, and at least 10% change after dense tracing). The EB neurons are ordered by type and colored by supertype (a label for their parent group). Throughout, we analyze input and output connectivity separately. The example neuron shown in **A** is circled in black.

C) Comparison of the input connectivity of the neuron shown in **A** before and after dense tracing. Each point is the relative weight of a connection between that EPG and a single other neuron. Relative weight refers to the fraction of the inputs that comes from the given partner (see Methods). The color denotes the supertype of the partner neuron. The grey line is a linear fit with 95% confidence intervals (the confidence interval is too small to be seen). The dashed line is the identity line.

D) Slope of the linear fits (similar to the one in **C**) with 95% confidence intervals for all neurons considered. Many confidence intervals are too small to be seen. The example shown in A is circled in black.

Figure 3—figure supplement 1

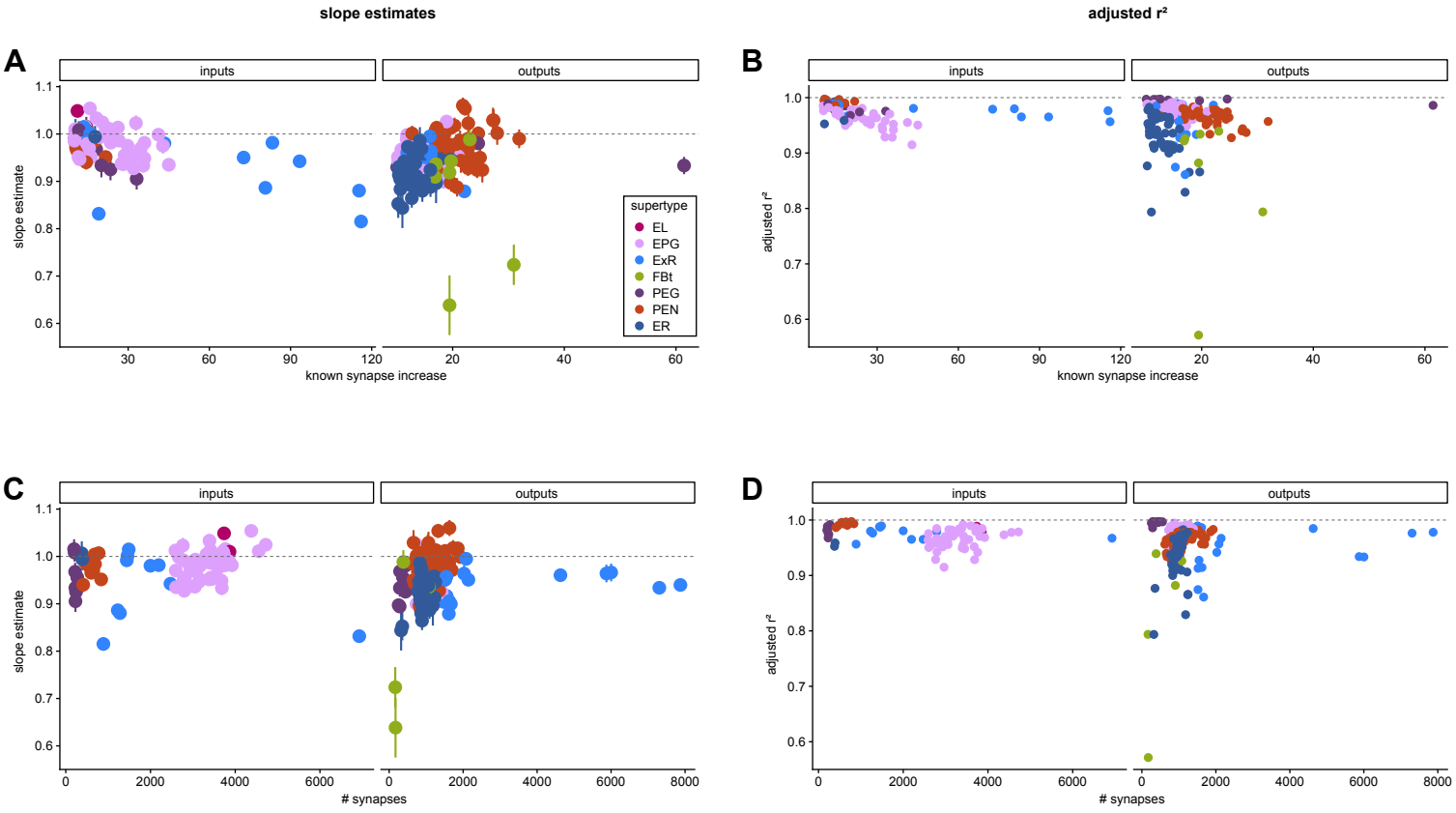


Figure 3—figure supplement 1: Influence of the amount of change from tracing on fit results.

A) Influence of the percentage change in the number of input synapses (left) and output synapses (right) made with known partners between the old and the new dataset (the same quantity as plotted in **Aii**) on the slope of the fit for each neuron considered.

B) Influence of the percentage change in the number of input synapses (left) and output synapses (right) made with known partners between the old and the new dataset (the same quantity as plotted in **Aii**) on the quality of the fit as measured with the corrected r2 for each neuron considered.

C) Influence of the total number of input synapses (left panel) or output synapses (right panel) to known partners in the new dataset on the slope of the fit for each neuron considered.

D) Influence of the total number of input synapses (left panel) or output synapses (right panel) to known partners in the new dataset on the quality of the fit as measured with the corrected r2 for each neuron considered.

Figure 4: PB glomeruli 3 comparisons

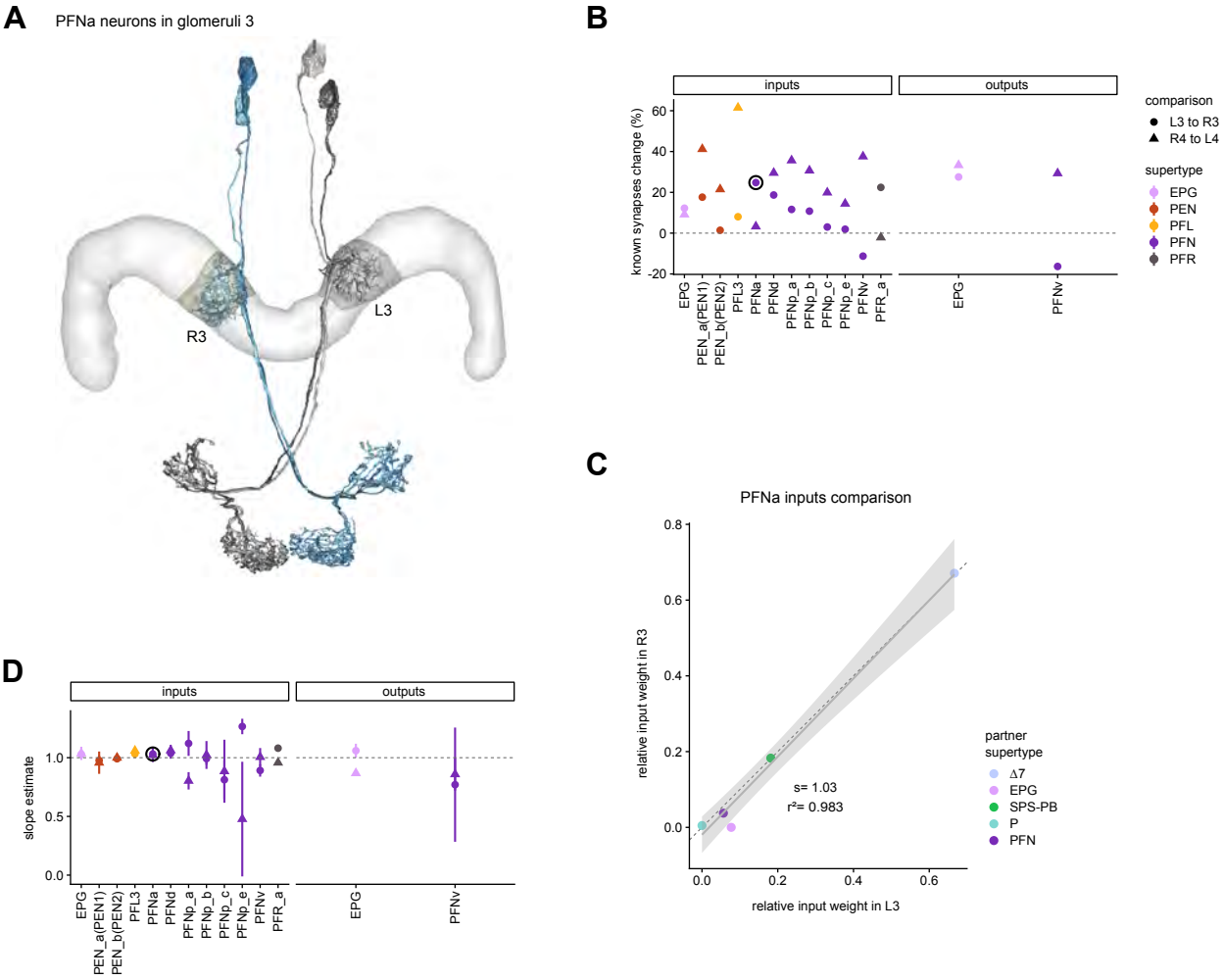


Figure 4: Differences in connectivity between mirror-symmetric PB glomeruli

We compare glomeruli that are densely proofread (L4/R3) or not (R4/L3). R or L refer to the right or left half of the PB, respectively. Each half of the PB is made up of 9 distinct glomeruli, with glomerulus 1 at the center of the brain and glomerulus 9 at the periphery.

A) Sample PFNa neurons that each arborize in a single PB glomerulus. Two arborize in L3, and the other two in its mirror symmetric glomerulus, the densely proofread PB glomerulus R3.

B) Percentage increase in input connectivity (left) and output connectivity (right) to known partners for neuron types innervating single columns between R4 and L4 or L3 and R3.
Types were selected if they had neuron instances that innervate all 4 of these glomeruli, with each instance having at least an average of 20 synapses per glomerulus and at least 80% of their PB synapses in the given glomerulus. For a given type, circles denote the L3 to R3 comparison and triangles the R4 to L4 comparison.

C) Comparison of input connectivity for the type shown in A in R3 and L3. Each point is the relative weight of a connection between that type and another neuron type. The color denotes the supertype of the partner type. The grey line is a linear fit with 95% confidence intervals. The dashed line is the identity line.

D) Slope of the linear fit (similar to the one in C) with 95% confidence intervals for all types.

Figure 4—figure supplement 1: comparing PB glomeruli at equivalent tracing levels

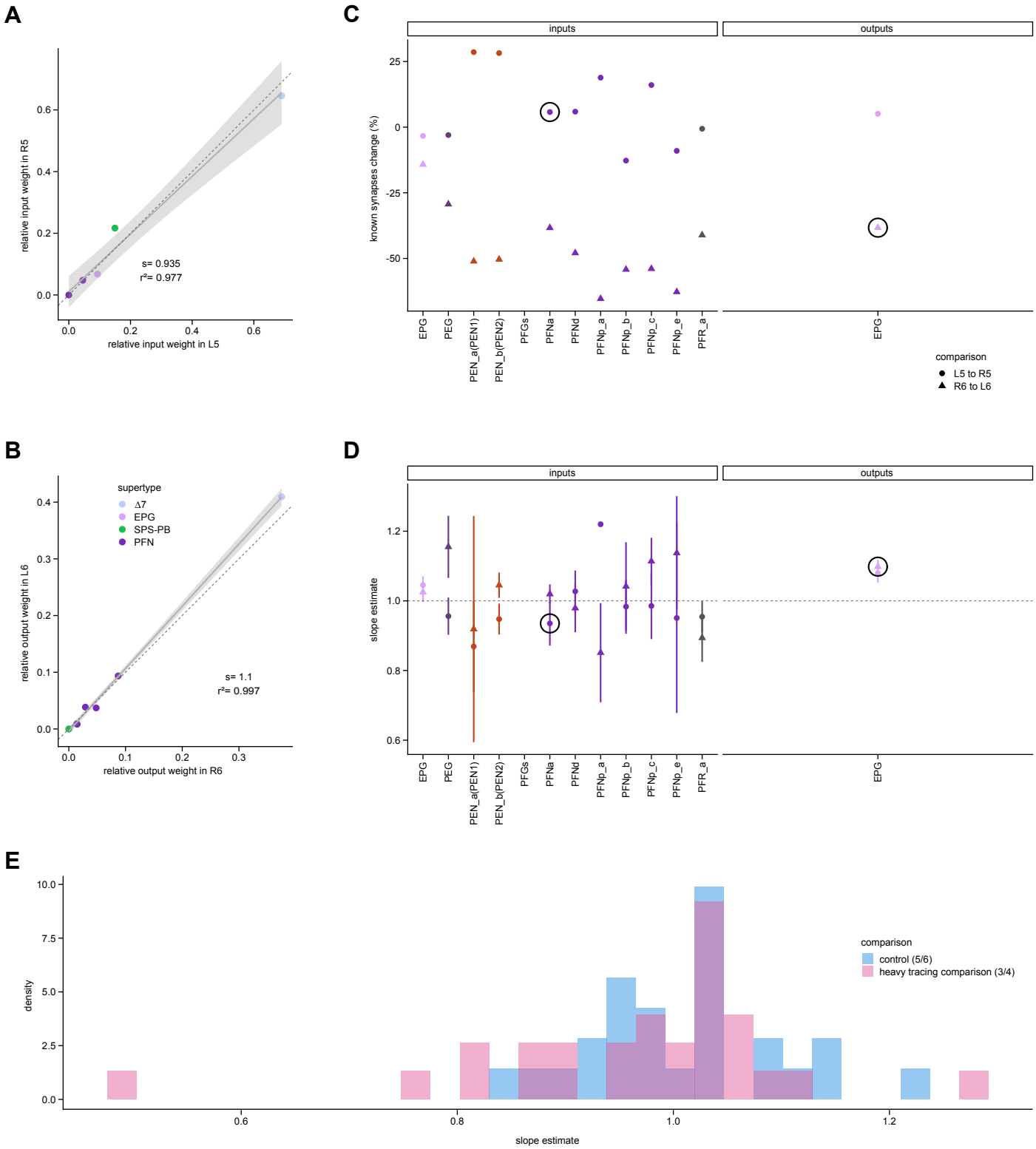


Figure 4—figure supplement 1: Comparing PB connectivity in glomeruli with similar levels of tracing

We compare glomeruli that are densely proofread (L4/R3) or not (R4/L3). R or L refer to the right or left half of the PB, respectively. Each half of the PB is made up of 9 distinct glomeruli, with glomerulus 1 at the center of the brain and glomerulus 9 at the periphery.

A) Similar to **Figure 4C**, but for PB glomeruli L5-R5.
B) Similar to **Figure 4C**, but for EPGs in L6-R6.
C) Similar to **Figure 4B** and **4D**, but for glomeruli L5-R5/R6-L6.
D) Distribution of slopes for the fits for the equally traced (glomeruli 5 and 6) and the densely vs. sparser traced (glomeruli 3 and 4) conditions.

Figure 5: Comparison between column 3 and the rest of the FB

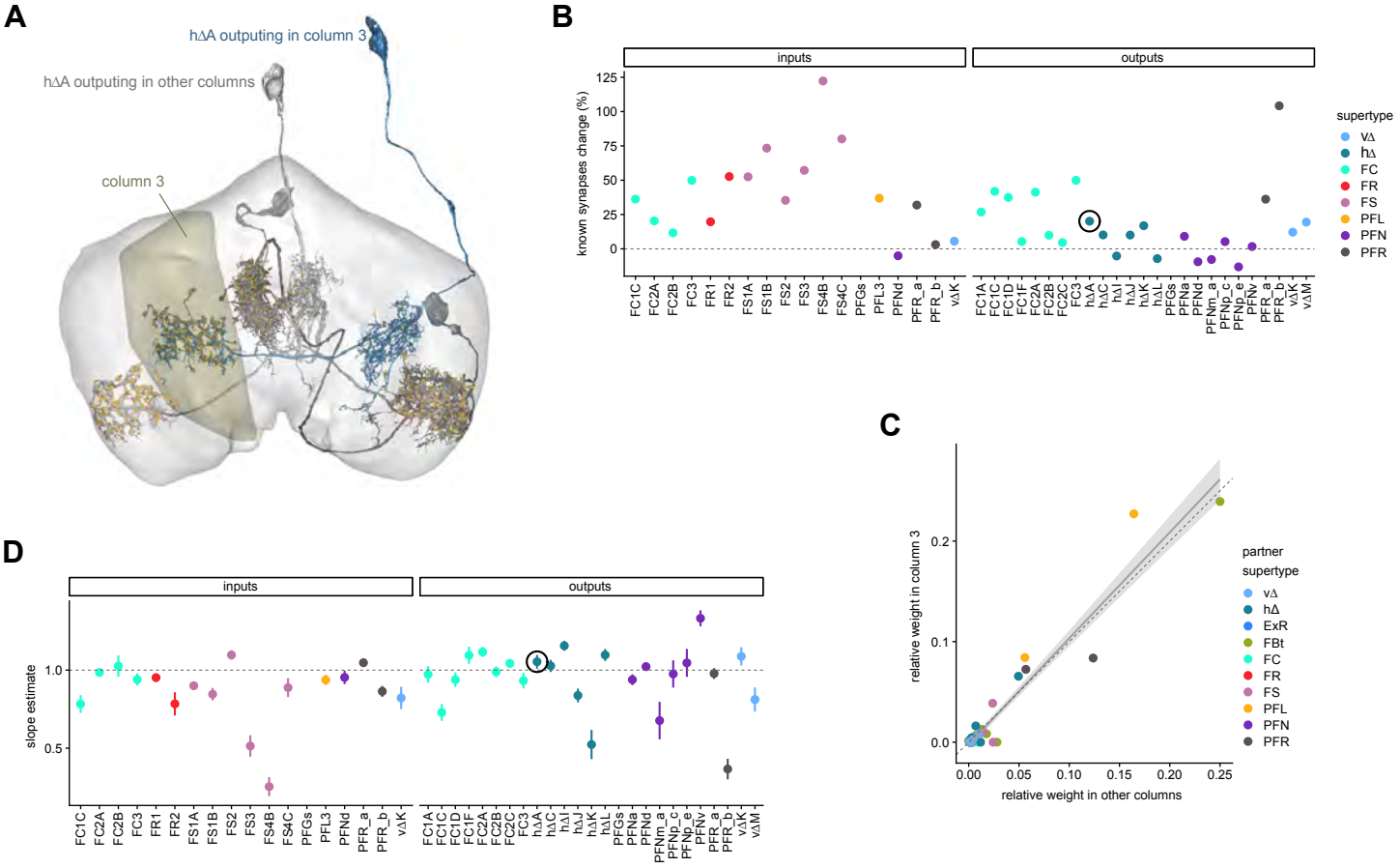


Figure 5: Differences in connectivity between a densely proofread FB column (C3) and other columns

The FB was manually divided into 8 columns (see later sections for more detail).

A) Similar Sample hΔA neurons. One (in blue) has almost all of its output synapses in the third column of the FB. The other 4 avoid column 3 altogether. Output synapses are in orange

B) Comparison of the average number of synapses to known partners per type between neuron instances innervating the heavily traced column 3 and instances innervating other columns. Types are selected as having instances innervating column 3 with at least an average of 200 synapses of a given polarity in the FB and having at least 80% of those synapses in column 3. They are compared to neurons of the same type with no synapses in column 3 (e.g., the hΔA neurons in grey in A). Plotted are the percentage increases in input connectivity (left) or output connectivity (right) to known partners for neurons in FB C3 versus other columns, by type.

C) Comparison of output connectivity for the type shown in A between neuron instances innervating column 3 and instances avoiding column 3. Each point is the average relative weight of a connection between that type and another neuron type. The color denotes the supertype of the partner type. The grey line is a linear fit with 95% confidence intervals, the dashed line is the identity line.

D) Slope of the linear fit (similar to the one in C) with 95% confidence intervals for the types considered.

Figure 6: AOTU

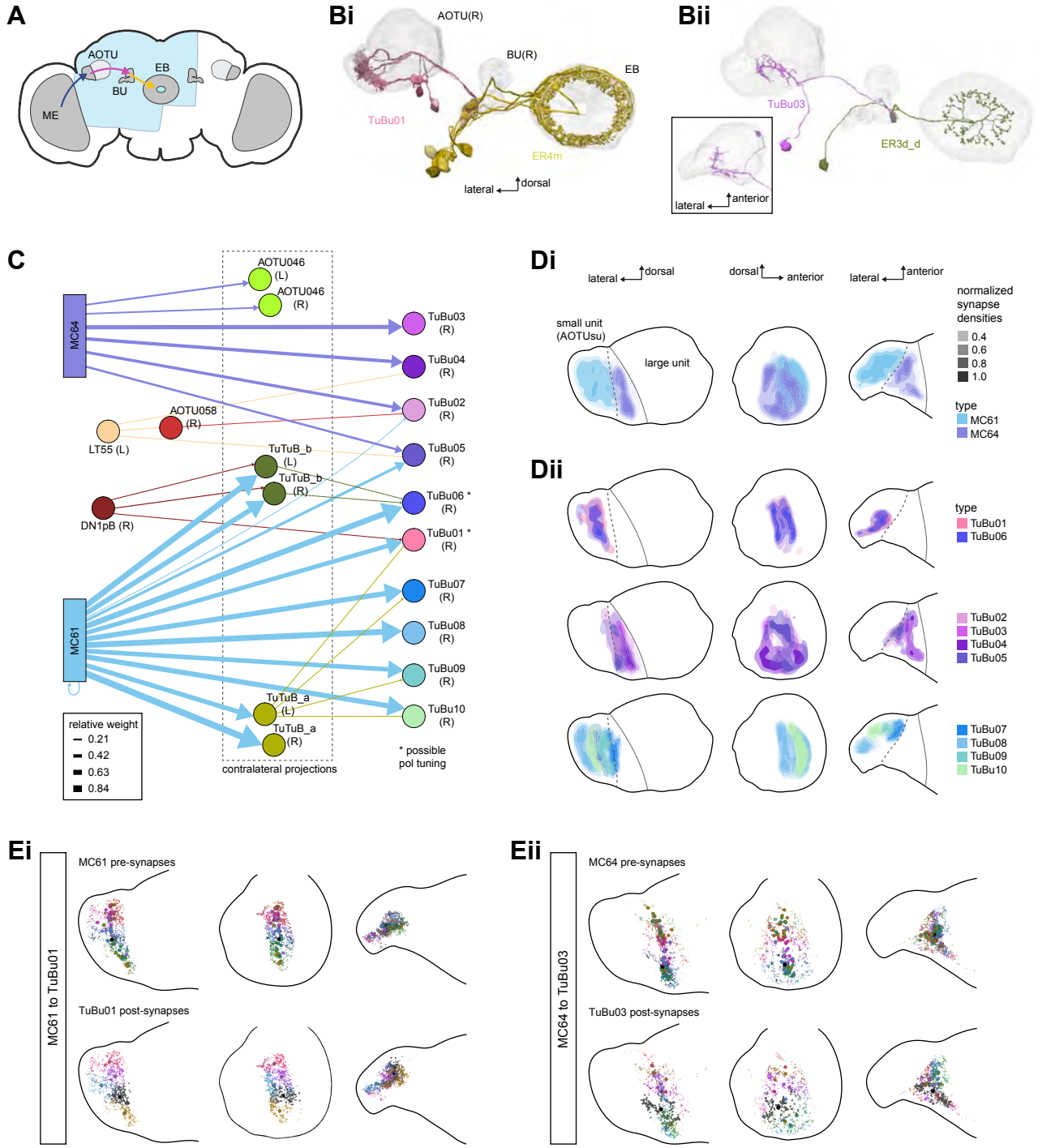


Figure 6: Overview of the anterior visual pathway and organization of the small unit of the AOTU

A) Schematic of the fly brain indicating the neuropils that are part of the anterior visual pathway (AVP), which starts at the medulla (ME) and projects via the anterior optic tubercle (AOTU) and the bulb (BU) to the ellipsoid body (EB). The AVP only passes through the smaller subunit of the AOTU (small unit of the AOTU, or AOTUsu). The light blue shaded region indicates the coverage of the hemibrain dataset.

B) Morphological renderings of a subset of neurons that are part of the AVP. Bi and Bii highlight two of several parallel pathways. **Bi**) TuBu01 neurons tile a subregion of the AOTUsu and project to the BU, where they form glomeruli and provide input to ER4m neurons. ER4m neurons project to the EB. All TuBu01 and ER4m neurons from the right hemisphere are shown. **Bii**) TuBu03 neurons also arborize in the AOTU, but these neurons target different regions of both the AOTU and BU and form larger arbors in the AOTU than do TuBu01 neurons. TuBu03 also form glomeruli in the BU, where they connect to ER3d_d. Inset shows the TuBu03 arbor in the AOTU as seen from the ventral direction.

C) Connectivity graph showing inputs to the TuBu neurons in the AOTU (significant inputs were selected using a 0.05 cutoff for relative weight (5%)). AOTU046 neurons are included here, as they provide input to TuBu neurons in the BU (see **Figure 7 & 8**). TuBu are colored from pink to green based on the regions they target in the BU (see **Figure 7**). The dashed rectangle marks neuron types that also project to the contralateral AOTU. Asterix marks TuBu types with likely tuning to polarized light based on their morphology and connectivity (see text).

D) Projections of the normalized synapse densities for medullar columnar types (**Di**) and each TuBu type (**Dii**) along the dorsal-lateral (left), the dorsal-anterior (center), and the anterior-lateral (right) plane, respectively. The synapse locations of MC61 and MC64 define two subregions of the AOTUsu, which are marked with a dashed line. Projections for the 10 TuBu types were split up in subplots for ease of readability. Types that arborize in similar regions were grouped together. Note the columnar organization of TuBu01 and TuBu06-10 as opposed to the more diffuse projections of TuBu02-05.

E) Projections of individual synapse locations from medullar columnar to TuBu neurons. **Ei**: Synapses from MC61 onto TuBu01 neurons. Projections are shown along the same planes as in D. Synapse locations are color-coded by the identity of the presynaptic neuron (MC61, top) or the postsynaptic neuron (TuBu01, bottom). The large, black-outlined dots indicate the center of mass for synapses from an individual neuron. Note that there are many more MC61 than TuBu01 neurons. **Eii**: Same as Ei, but for synapses from MC64 to TuBu03.

AVP: anterior visual pathway, ME: medulla, AOTU: anterior optic tubercle, BU: bulb, EB: ellipsoid body.

Figure 6—figure supplement 1

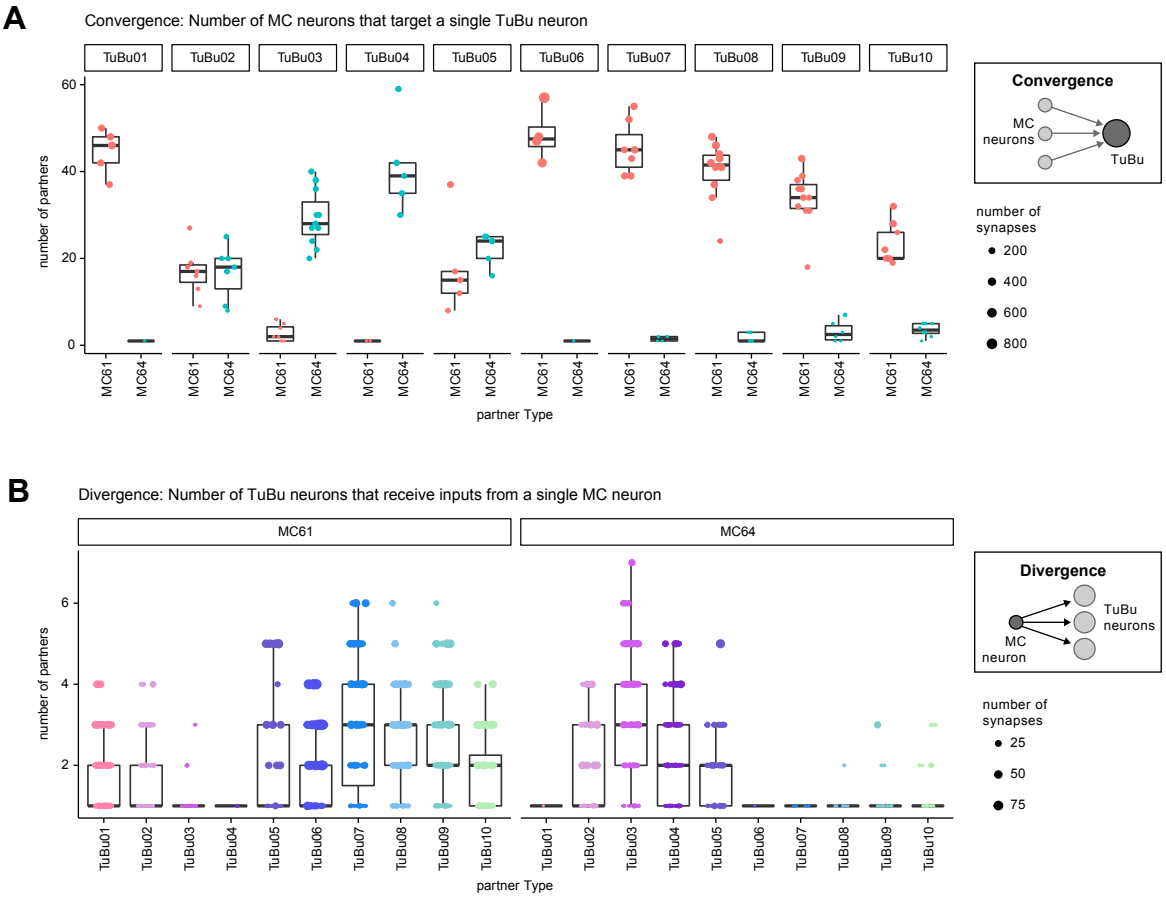
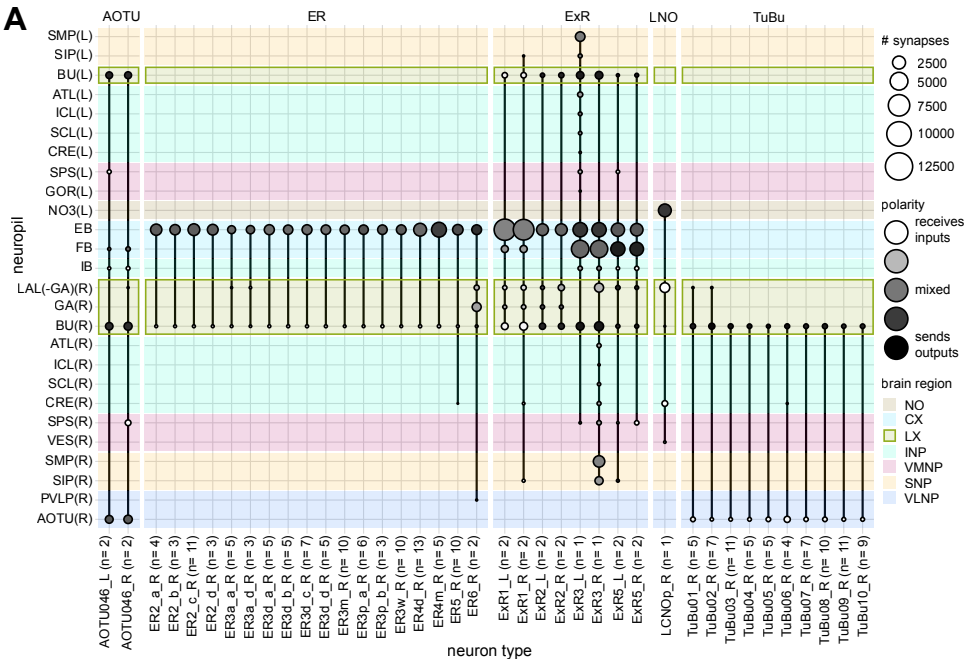


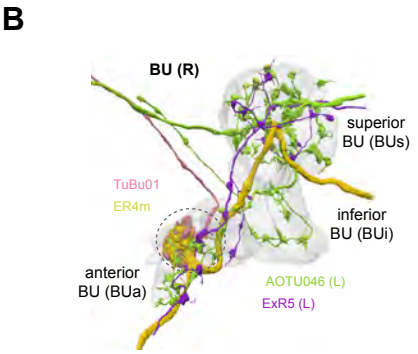
Figure 6—figure supplement 1

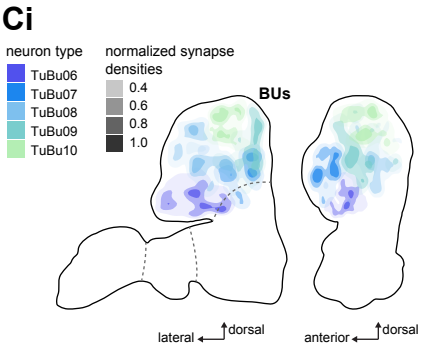
A) Quantification of the level of convergence from MC to TuBu neurons in the AOTU (see schematic on the right). Each dot represents the number of distinct MC neurons that give input to a given TuBu neuron. The total number of synapses that a TuBu neuron receives from all MC neurons of a given type is encoded in the dot size. Boxplots show interquartile range and medians. A single TuBu neuron receives input from 20-50 MC neurons of the primary MC input type.

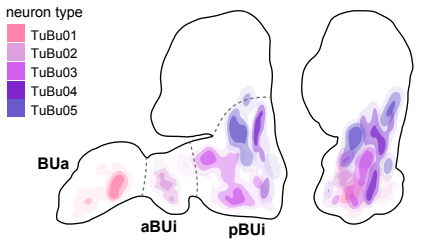
B) Quantification of the level of divergence in the connections from MC to TuBu neurons (see schematic on the right). Here a single dot represents the number of distinct TuBu neurons of a given type that a single MC neuron gives inputs to. Dot size represents total number of synapses from a MC neuron to the respective TuBu neuron.

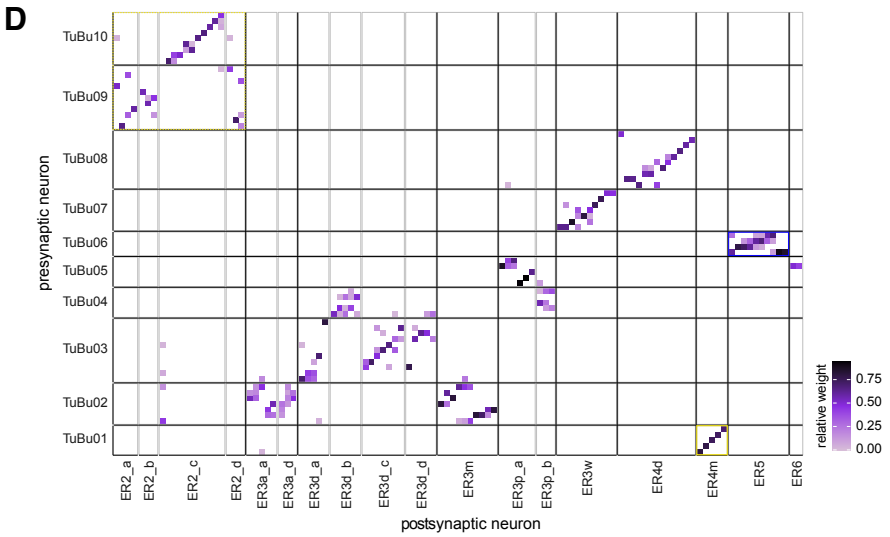
Figure 7: BU

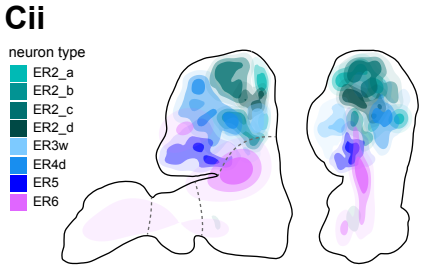

A
AOTU
ER
ExR
LNO
TuBu
neuropil
SMP(L)
SIP(L)
BU(L)
ATL(L)
ICL(L)
SCL(L)
CRE(L)
SPS(L)
GOR(L)
NO3(L)
EB
FB
IB
LAL(-GA)(R)
GA(R)
BU(R)
ATL(R)
ICL(R)
SCL(R)
CRE(R)
SPS(R)
VES(R)
SMP(R)
SIP(R)
PVLP(R)
AOTU(R)
neuron type
synapses
2500
5000
7500
10000
12500
polarity
receives inputs
mixed
sends outputs
brain region
NO
CX
LX
INP
VMNP
SNP
VLNP

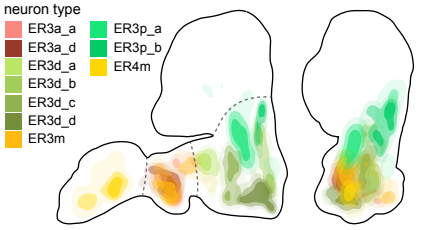

B
BU (R)
superior BU (BUs)
TuBu01
ER4m
inferior BU (BUi)
anterior BU (BUa)
AOTU046 (L)
ExR5 (L)


Ci
neuron type
TuBu06
TuBu07
TuBu08
TuBu09
TuBu10
normalized synapse densities
0.4
0.6
0.8
1.0
BUs
lateral
dorsal
anterior
dorsal


neuron type
TuBu01
TuBu02
TuBu03
TuBu04
TuBu05
BUa
aBUi
pBUi


D
presynaptic neuron
TuBu10
TuBu09
TuBu08
TuBu07
TuBu06
TuBu05
TuBu04
TuBu03
TuBu02
TuBu01
postsynaptic neuron
ER2_a
ER2_b
ER2_c
ER2_d
ER3a_a
ER3a_d
ER3d_a
ER3d_b
ER3d_c
ER3d_d
ER3m
ER3p_a
ER3p_b
ER3w
ER4d
ER4m
ER5
ER6
relative weight
0.75
0.50
0.25
0.00


Cii
neuron type
ER2_a
ER2_b
ER2_c
ER2_d
ER3w
ER4d
ER5
ER6


neuron type
ER3a_a
ER3a_d
ER3d_a
ER3d_b
ER3d_c
ER3d_d
ER3m
ER3p_a
ER3p_b
ER4m

Ei
TuBu06
ER5
Eii
Eiii

Figure 7: The BU is more than just a relay station of visual information

A) Region arborization plot of cell types that innervate the bulb (BU), showing average pre- and postsynaptic counts by region. The following types were excluded based on their small number of synapses in the BU: ExR7, SMP238, CRE013, LHCENT11, LHPV5l1.

B) Morphological rendering of one AOTU046 and one ExR5 neuron, which both arborize widely within the BU, as well as one TuBu01 and one ER4m neuron, which form a glomerulus (dashed circle). Different anatomical zones of the BU are labeled.

C) Projections of the normalized synapse densities for TuBu types (**Ci**) and ER types (**Cii**) along the dorsal-lateral (*left*) and the anterior-lateral (*right*) planes of the BU, respectively. Borders between different anatomical zones are indicated with dashed lines. To easy readability, synapse densities of TuBu and ER types that arborize in the BUs versus the BUi or BUa are displayed separately. All populations of neurons, except ER6, form glomeruli.

D) Neuron-to-neuron connectivity matrix of connections from TuBu neurons to ER neurons. Neurons were grouped according to type and, within a type, ordered such that most connections lie on a diagonal. The yellow boxes mark connections between (putatively) polarized light tuned neurons. The blue box marks connections of sleep-related neurons.

A) Morphological rendering of the glomeruli formed by TuBu06 and ER5. **Ei**: All TuBu06 and ER5 neurons. **Eii**: Same as Ei but just TuBU06 neurons. **Eiii**: Same as Ei, but with only one ER5 neuron shown to highlight how a single ER neuron can target multiple glomeruli. Top view shown on the right.

BUs: superior bulb, BUi: inferior bulb; BUa: anterior bulb; pBUi: posterior inferior bulb, aBUi: anterior inferior bulb.

Figure 7—figure supplement 1: Connectivity motifs between TuBu and ring neurons

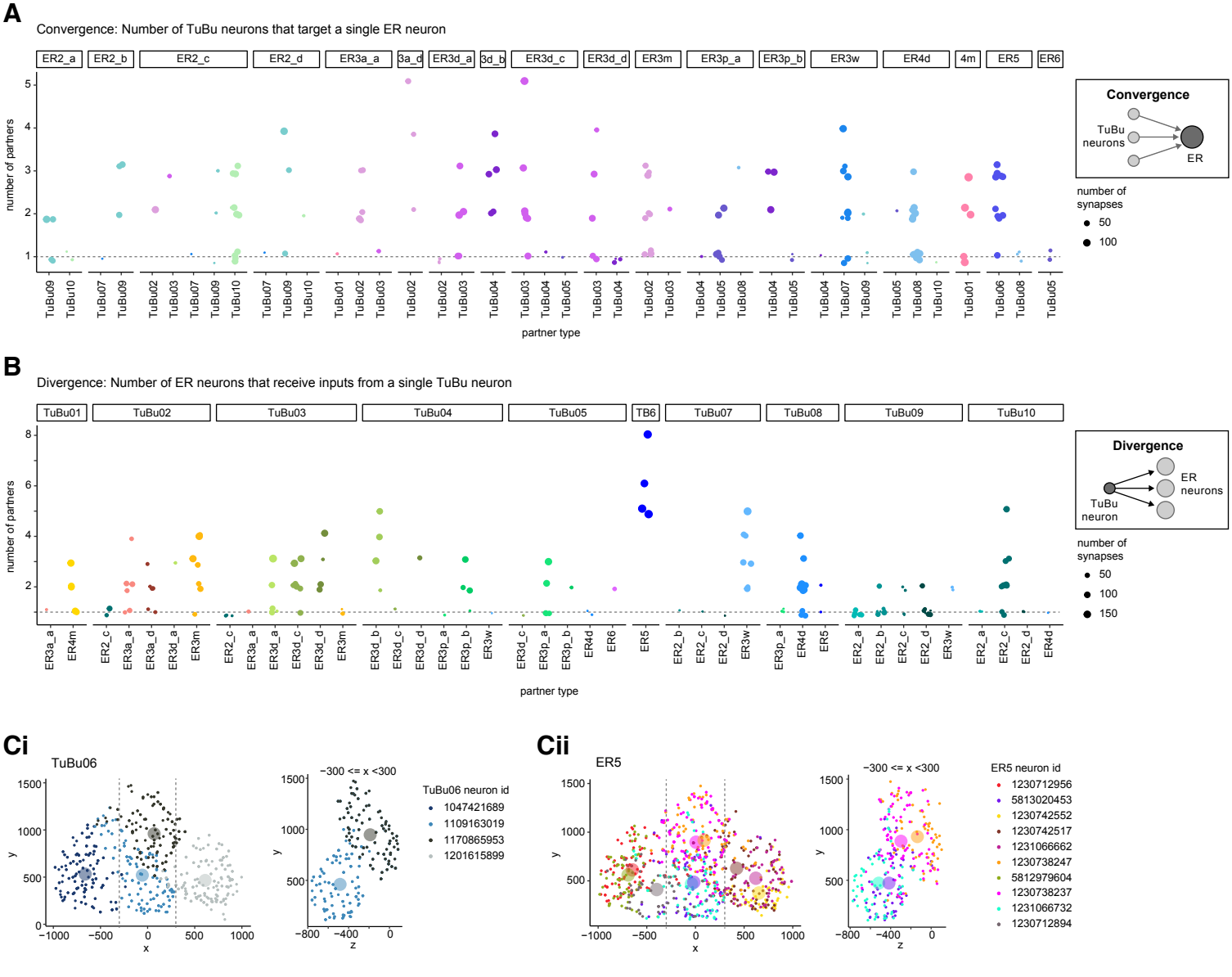


Figure 7—figure supplement 1

A) Quantification of the level of divergence in the connections from TuBu to ER neurons. Visualization as in **Figure 6—figure supplement 1A**.

B) Quantification of the level of convergence and from TuBu to ER neurons in the right BU. Visualization as in **Figure 6—figure supplement 1B**.

C) Scatter plot of synapse locations for TuBu06 neurons (**Ci**) and ER5 neurons (**Cii**) in the right BU. Synapses are color coded based on body id. Synapse locations were projected onto projected onto the x/y axis (left) and z/y axis (right). For the z/y projection only a thin slice as indicated by the dashed lines in the x/y plot, is considered.

Figure 8: Source of contralateral visual information

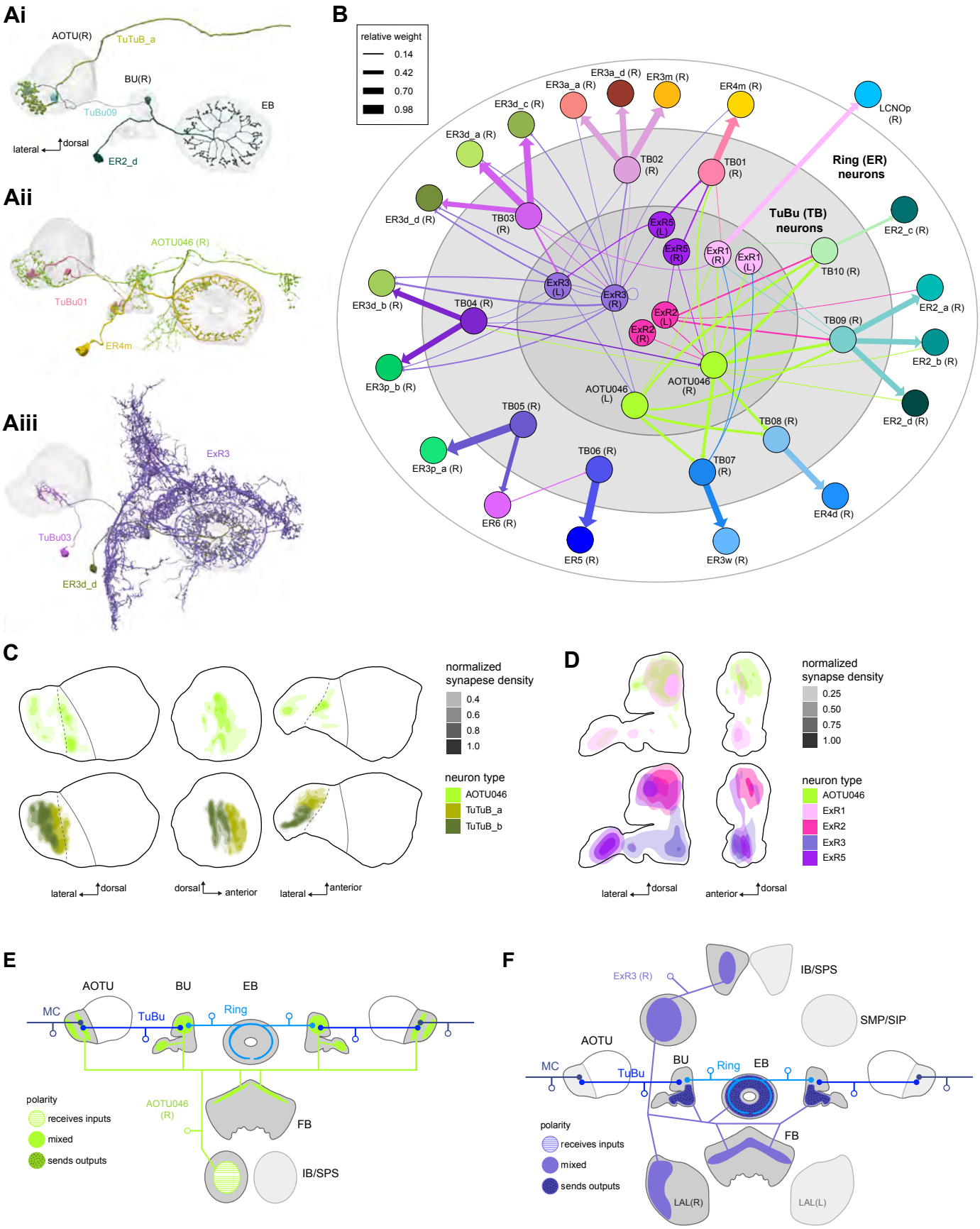


Figure 8: Source of contralateral visual information

- **A)** Morphological renderings of neurons in the anterior visual pathway together with neurons that connect to the contralateral AOTU and/or BU. **Ai**: TuBu09, ER2_d and TuTuB_a. **Aii**: TuBu01, ER4m and AOTU046. **Aiii**: TuBu03, ER3d_d and ExR3.
- **B)** Connectivity graph of TuBu and ER neurons as well as other neurons, ExR and AOTU046, that provide input to TuBu and ER neurons in the right BU. To highlight the organizational principles of connectivity in the BU, the nodes of ER neurons are placed in an outer ring, TuBu (for brevity named TB here) in a middle ring, and ExR and AOTU046 in a central circle.
- **C)** Projections of the normalized synapse densities of AOTU046 and TuTuB neurons in the right AOTU. Visualization as in **Figure 6D**.
- **D)** Projections of the normalized synapse densities of AOTU046 and ExR neurons in the right BU. Visualization as in **Figure 7C**.
- **E)** Schematic of the projection pattern of the right AOTU046 neurons, piecing together innervations on the left hemisphere from the left AOTU46 neurons. Qualitative indication of input/output ratios per region are given based on region innervation plots shown in **Figure 7A**.
- **F)** Schematic as in D, but for the right ExR3 neuron.

Figure 8—figure supplement 1

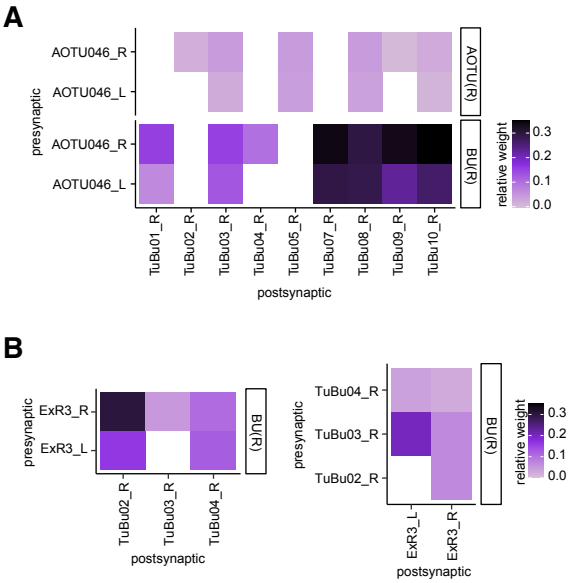


Figure 8—figure supplement 1:

A) Type-to-type connectivity matrices from AOTU046 to TuBu neurons for the right AOTU and BU. Connectivity shown on per-type level.

B) Type-to-type connectivity matrices as in **A**, but for ExR3 input to TuBu (left) and TuBu input to ExR3 (right) in the right BU.

Figure 9: Putative antennal mechanosensory input to ring neurons

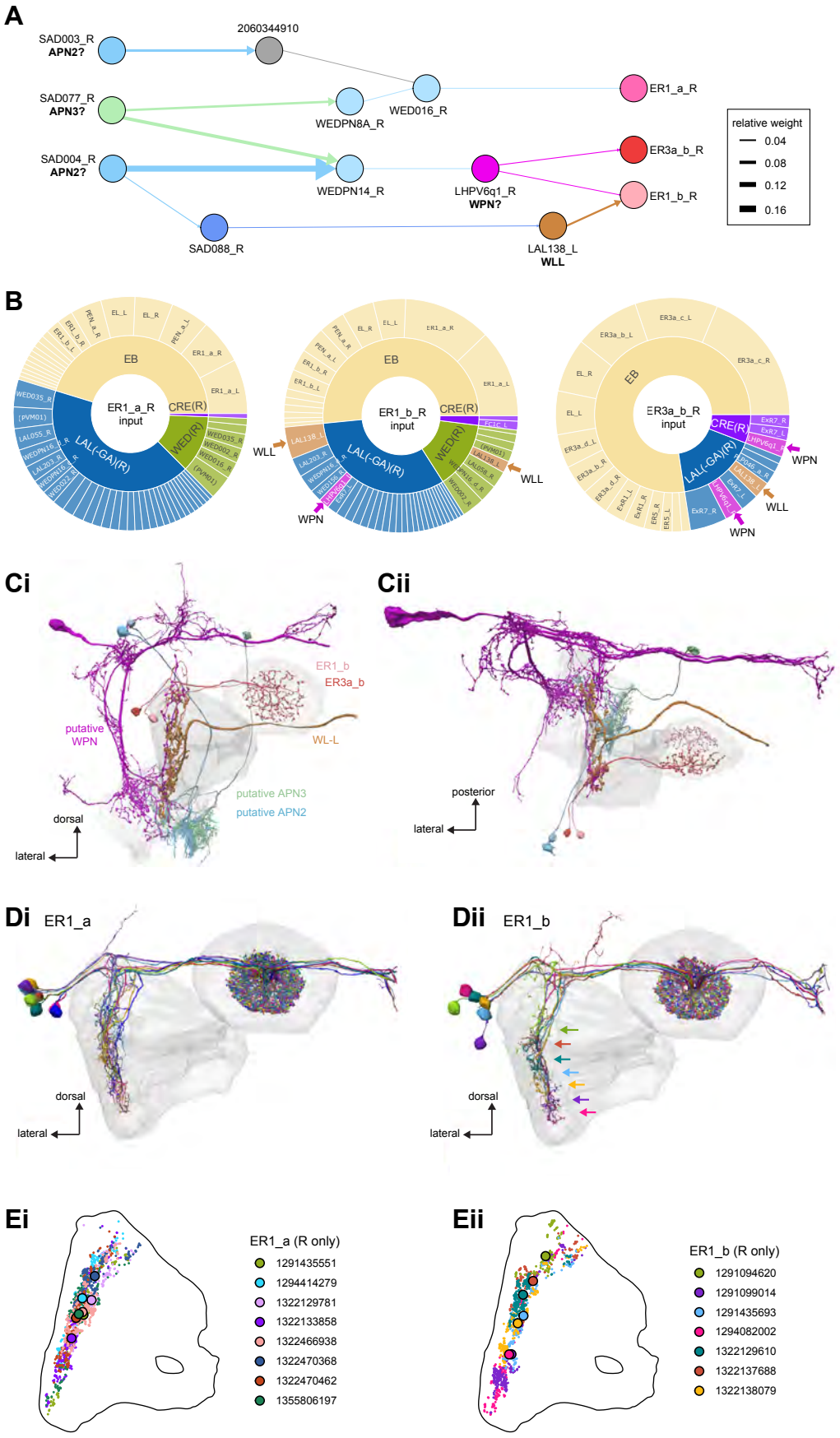


Figure 9: Mechanosensory input to the EB

A) Connectivity graph of paths from putative APN2 and APN3 to ER neurons. Only pathways with a minimal total weight of 1E-05 and a maximum length of 5 were considered.

B) Hierarchical pie charts showing the fraction of inputs from various neuron types separated by input region for ER1_a (left), ER1_b (center) and ER3a_b (right) neurons. The fractions represent the average per type (computed only for neurons from the right hemisphere). Arrows highlight inputs from WPN (LHPV6q1) and WL-L (LAL138).

C) Morphological renderings of putative APN2 (SAD003, SAD004), APN3 (SAD077), WPN (LHPV6q1) and WL-L (LAL138) neurons as well as ER1_b and ER3a_b. **Ci**: Frontal view. **Cii**: Top view.

D) Morphological renderings of ER1_a (**Di**) and ER1_b (**Dii**). Only neurons with cell bodies in the right hemisphere are shown. Individual neurons are colored differently.

E) Projections of synapse locations of the neurons shown in **D**. Synapses are colored by neuron identity (see legend). Larger, black-outlined dots mark the mean synapse position (center of mass) of each neuron. Synapses of individual ER1_b neurons separate along the dorsal-ventral axis (**Eii**) whereas synapses of ER1_a neurons are more spatially mixed (**Ei**).

Figure 9—figure supplement 1

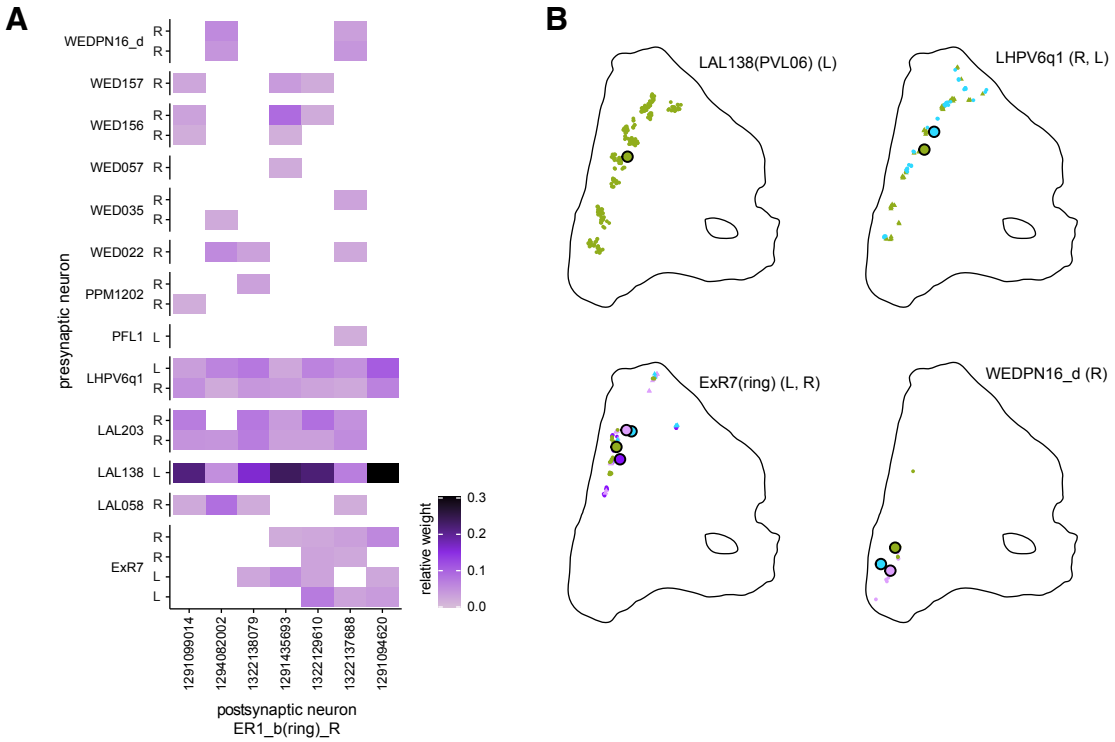


Figure 9—figure supplement 1: Organization of inputs to ER1_b in the LAL

A) Neuron-to-neuron connectivity matrix for inputs to ER1_b neurons in the LAL. The ER1_b neurons are sorted by their mean synapse location along the dorso-ventral axis of the LAL.

B) Synapse positions of individual neurons from four major input types of ER1_b. Visualization as in **Figure 9E**.

Figure 10: Overview of the organization of the EB

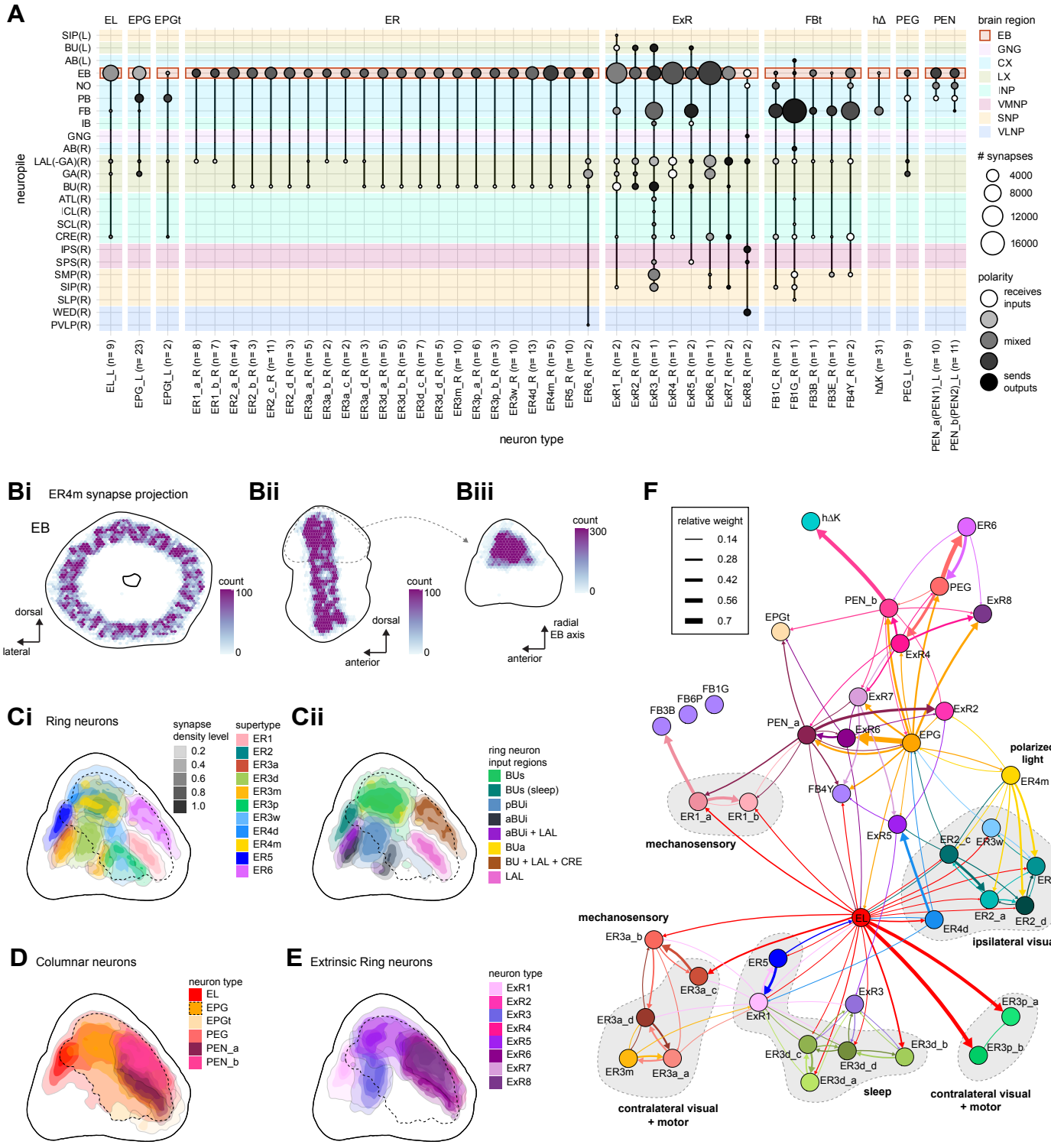


Figure 10: Overview of the organization of the ellipsoid body

A) Region arborization plot of neuron types that innervate the ellipsoid body (EB), showing average pre- and postsynaptic counts by region.

B) Two-dimensional histograms of synapse counts of ER4m after projection onto the EB cross sections along the dorso-lateral (**Bi**), dorso-anterior (**Bii**) and anterior-radial axes (**Biii**). The dashed line in **Bii** outlines the cross section shown in **Biii**.

C) Normalized synapse densities of ring neurons onto the EB cross section along the anterior-radial axes (see dashed outline in Bii, solid outline in Biii). **Ci**: The synapse densities are color-coded by ring neuron type. **Cii**: The synapse densities are color-coded by input regions. The dashed line indicates the outline of the EPG synapse density as seen in **D**, for reference.

D) Same as in **Ci**, but for columnar EB neurons.

E) Same as in **Ci**, but for ExR neurons.

F) Connectivity graph of neurons innervating the EB. Relative weight as measured on a type-to-type level has been mapped to the edge width. Grey shapes indicate groups of neuron types that likely share similar functional tuning based on existing literature. Only connections with a minimal relative weight of 0.05 are shown.

Figure 10—figure supplement 1: ER (ring) neurons

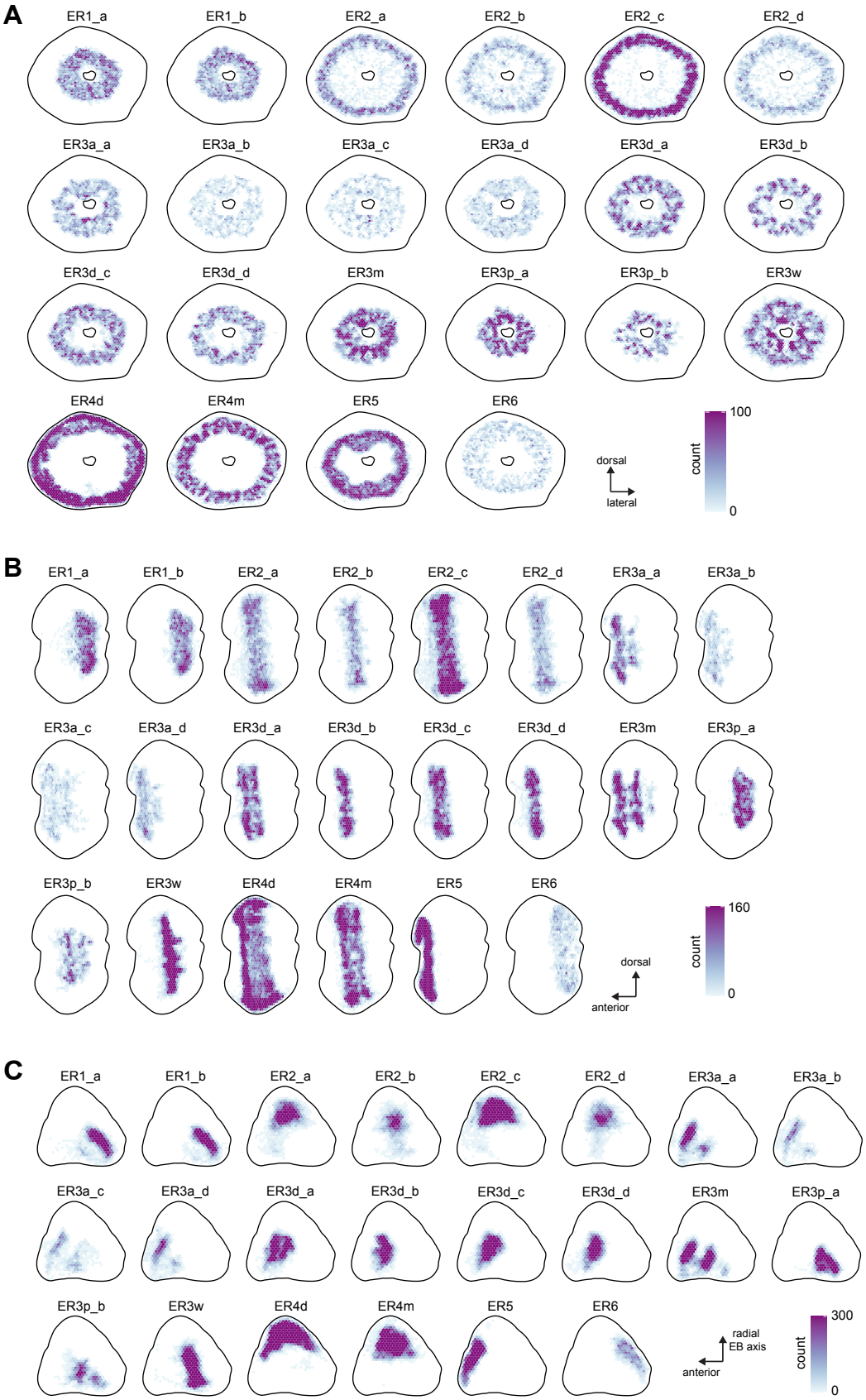


Figure 10—figure supplement 1

Two-dimensional histograms of synapse counts of all ring neurons after projection onto the EB cross sections along the dorso-lateral (**A**), dorso-anterior (**B**) and anterior-radial axes (**C**).

Figure 10—figure supplement 2: Columnar neurons

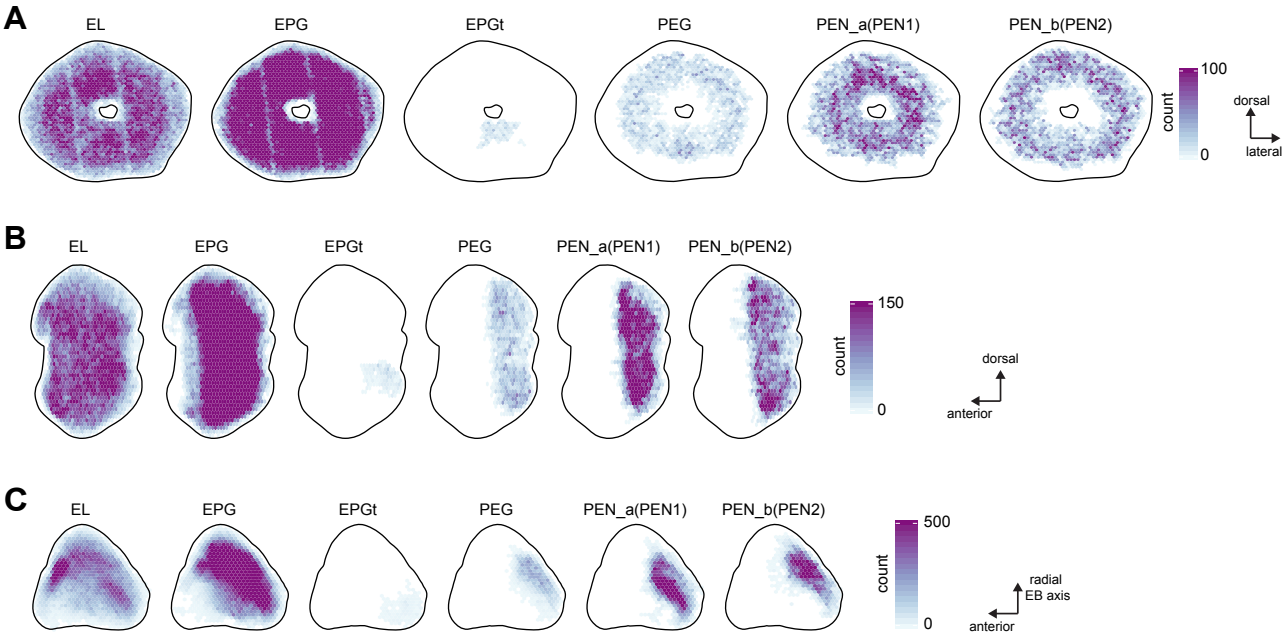


Figure 10—figure supplement 2

Two-dimensional histograms of synapse counts of all EB columnar neurons after projection onto the EB cross sections along the dorso-lateral (**A**), dorso-anterior (**B**) and anterior-radial axes (**C**).

Figure 10—figure supplement 3: ExR neurons

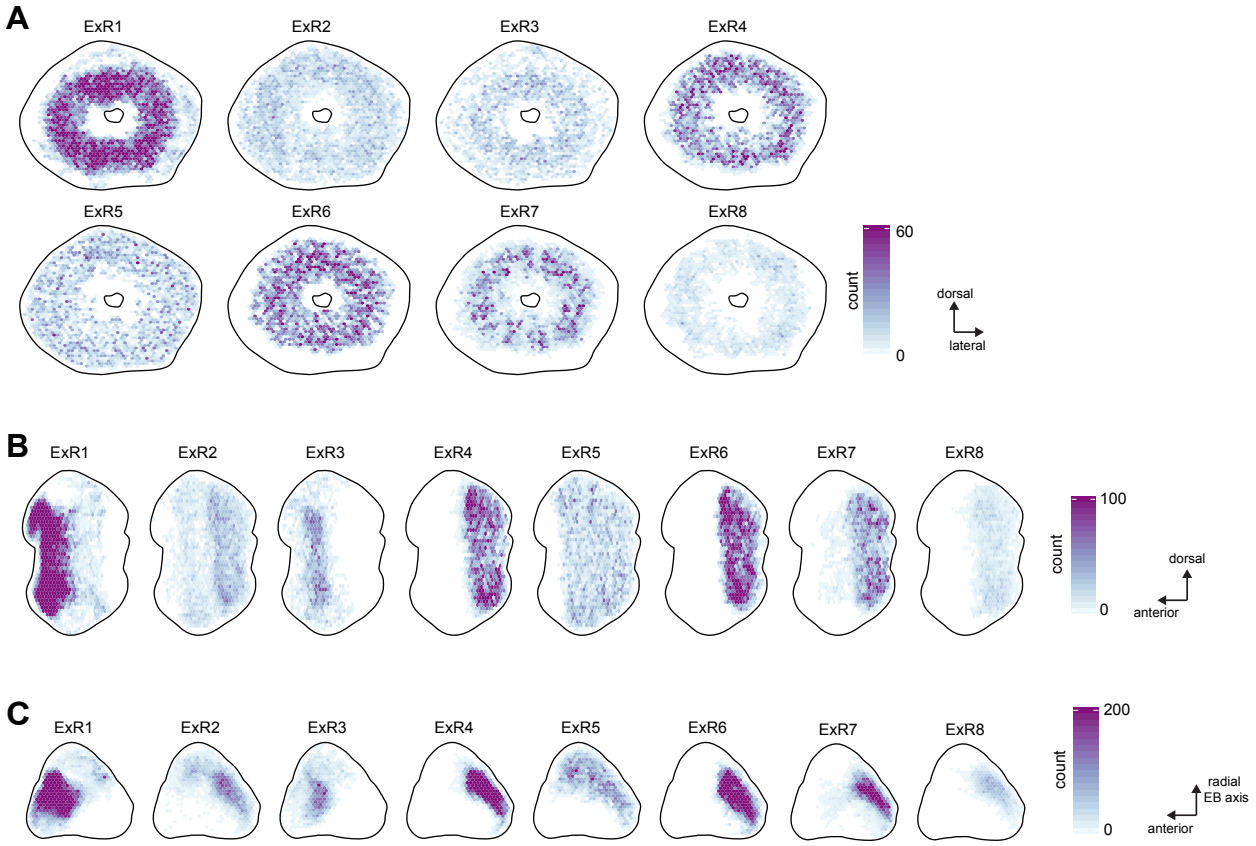


Figure 10—figure supplement 3

Two-dimensional histograms of synapse counts of all ExR neurons after projection onto the EB cross sections along the dorso-lateral (**A**), dorso-anterior (**B**) and anterior-radial axes (**C**).

Figure 10—figure supplement 4:
Anterior-radial cross sections along EB circumference

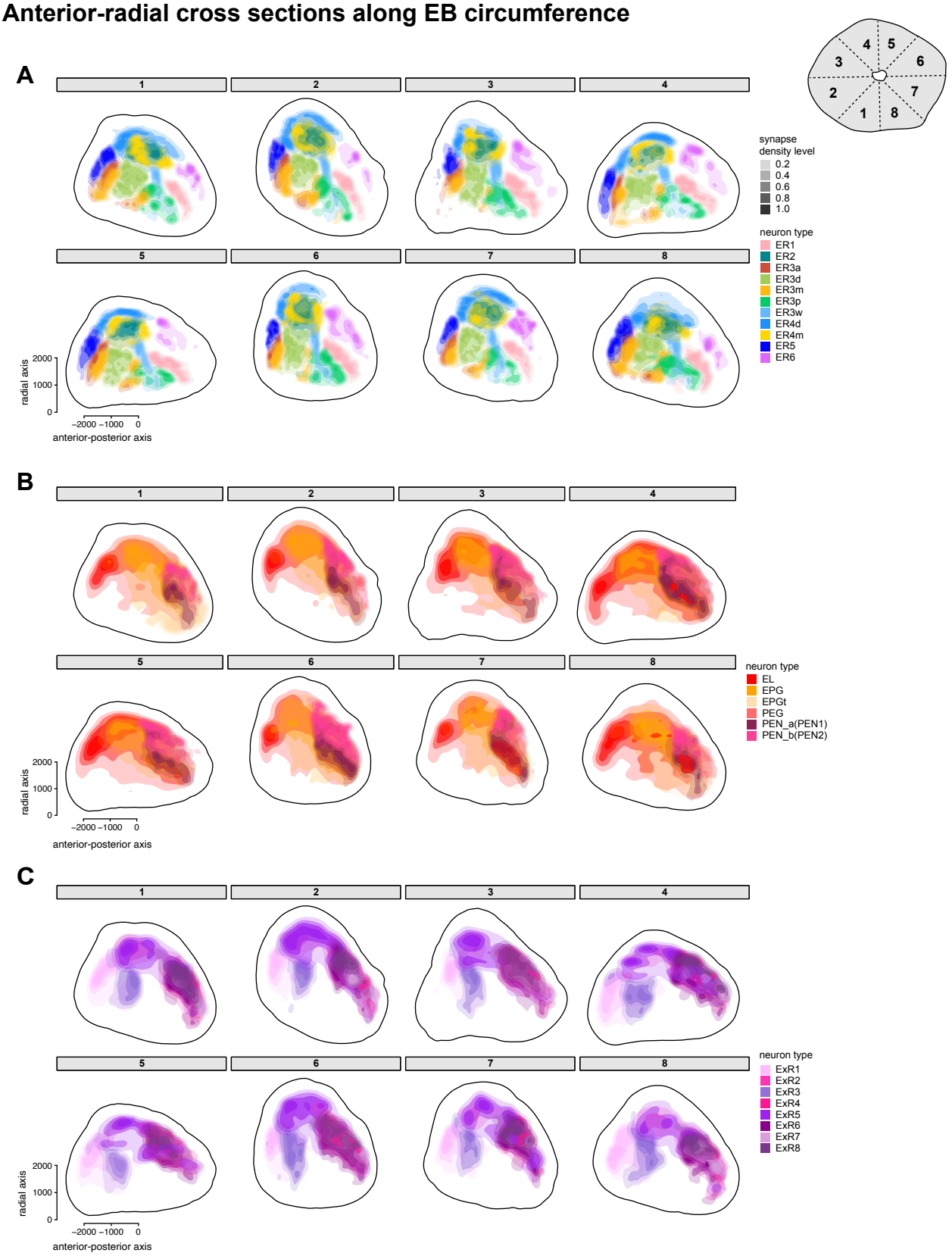
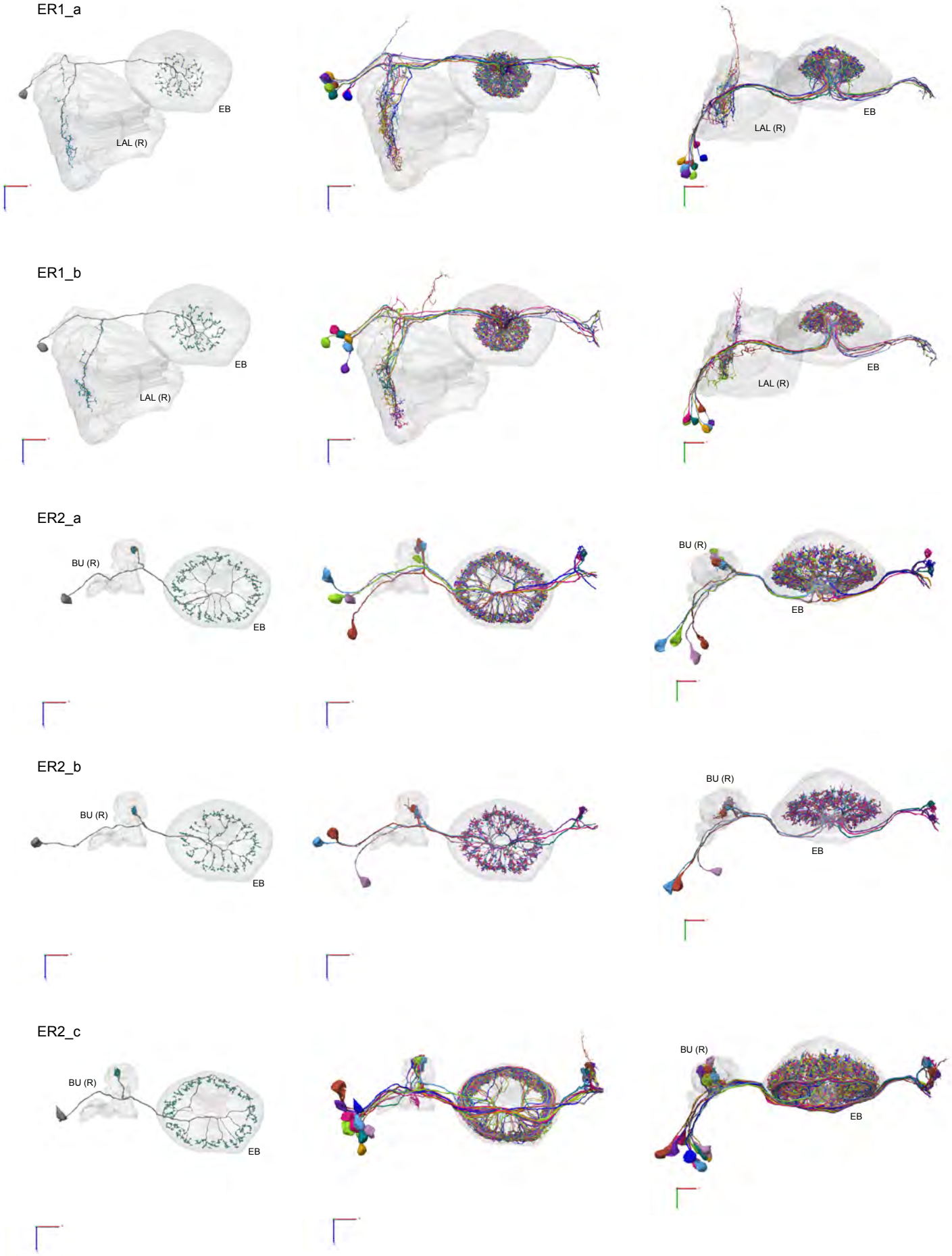


Figure 10—figure supplement 4

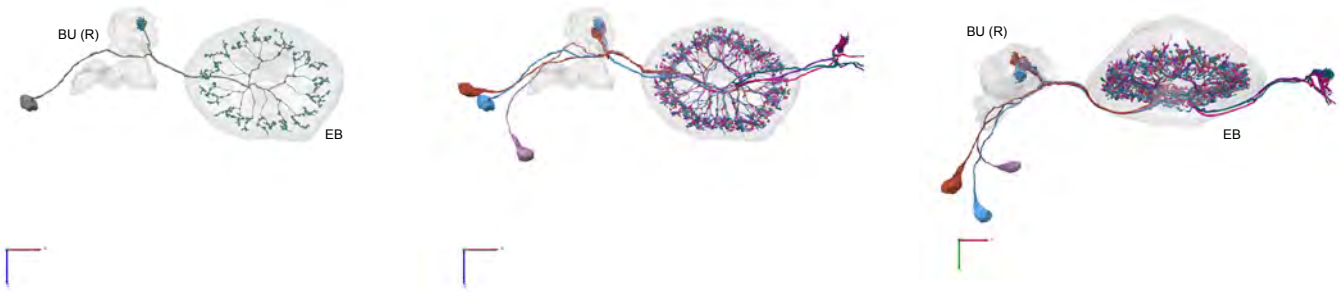
Normalized synapse densities of ring neurons onto the EB cross section along the anterior-radial axes. 8 projections for wedge-shaped cross sections around the EB circumference are shown (see schematic for reference). Illustration of the position of cross sections on the upper right corner of panel **A**. Synapse densities color-coded by neuron type.

A) Ring neuron types
B) EB columnar neuron types
C) ExR neuron types

Figure 10—figure supplement 5: Anatomy of ER (ring) neurons

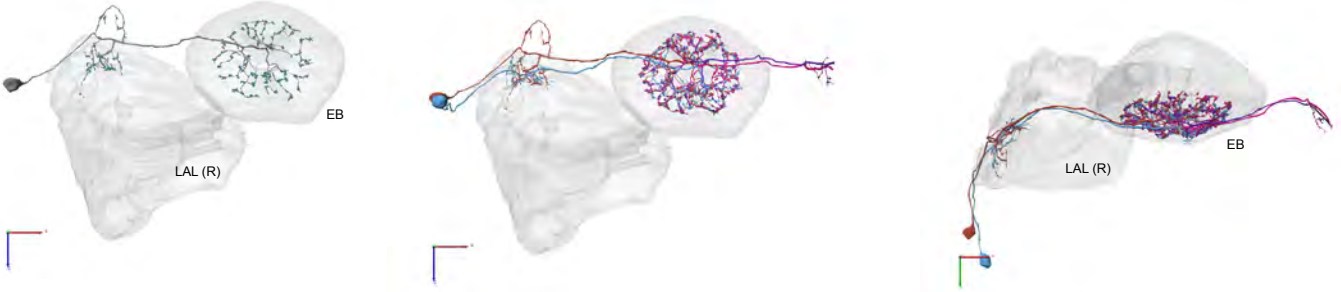


ER2_d

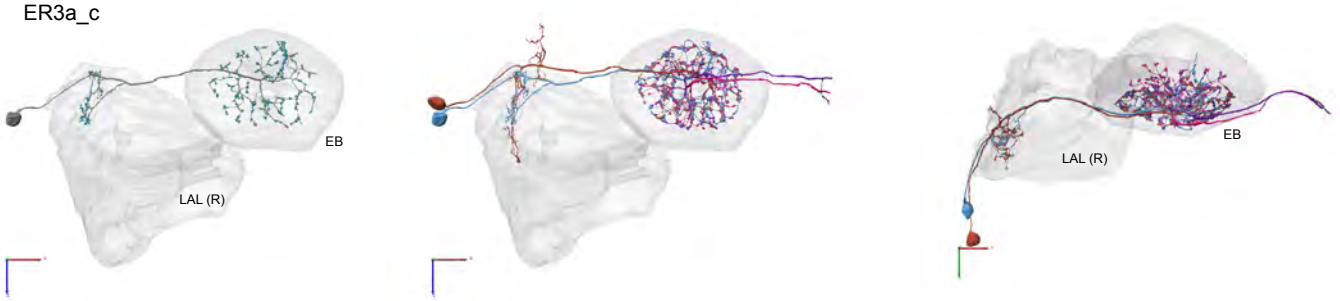


ER3a_a

ER3a_b



ER3a_c



ER3a_d

ER3d_a

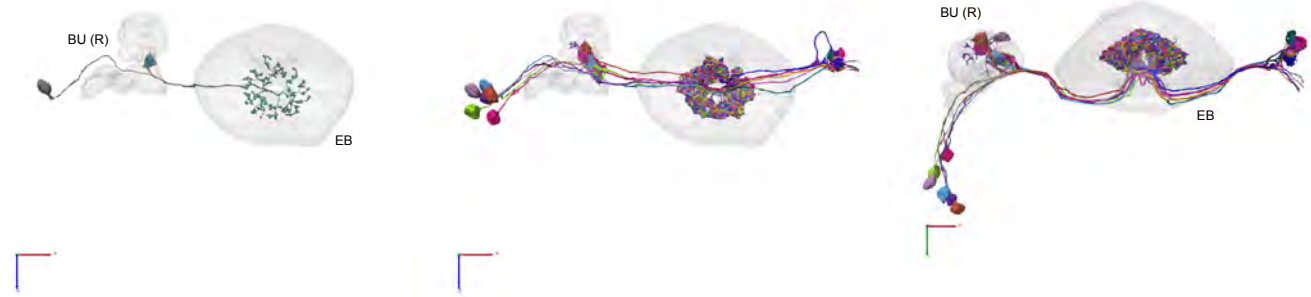
ER3d_b

ER3d_c

ER3d_d

ER3m

ER3p_a

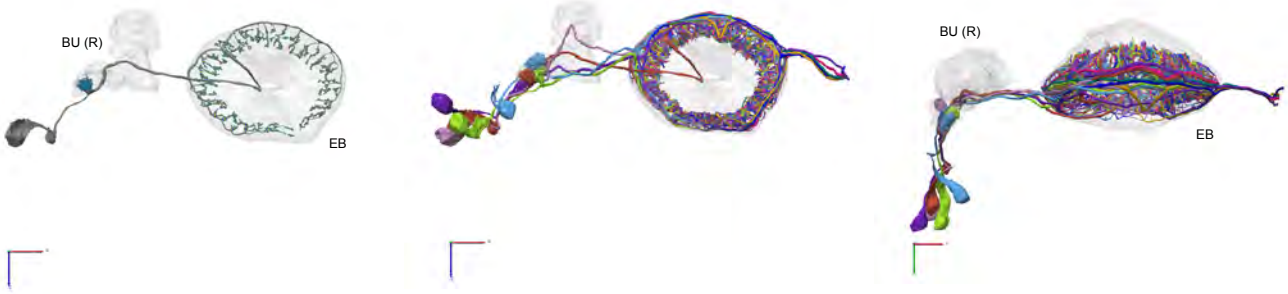


ER3p_b

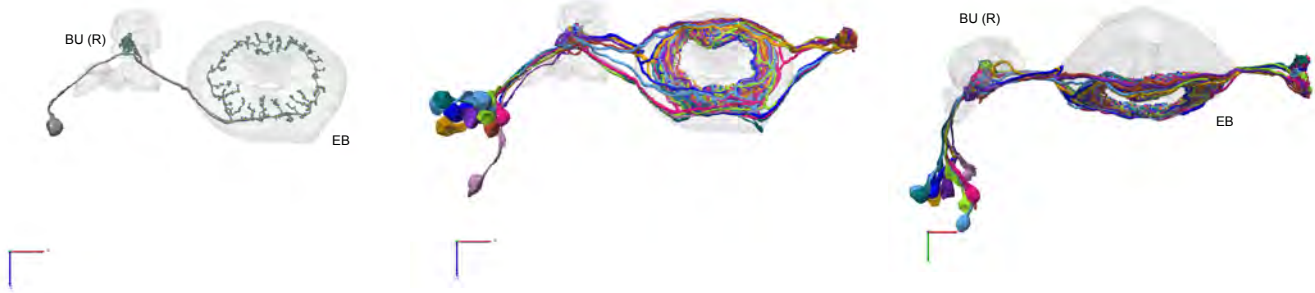
ER3w

ER4d

ER4m



ER5



ER6

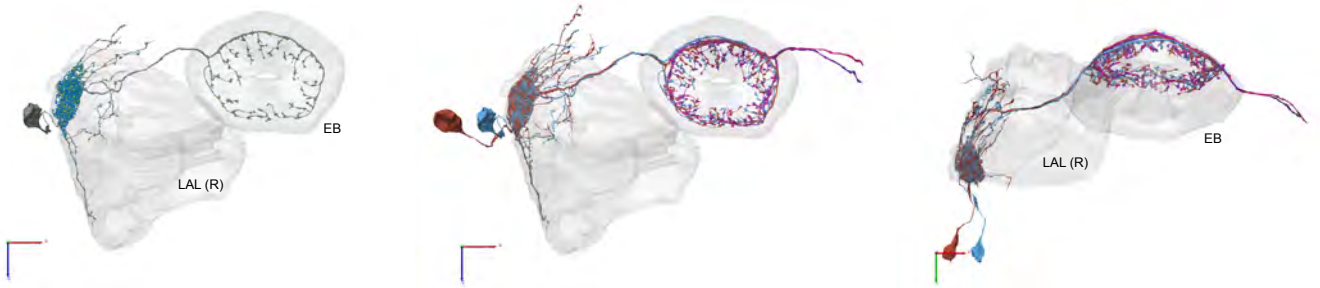


Figure 10—figure supplement 5: Morphological renderings of ring neurons

Morphological renderings of all ring neuron types and their primary regions of innervation. Left column: Rendering of a single ring neuron from right hemisphere population with blue dots marking the location of postsynapses and yellow dots those of presynapses. Middle and right column: Two views of the full population of ring neurons for each type.

Figure 11: Ring neuron to columnar connectivity

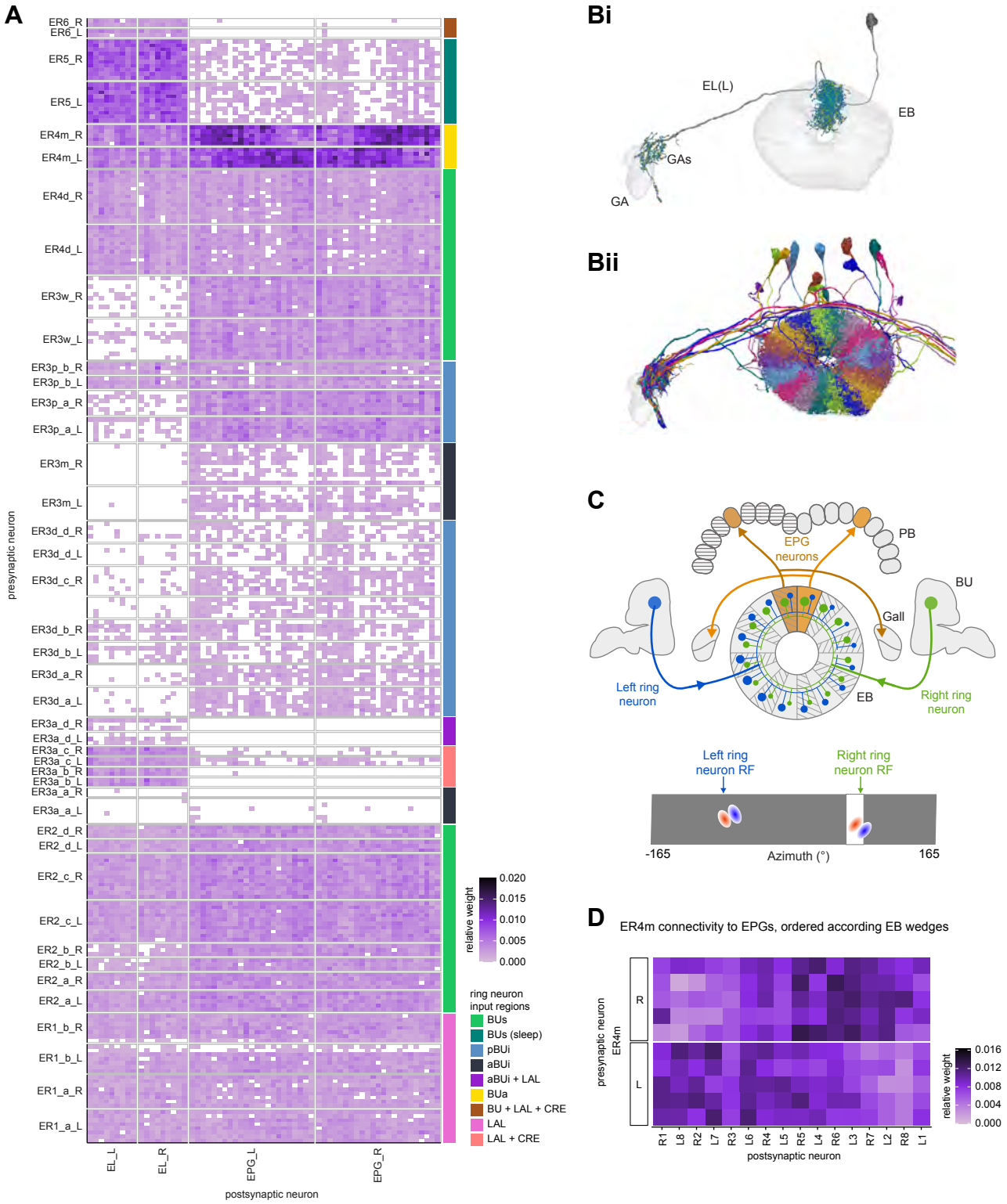


Figure 11: Ring neuron to columnar connectivity

A) Neuron-to-neuron connectivity matrix for connections from ring neurons to EL and EPG neurons in the EB on a single neuron level. The boxes on the right side are colored according to the ring neuron's input region.

B) Morphological renderings of EL neurons and renders of innervated ROIs. Note that EL neurons target a small region next to the gall (GA), called the gall surround (GAs). **Bi**: Single left hemisphere EL neuron with blue dots marking the location of postsynapses and yellow dots those of presynapses. **Bii**: Full population of EL neurons.

A) Schematic illustrating variation in synaptic strength in ring neuron to EPG connections due to neural plasticity. Top: Connectivity between ring neurons and EPGs. Bottom: Illustration of receptive fields (RF) of single ring neurons.

B) Connectivity matrix of ER4m inputs to EPG wedges in the EB. EPG neurons are sorted and averaged according to the EB wedge they innervate.

Figure 11—figure supplement 1

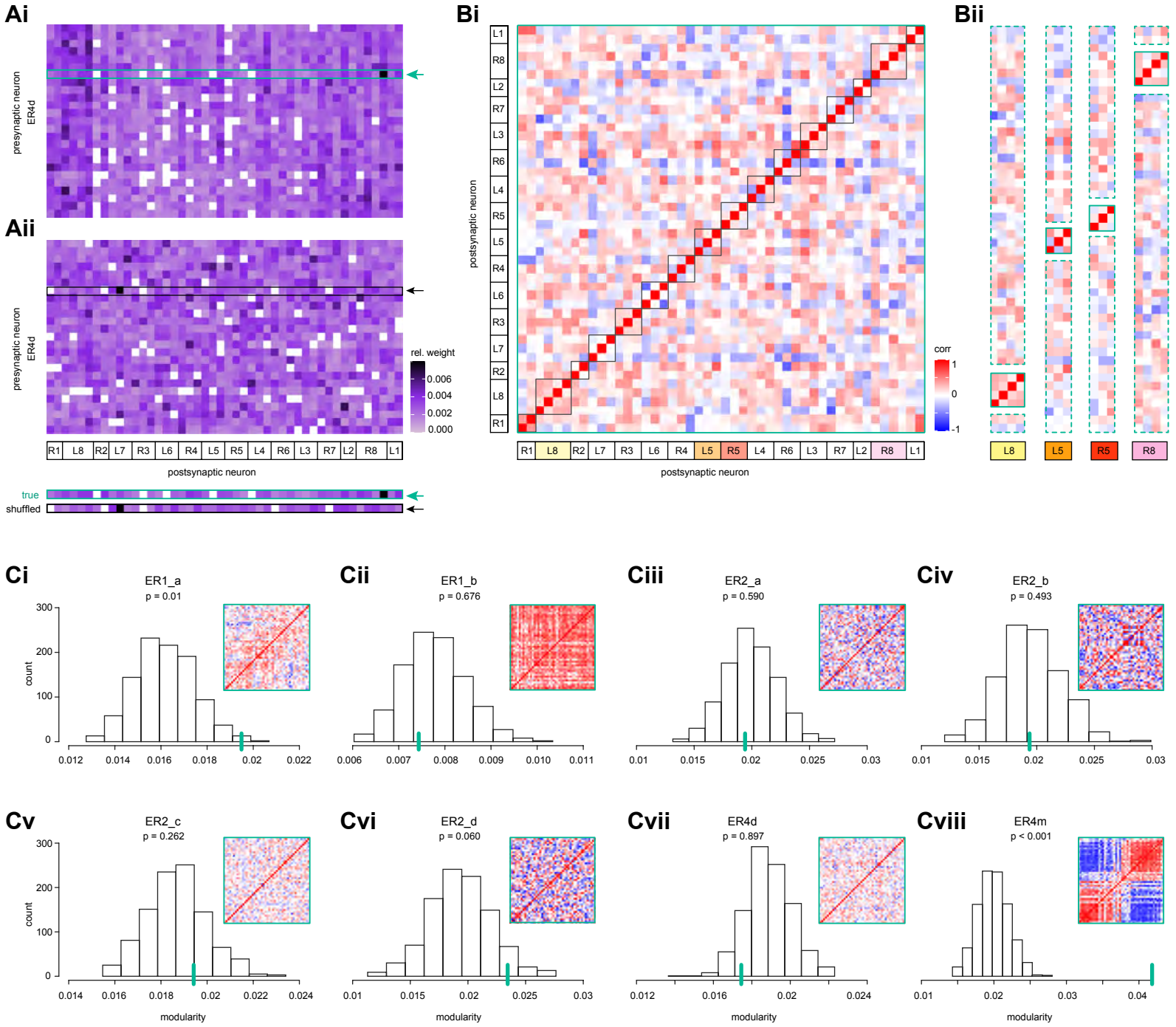


Figure 11—figure supplement 1: Wedge-specific modularity of inputs from ring neurons to EPG neurons

A) Neuron-to-neuron connectivity matrix for connections from ER4d neurons to EPG neurons in the EB, shown for the matrix that preserves (**Ai**) versus shuffles (**Aii**) the individual EPG neurons onto which individual ER4d neurons synapse (highlighted boxes). EPG neurons are ordered according to the EB wedge that they innervate.

B) Pairwise Pearson's correlation measured between individual EPG neurons according to the pattern of their ER4d neuron inputs. Solid red boxes highlight clusters of EPG neurons that innervate the same EB wedge. Highlighted wedges in **Bi** are shown in **Bii**. The modularity of the matrix in **Bi** measures whether individual EPG neurons are more correlated with those EPG neurons within the same wedge (solid boxes in **Bi** and **Bii**) than would be expected based on their average correlation with neurons across all wedges (dashed boxes in **Bii**).

C) Modularity of connectivity from different ring neuron types onto EPGs. Histograms show the distribution of modularity values computed for 1000 shuffled versions of each connectivity matrix (one example of which is shown in **Aii**). Insets show the correlation matrix of the measured (unshuffled) connectivity matrix; the modularity of this matrix is marked by a red line on the histogram. P-values indicate the fraction of shuffles that produced higher modularity than that of the measured connectivity matrix.

Figure 12: EPG morphology analysis

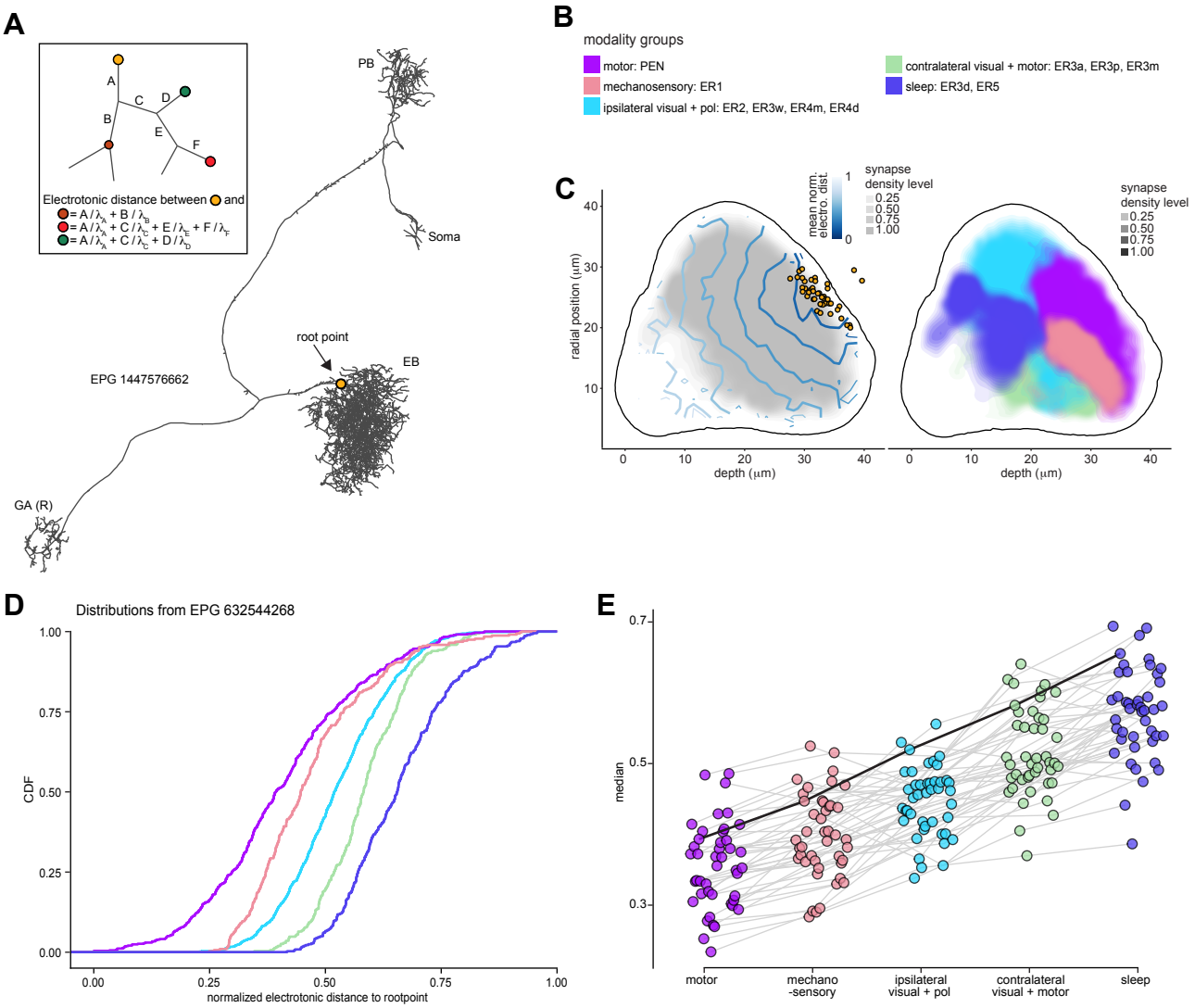


Figure 12: Morphology analysis of ring neuron connectivity to EPG and EL neurons

A) Skeleton of a single EPG (1447576662) with the selected root point indicated in yellow. Inset: Schematic indicating how the electrotonic distance from a point on the skeleton to the root point is calculated. The Euclidean metric is used to calculate the length of each segment (A, B, C, D, E, F) and λ_* (for $*$ = A, B, C, D, E, F) are the length constants of the edges (see Materials and Methods).

B-E) Localization of synaptic inputs, split according to modality, to EPGs in the EB along the dendritic tree.

B) The modality groups, neuron type grouping, and the colormap that will be used for modality groups for the rest of the panels in this figure.

C) Density of synapse locations onto EPGs in the radial vs. depth plane for all EPGs included in the analysis (n = 44). The black outline approximates the EB outline in this plane. Left: Synapse locations are shown in gray (included here are synapses from partner types: ER, ExR, PEG, PEN, EPG, EPGt). Overlaid contour lines indicate the distribution of the mean of the normalized electrotonic distance from the root. The yellow points indicate where the root points of the EPGs are located in this plane. Right: Synapse locations from selected inputs separated and color-coded based on input modality (see **B** for input assignment to modality).

D) Cumulative density function (CDF) of the distribution of the normalized electrotonic distance to root for synapses separated by input modalities for a single EPG (632544268).

E) Medians of the normalized electrotonic distance distributions grouped by modality. The connecting lines indicate the points corresponding to each individual EPG (n = 44), with the black line corresponding to the EPG whose CDF's are shown in **D**.

Figure 12—figure supplement 1: EPG morphology analysis

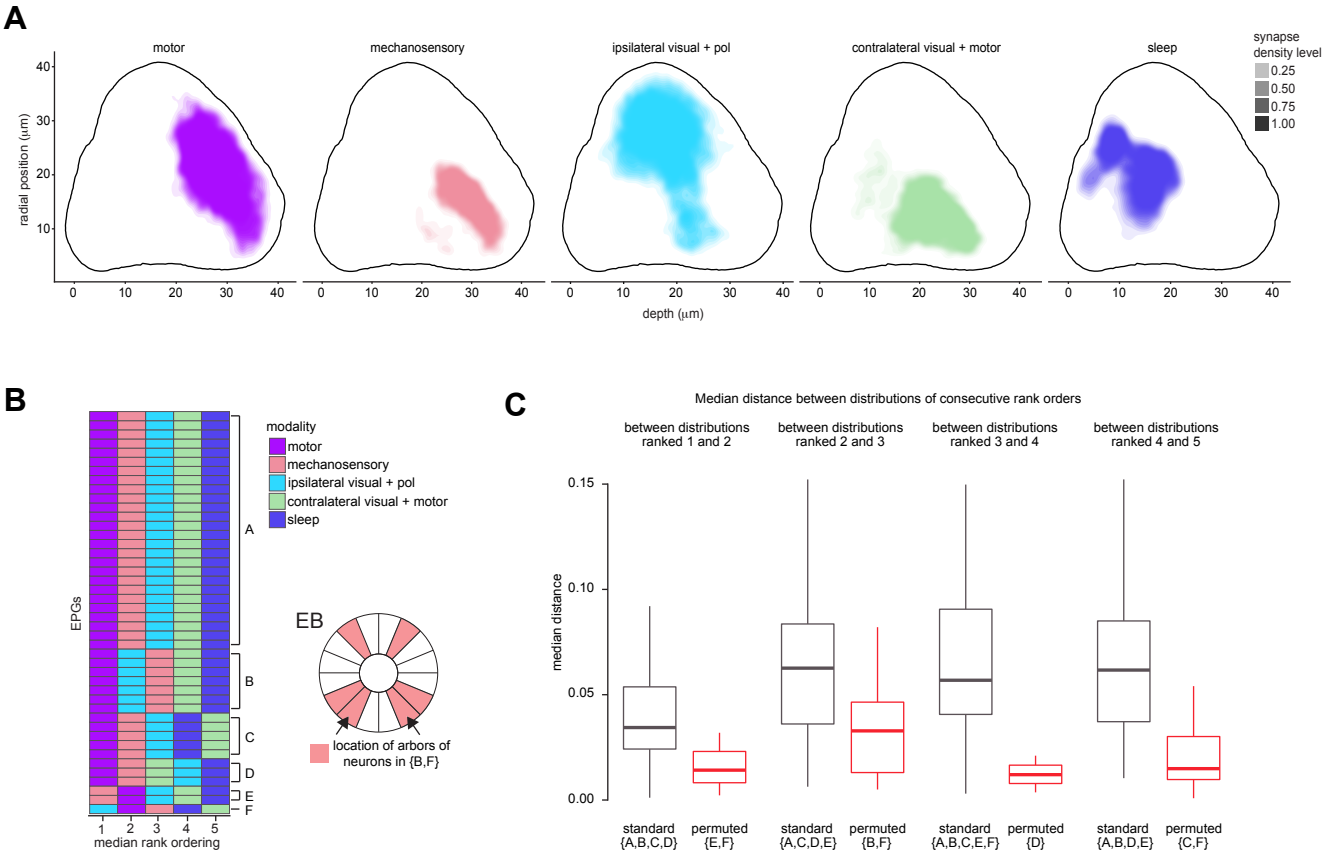


Figure 12—figure supplement 1

A) Synapse densities of each modality type separated in order to see where overlap occurs (most notably, between motor and mechanosensory).

B) Rank ordering of the input modalities determined via the location of their median for each EPG included in the analysis (n = 44). Group A indicates the most common ("standard") ordering. Most other groups are only a single permutation from group A (for example, group B is one permutation, (2,3), from group A). The only exception to this is group F which consists of a single neuron and is separated by three permutations from group A ((2,3), (1,2), and (4,5)). Schematic on the right shows where the neurons innervate the EB for groups that contain the (2,3) permutation (which shows the largest separation in distributions of all the permutations, see **C**) from the standard ordering (groups B and F). Each shaded region indicates the arbor locations of one neuron, with the exception of the regions indicated by the arrows which contains arbors of two neurons.

C) Box plot showing the distance between the median of the modalities with consecutive rank order distributions. Neurons are included in the standard group if they do not show a permutation between the rank orders considered and in the permuted group otherwise. The groups included in each boxplot are given by letter below the label of standard or permuted (note, these match the group labelling in **B**.

Figure 12—figure supplement 2: Medians by ring neuron type

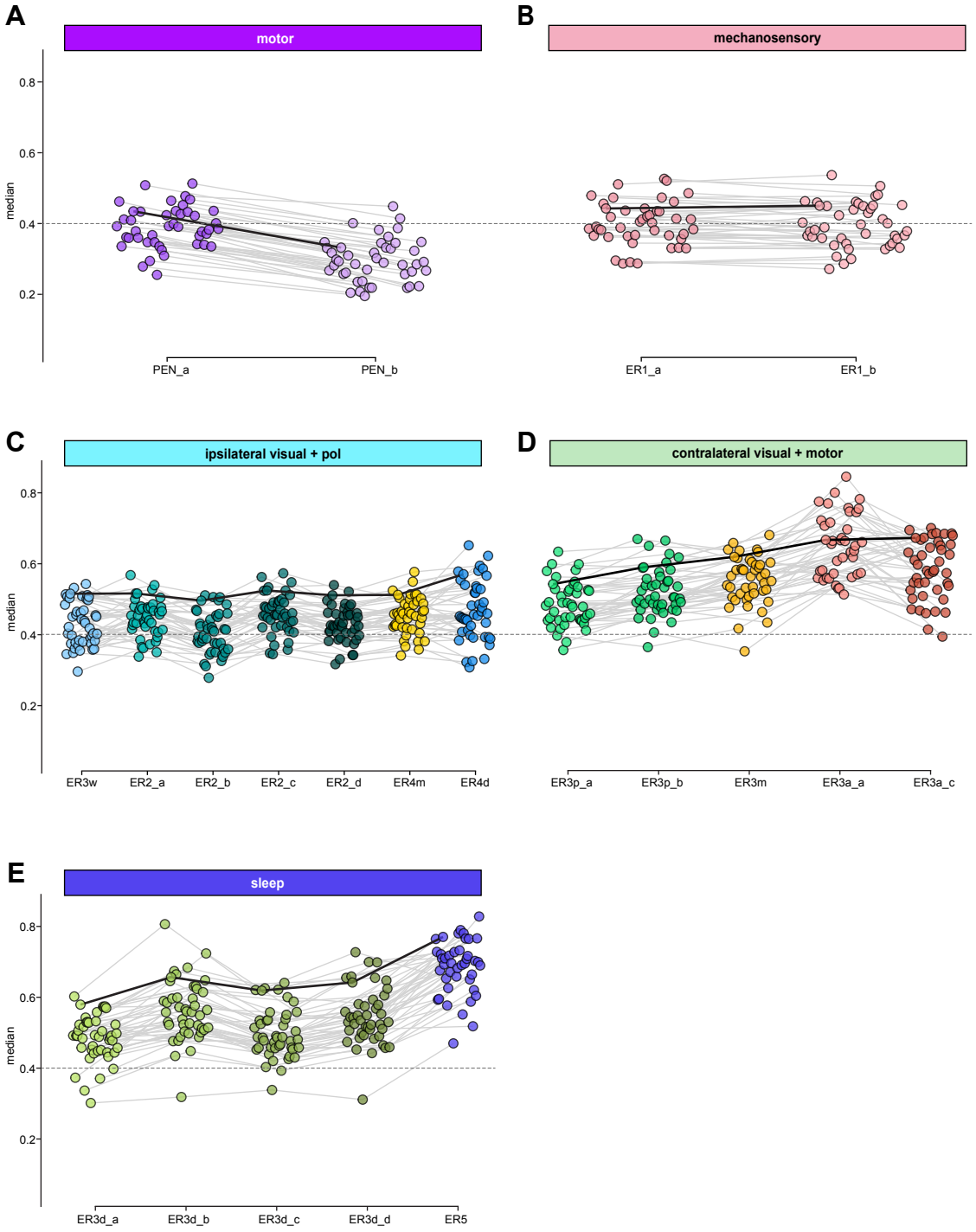


Figure 12—figure supplement 2

Medians of the normalized electrotonic distance distributions grouped by neuron type within each modality group. The connecting lines indicate the points corresponding to each individual EPG (n = 44), with the black line corresponding to the EPG whose CDF's are shown in **Figure 12D**.

A) Motor group.
B) Mechanosensory group.
C) Ipsilateral visual and polarization sensitive group.
D) Contralateral visual and motor group.
E) Sleep group.

Figure 12—figure supplement 3: EL morphology analysis

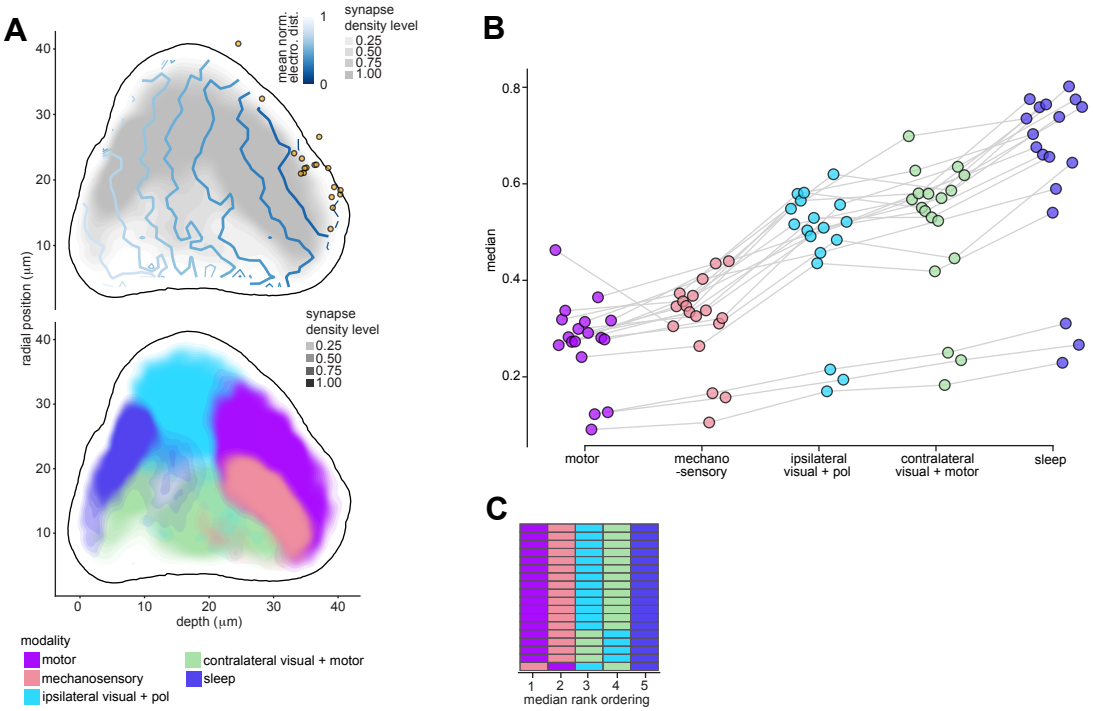


Figure 12—figure supplement 3

Same as **Figure 12C,E** and **Figure 12—figure supplement 1B**, but for synapses onto EL neurons instead of EPG neurons.

Figure 13: Inter-ring neuron connectivity

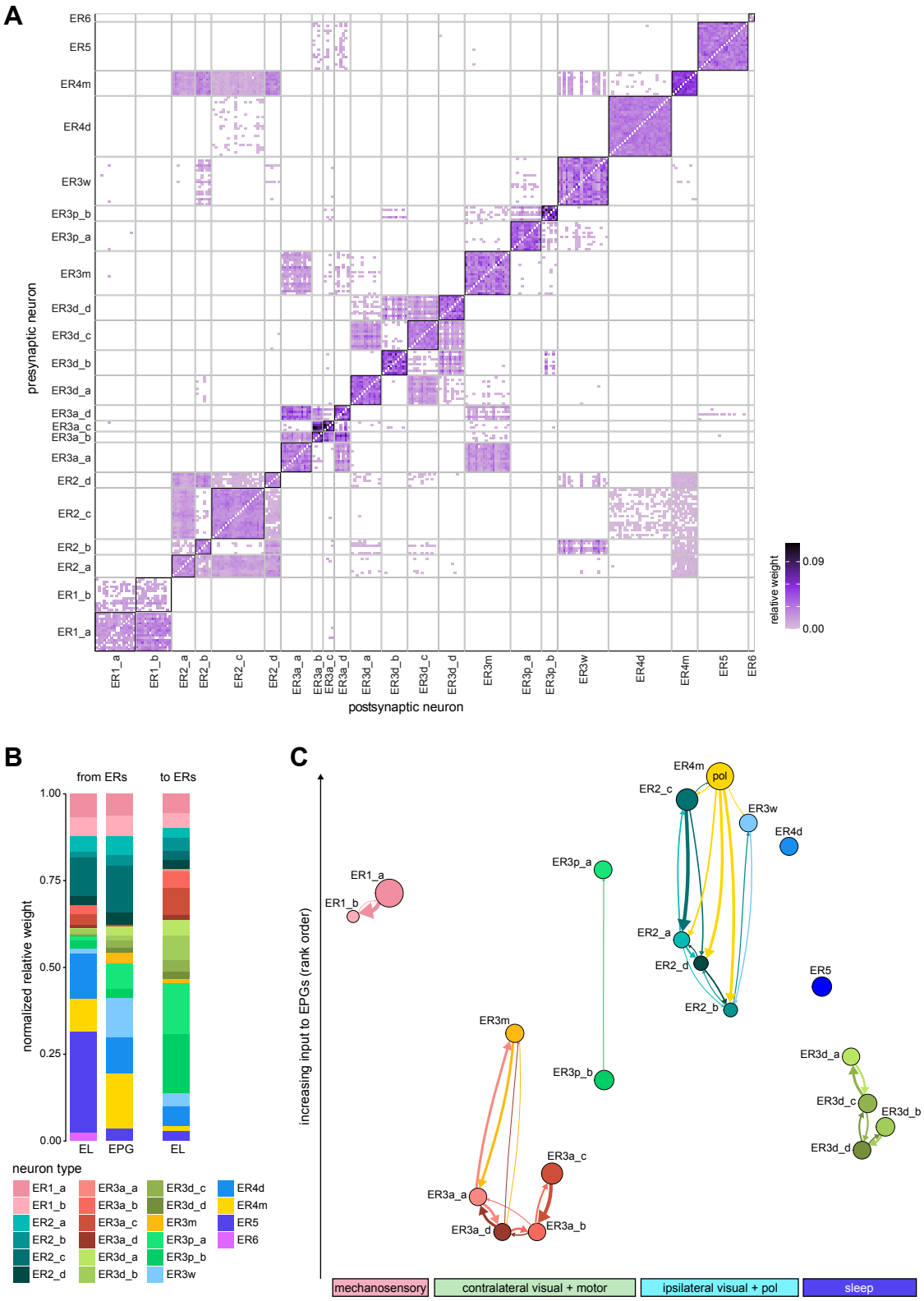


Figure 13: Inter-ring neuron connectivity

A) Connectivity matrix for connections between ring neurons in the EB on single neuron level. Black boxes mark connections between neurons of the same type.

B) Normalized contributions of different ring neuron types to EL and EPG neurons (left) vs. normalized contributions of EL neurons to different ring neuron types (right, EPGs make very few synapses to ring neurons, see **Figure 13—figure supplement 1B**).

C) Connectivity graph of connections between ring neurons. The graph nodes are arranged along the x-axis to group ring neuron types with putative similar tuning. Vertices are ordered on the y-axis according to their rank-ordered connectivity strength to EPG neurons. Vertex size is scaled by the ratio of the sum of all outputs divided by the sum of all inputs. Only connections with a relative weight of at least 0.05 are shown.

Figure 13—figure supplement 1: Connectivity of other columnar neurons and ring neurons

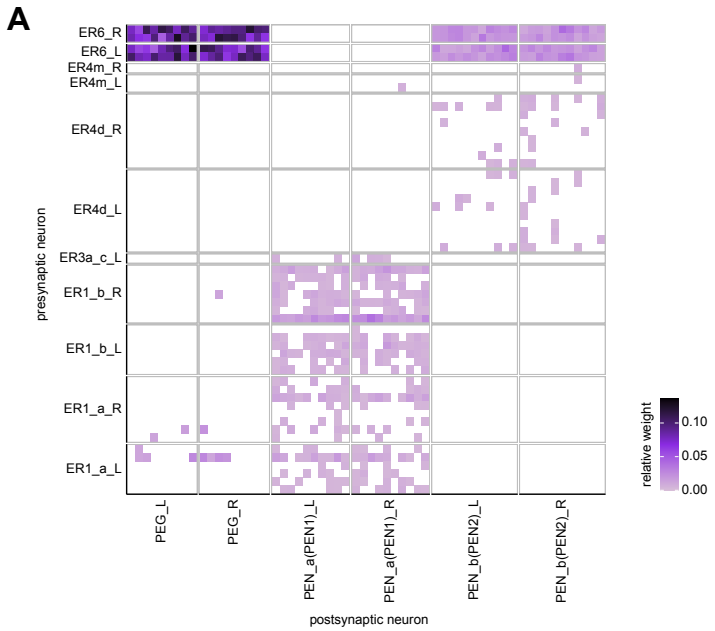


Figure 13—figure supplement 1: Connectivity between EB columnar neurons and ring neurons

A) Neuron-to-neuron connectivity matrix for connections from ring neurons to PEG and PEN neurons in the EB.

B) Same as **A**, but for connections from all columnar neurons (EL, EPG, PEG and PEN neurons) to ring neurons.

Figure 14: Overview of ExR neurons

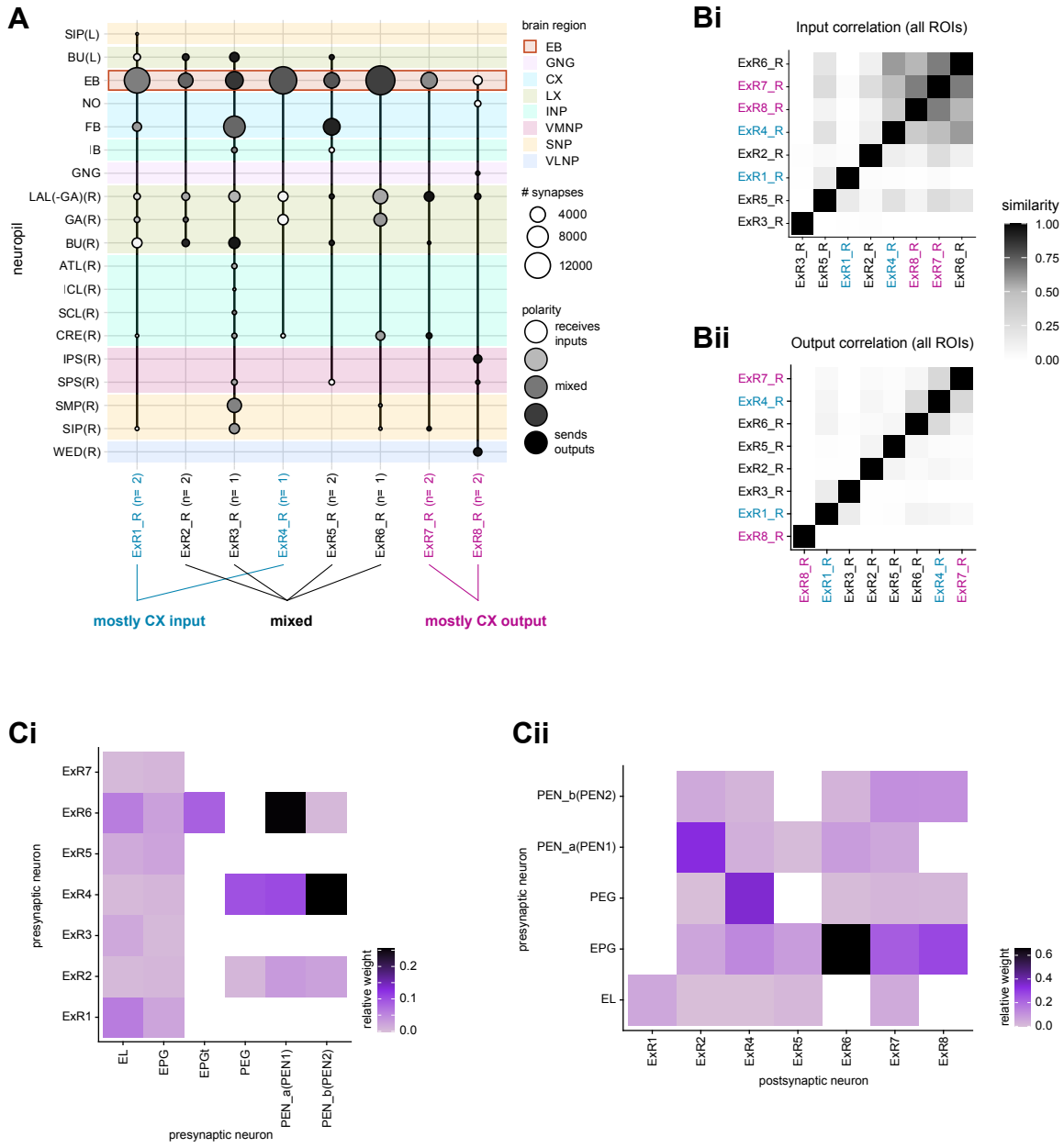


Figure 14: Overview of ExR neurons

A) Region arborization plot of all ExR types from the right hemisphere, showing average pre- and postsynaptic counts by region. Indicated below the plot is a qualitative categorization into three groups: mostly input to the EB (blue), mostly output from the EB (pink) and mixed (black).

B) Similarity matrices (see Methods) for ExR neurons based on all their inputs (**Bi**) and outputs (**Bii**). ExR type labels are colored according to groups in **A**.

C) Type-to-type connectivity matrix of ExR to EB columnar neurons (**Ci**) and EB columnar to ExR neurons (**Cii**).

Figure 14—figure supplement: ExR morphology

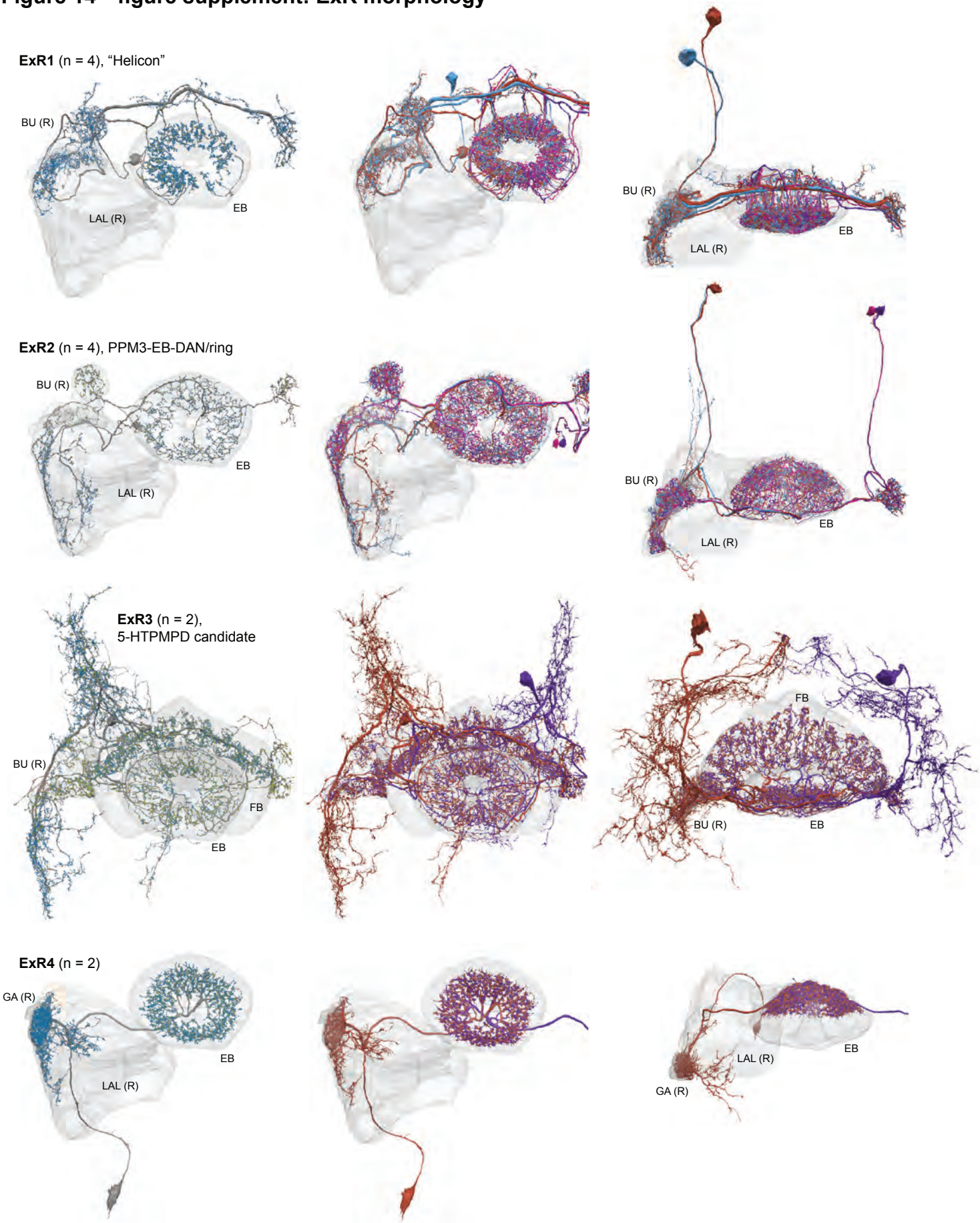


Figure 14—figure supplement: ExR morphology

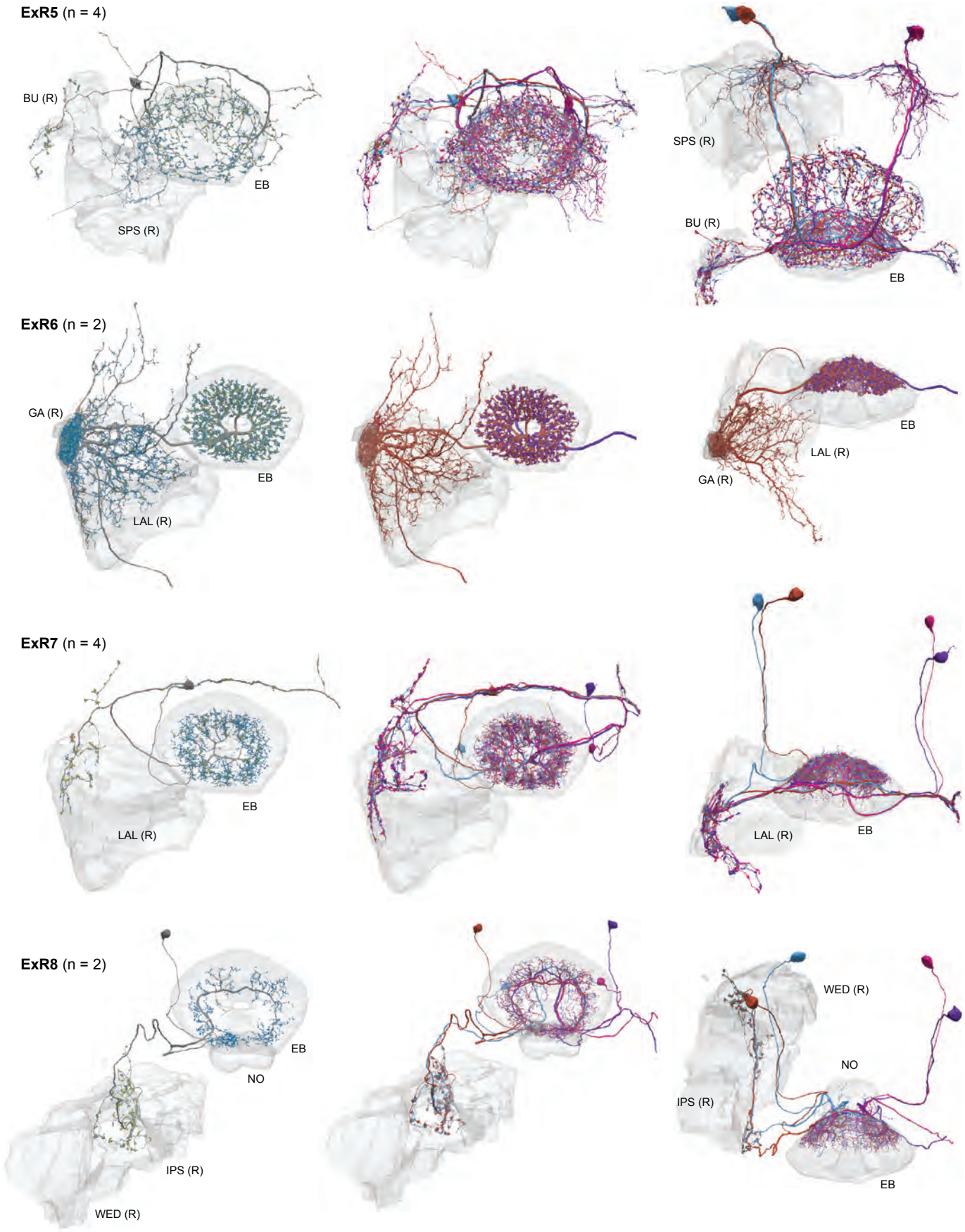


Figure 14—figure supplement 1

Morphological renderings of all ExR types. Some of the innervated neuropils are shown. The left column shows a single, right hemisphere ExR neuron for each type with presynapses marked by yellow dots and postsynapses marked by blue dots. The middle and right column shows morphological renderings of the full population.

Figure 14—figure supplement 2: Extended information on ExR partners

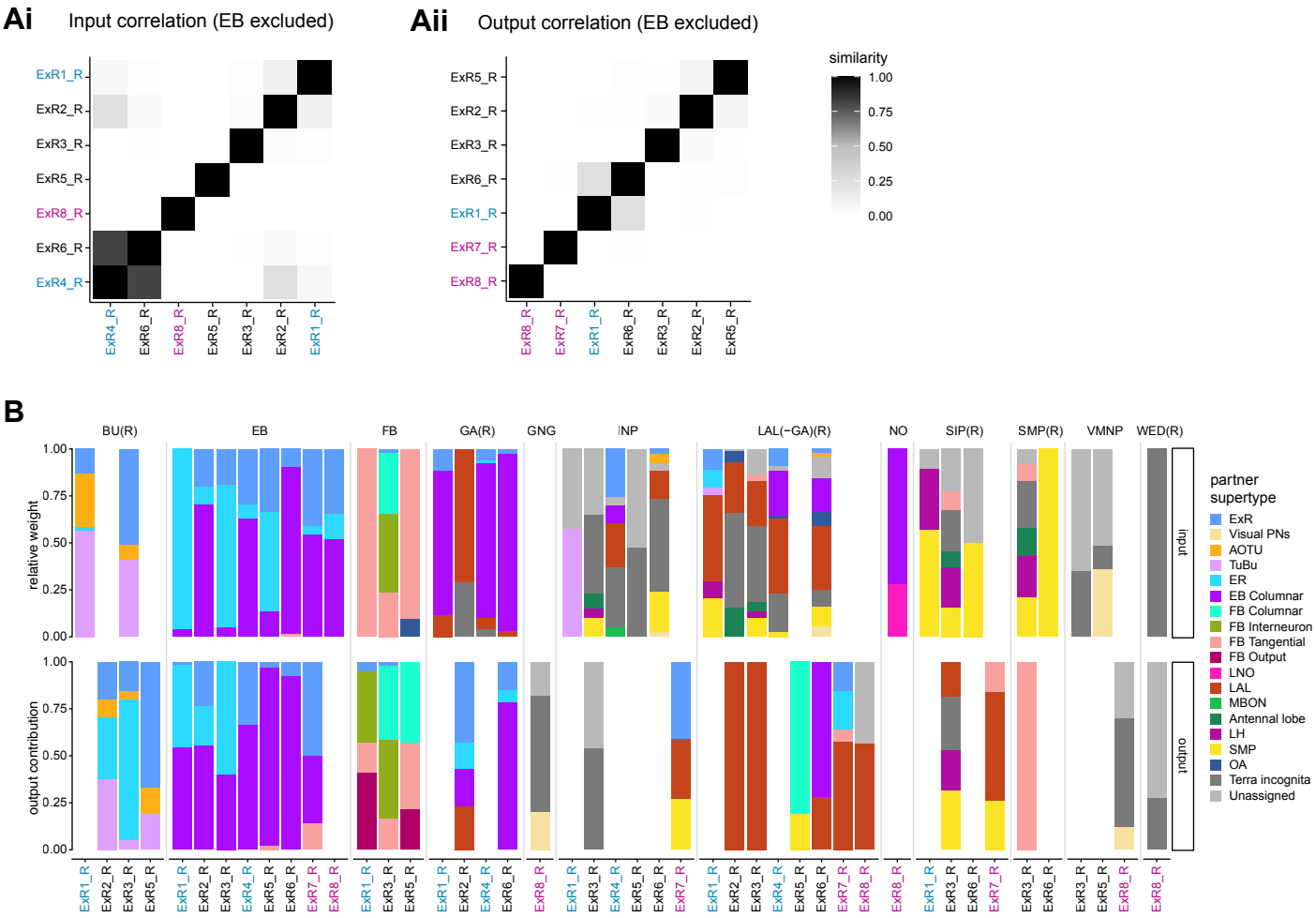


Figure 14—figure supplement 2

A) Similarity matrices (see Methods) as in **Figure 14B** but excluding inputs in the EB (**Ai**) or outputs in the EB (**Aii**).

B) Stacked bar graph illustrating the fraction of inputs from and outputs to ExR partners, grouped into supertypes and separated by brain region. Inputs and outputs are normalized per neuron type and brain region. The connectivity strength for inputs and outputs is measured by relative weight and output contribution, respectively. ExR type labels are colored according to groups in **Figure 14A**.

Figure 14—figure supplement 3: Connectivity between ExRs and columnar neurons in EB

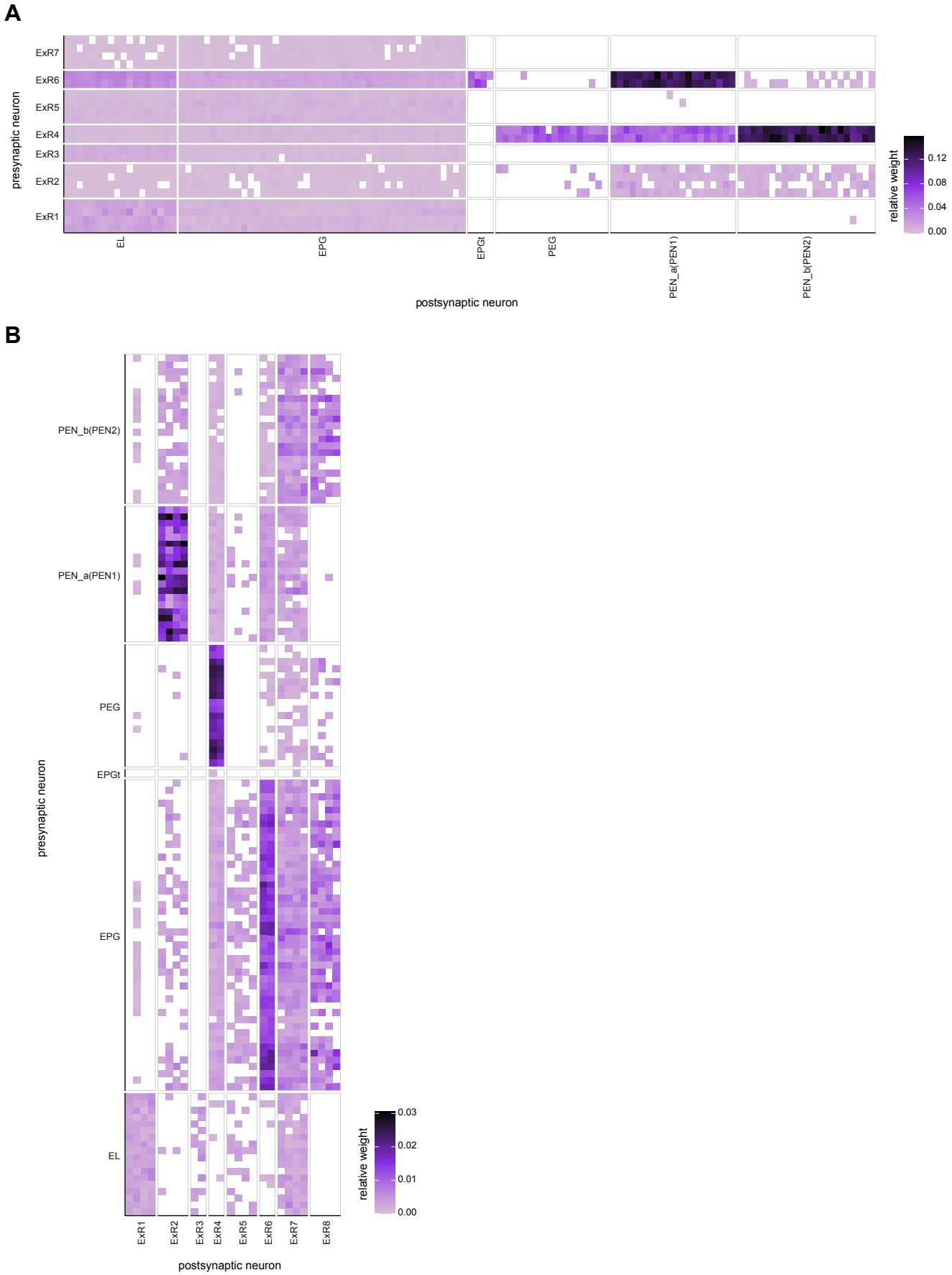


Figure 14—figure supplement 3

Neuron-to-neuron connectivity matrices for connections between ExR and columnar neurons in the EB.

A) Connections from ExR to columnar EB neurons.
B) Connections from columnar EB neurons to ExR.

Figure 15: ExR connectivity motifs between the EB and other regions

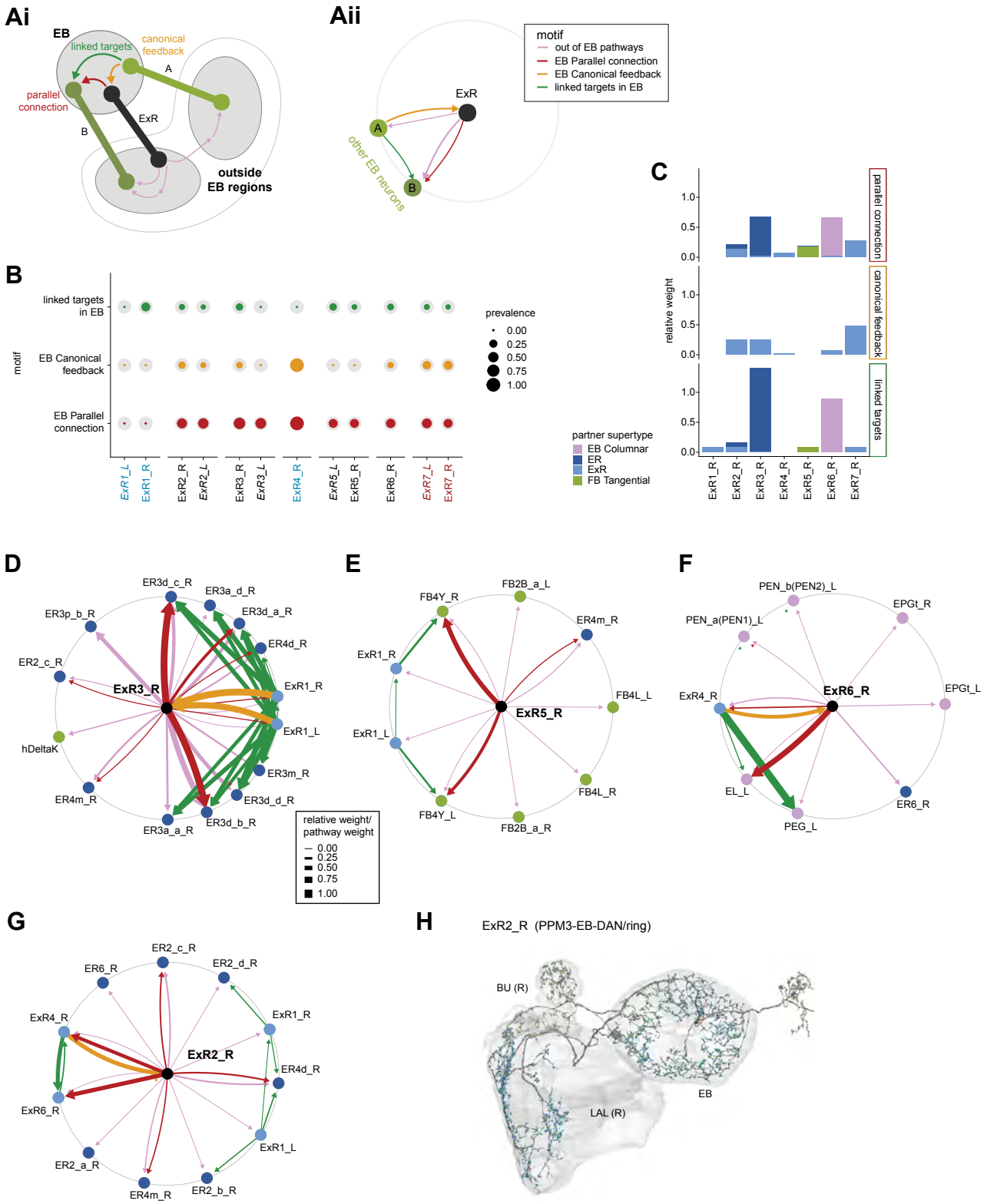


Figure 15: ExR connectivity motifs

A) Explanation of the ExR connectivity motif analysis, which compares connectivity within the EB to connectivity outside the EB. **Ai**) Schematic showing the three connectivity motifs that were investigated. Note that only ExR output pathways were considered for this analysis. **Aii**) Graph representation of the circuit motifs schematized in **Ai**.

B) Summary of motif prevalence across different ExR types.

C) Bar graph showing the contribution (measured by relative weight) of ExR partners in the EB to the observed connectivity motifs.

D) (**D-G**) Graph representation of connectivity motifs as depicted in **A** for ExR3_R (**D**), ExR5_R (**E**), ExR6_R (**F**), and ExR2_R (**G**).

H) Morphological rendering of one ExR2_R and some of the innervated ROIs. Blue dots mark postsynapses and yellow dots mark presynapses.

Figure 16: EPGs connect the EB to the PB

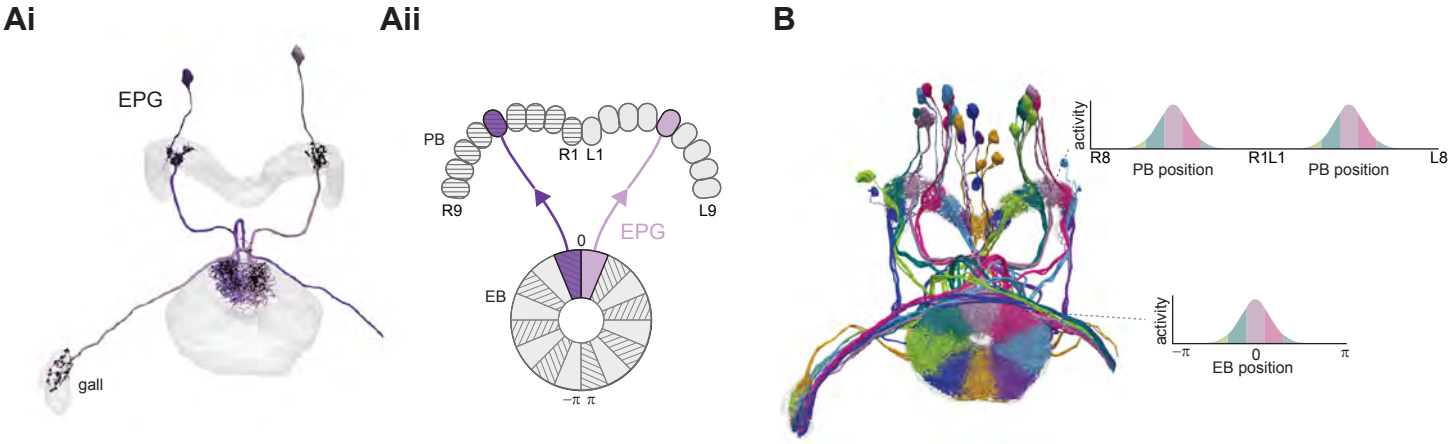


Figure 16: EPGs connect the EB to the PB

A) (left) A morphological rendering of two EPG neurons. Black dots are presynaptic sites. (right) Schematic showing where the EPG processes arborize in the EB and in the PB.

B) EPG processes in the EB alternatively output to either the right or the left PB. A fictive bump of activity in the EB will therefore split into both a right and a left bump of activity in the PB.

Figure 17: PEN_as connect the PB back to the EB, with a shift, forming feedback loops with the EPGs

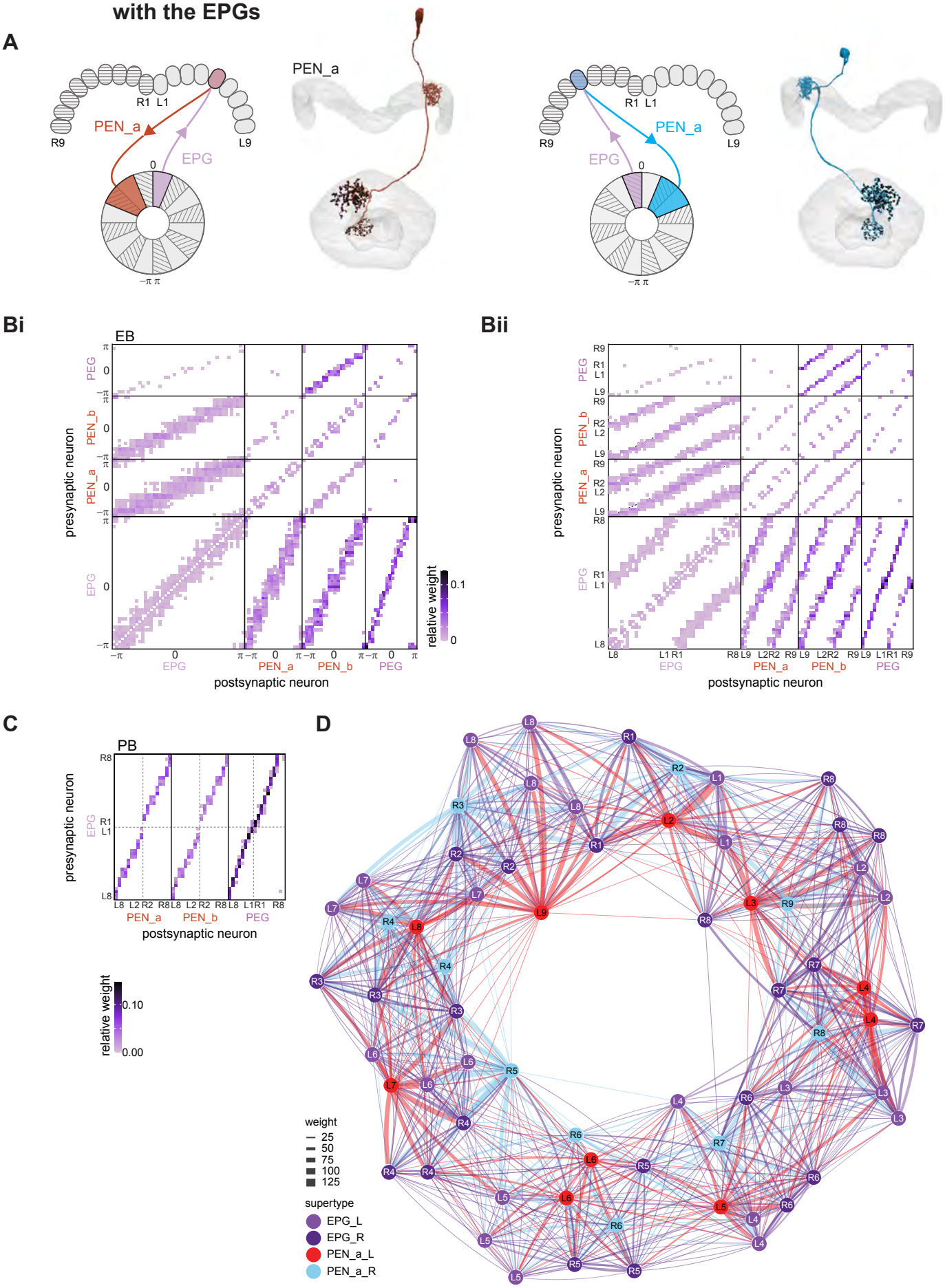


Figure 17: PEN_as connect the PB back to the EB, with a shift, forming feedback loops with the EPGs

A) (left) PEN_as on the left side of the PB project processes back to the EB that are counterclockwise shifted with respect to the EPG processes in the EB. (right) PEN_as on the right side of the PB project processes back the EB that are clockwise shifted with respect to the EPG processes in the EB.

B) Neuron-to-neuron connection table for EPG, PEN_a, PEN_b, and PEG neurons in the EB. The neurons are arranged according to their angular position in the EB (**Ai**) or according to their arrangement in the PB (**Aii**). Dotted lines are overlaid on the diagonal of the PEN to EPG quadrants to emphasize the offset in connectivity.

C) Neuron-to-neuron connection table for EPG, PEN_a, PEN_b, and PEG neurons in the PB.

D) A force-directed network layout of the EPG and PEN_a connections. Weight refers to the number of synapses between partners.

Figure 17—figure supplement 1:PEN_a and PEN_b connectivity

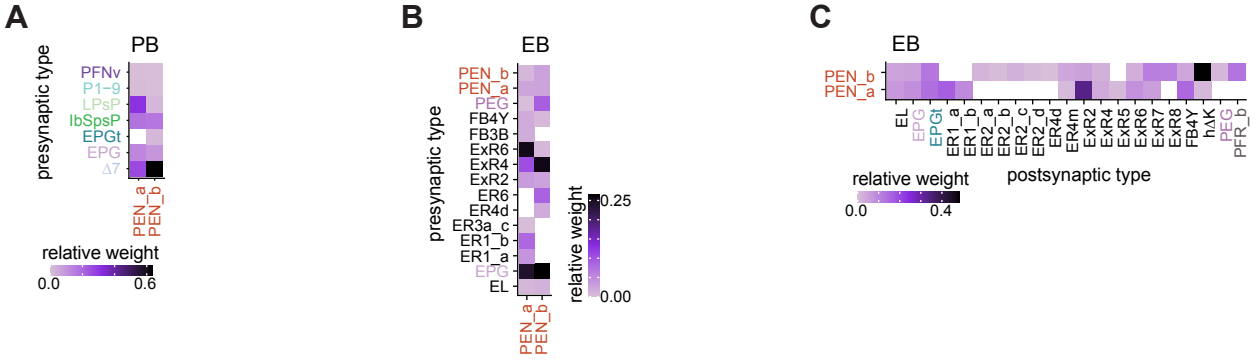


Figure 17—figure supplement 1: PEN_a and PEN_b connectivity

A) Type-to-type PEN_a and PEN_b input connection table in the EB.
B) Type-to-type PEN_a and PEN_b output connection table in the EB.
C) Type-to-type PEN_a and PEN_b input connection table in the PB.

Figure 18: EPGt neurons extend EPG-like connectivity

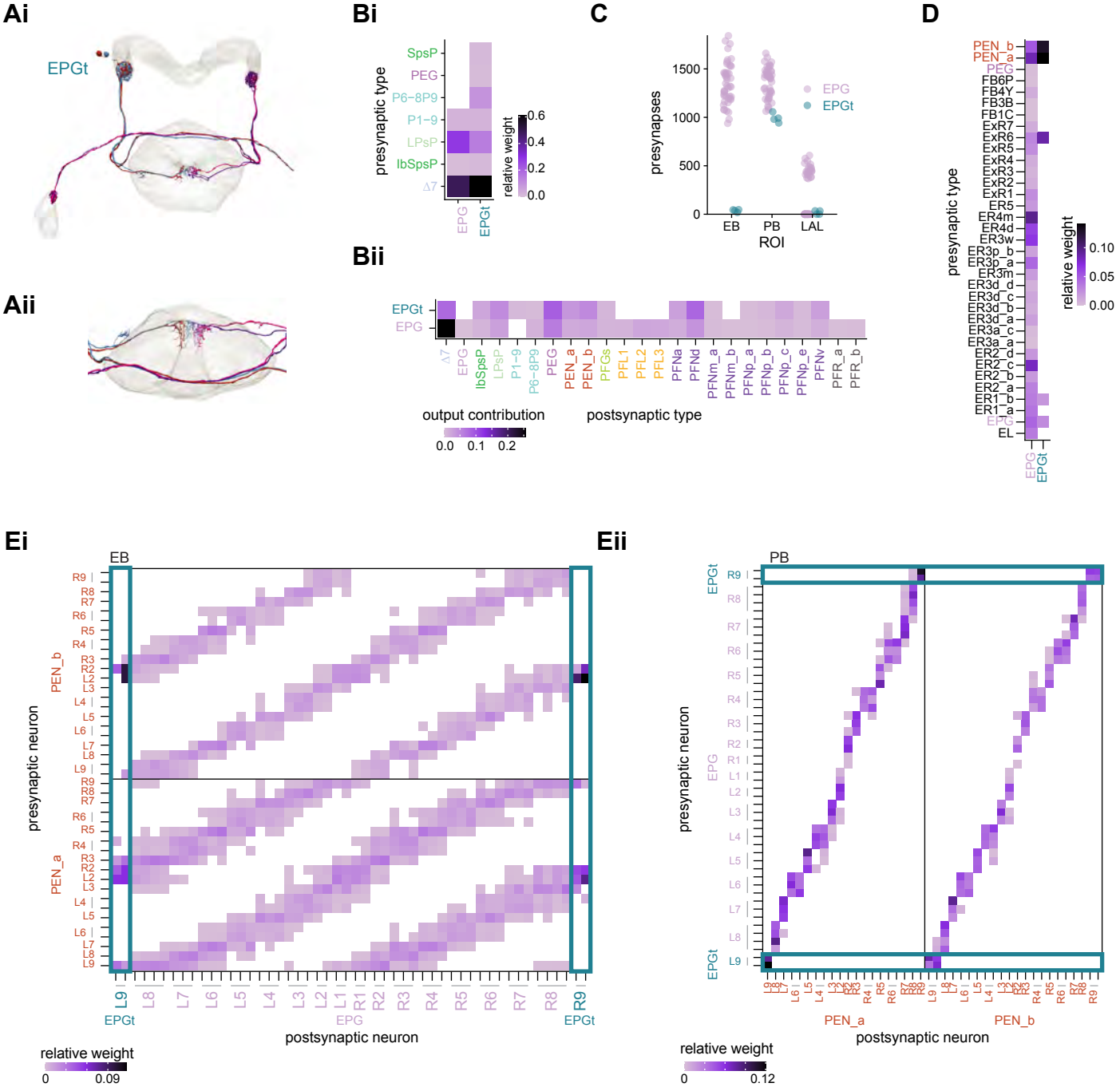


Figure 18: EPGt neurons extend EPG-like connectivity

A) Morphological renderings of all EPGt neuron. The EPGt neurons arborize only in glomeruli L9 and R9 and only at the bottom canal in the EB (**Ai**). A side view of the EB shows the position of EPGt processes in the EB (**Aii**).

B) Type-to-type connectivity matrix showing the inputs (**Bi**) and outputs (**Bii**) for the EPG and EPGt neurons in the PB.

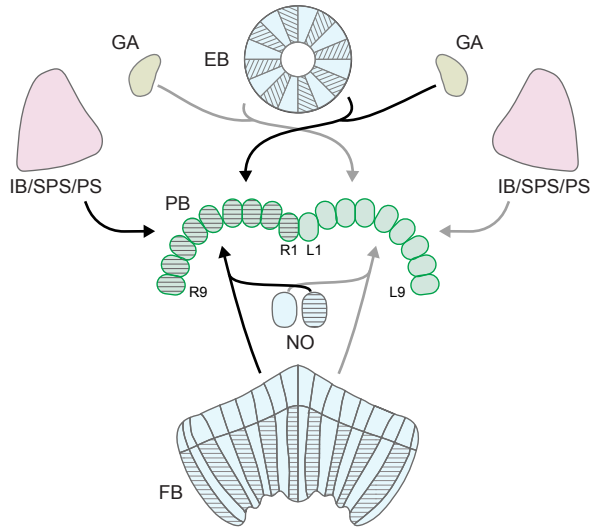
C) Total number of presynaptic sites for the EPGs and EPGts by brain region.

D) Type-to-type connectivity matrix showing the inputs to the EPG and EPGt neurons in the EB.

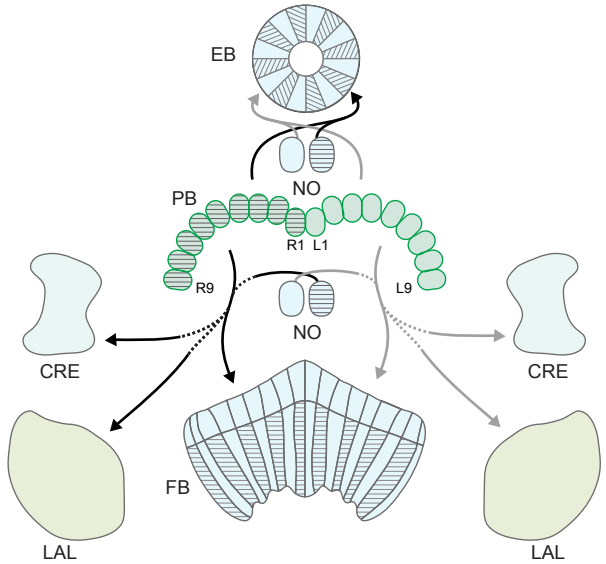
E) Neuron-to-neuron input connectivity from the PEN_a and PEN_b neurons to the EPG and EPGt neurons in the EB (**Ei**) and outputs from the EPGs and EPGts to the PEN_as and PEN_bs in the PB (**Eii**).

PB Figure 19: An overview of the protocerebral bridge

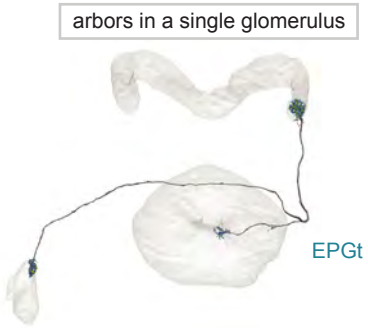
Ai



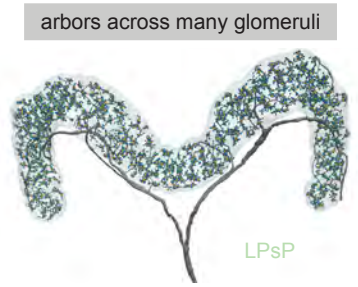
Aii



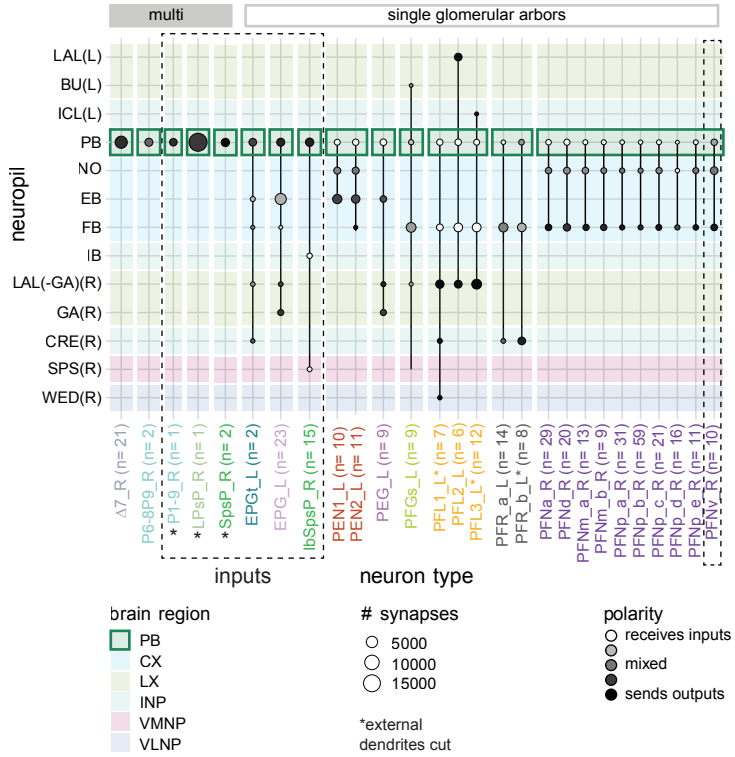
B



C



D



E

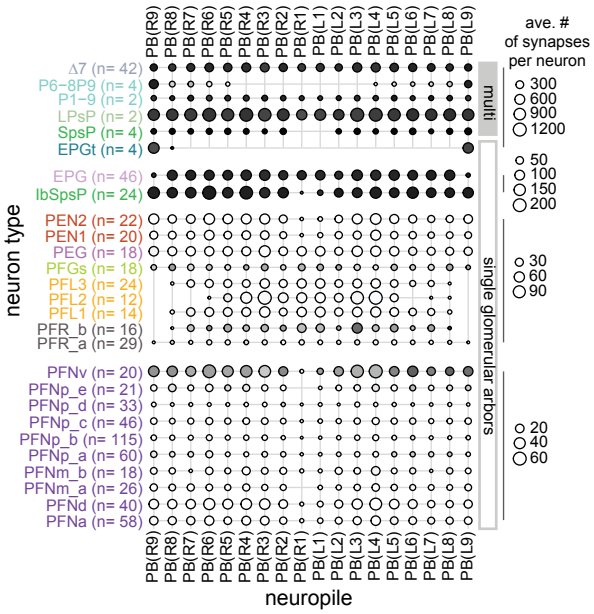


Figure 19: An overview of the protocerebral bridge

A) A diagram of the input (**Ai**) and output (**Aii**) pathways for the protocerebral bridge (PB). Connected brain regions include the ellipsoid body (EB), the inferior bridge (IB) the superior posterior slope (SPS), the posterior slope (PS), the crepine (CRE), the lateral accessory lobe (LAL), the fan-shaped body (FB) and the noduli (NO).

B) Morphological rendering of an EPGt neuron, which only arborize in a single glomerulus in the PB. Yellow dots mark presynaptic site. Blue dots mark postsynaptic sites.

C) Morphological rendering as in (**B**) of an LPsP neuron, which has arbors throughout the PB. Yellow dots mark presynaptic site. Blue dots mark postsynaptic sites.

D) Region arborization plot for each neuron type that contains arbors in the PB. Neuron types that provide input to the PB are denoted by the dashed vertical boxes. The horizontal boxes at top indicate which neurons arborize in multiple glomeruli (filled gray boxes) and which arborize in single glomeruli (gray outline).

E) The average number of synapses per neuron in each PB glomerulus for each neuron type that contains arbors in the PB.

Figure 20: E-PG to Δ7 connectivity forms a cosine-like profile

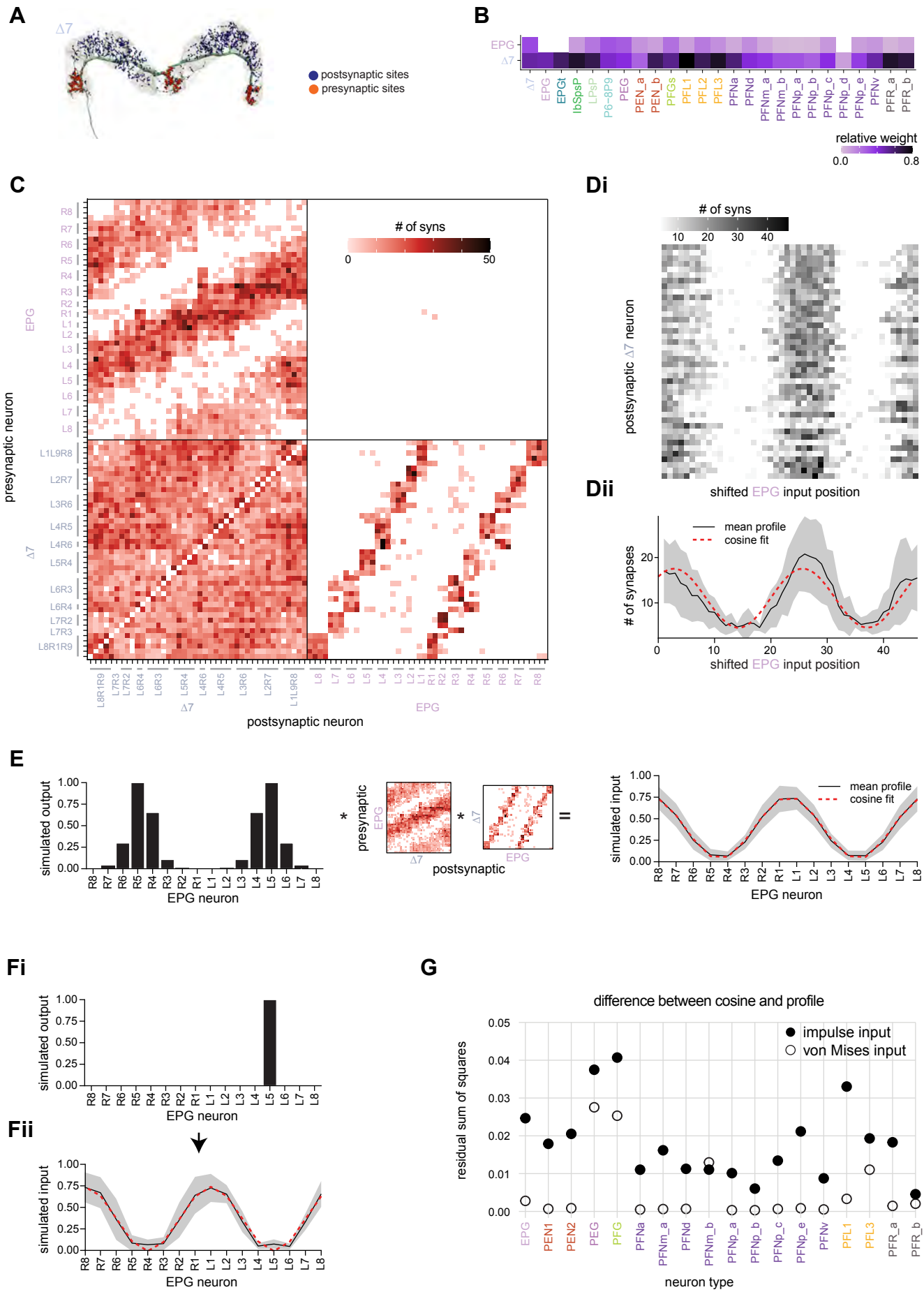


Figure 20: E-PG to Δ7 connectivity forms a cosine-like profile

A) A morphological rendering of a Δ7 neuron that outputs to glomeruli L8, R1, and R9.

B) Type-to-type connectivity table from EPGs and Δ7s to themselves and to all other PB neurons.

C) Synaptic connectivity matrix between EPG and Δ7 neurons.

D) The relative position of the EPG inputs to Δ7 outputs in the upper left quadrant of the matrix in A, circularly shifted to align the peaks (**Di**). Note that this matrix is rotated 90° from the quadrant in A. The mean and standard deviation across aligned Δ7 neurons (**Dii**). A cosine fit to the mean profile is shown with the dotted red line.

E) (left) A simulated von Mises bump profile in the EB leads to symmetric von Mises profiles in the right and left PB. (middle) The profile is multiplied by the EPG to Δ7 synaptic connectivity and then by the Δ7 to EPG connectivity to simulate the Δ7 input onto the EPGs. (right) The normalized mean Δ7 to EPG input profile, averaged across all possible bump positions and assuming a von Mises input. The standard deviation is shown in gray, and a cosine fit to the mean is shown with the dotted red curve.

F) A simulated impulse profile to one glomerulus in the PB (**Fi**) and the resulting simulated activity profile in the Δ7s (**Fii**). The procedure follows that used in **E**.

G) The residual sum of squares error between a cosine and the mean Δ7 input to a given neuron type assuming either a von Mises (black outline) or an impulse (black fill) input from the EPG neurons.

Figure 20—figure supplement 1: EPG and Δ7 neuron-to-neuron connectivity to PEG, PEN, PFL, and PFR neurons

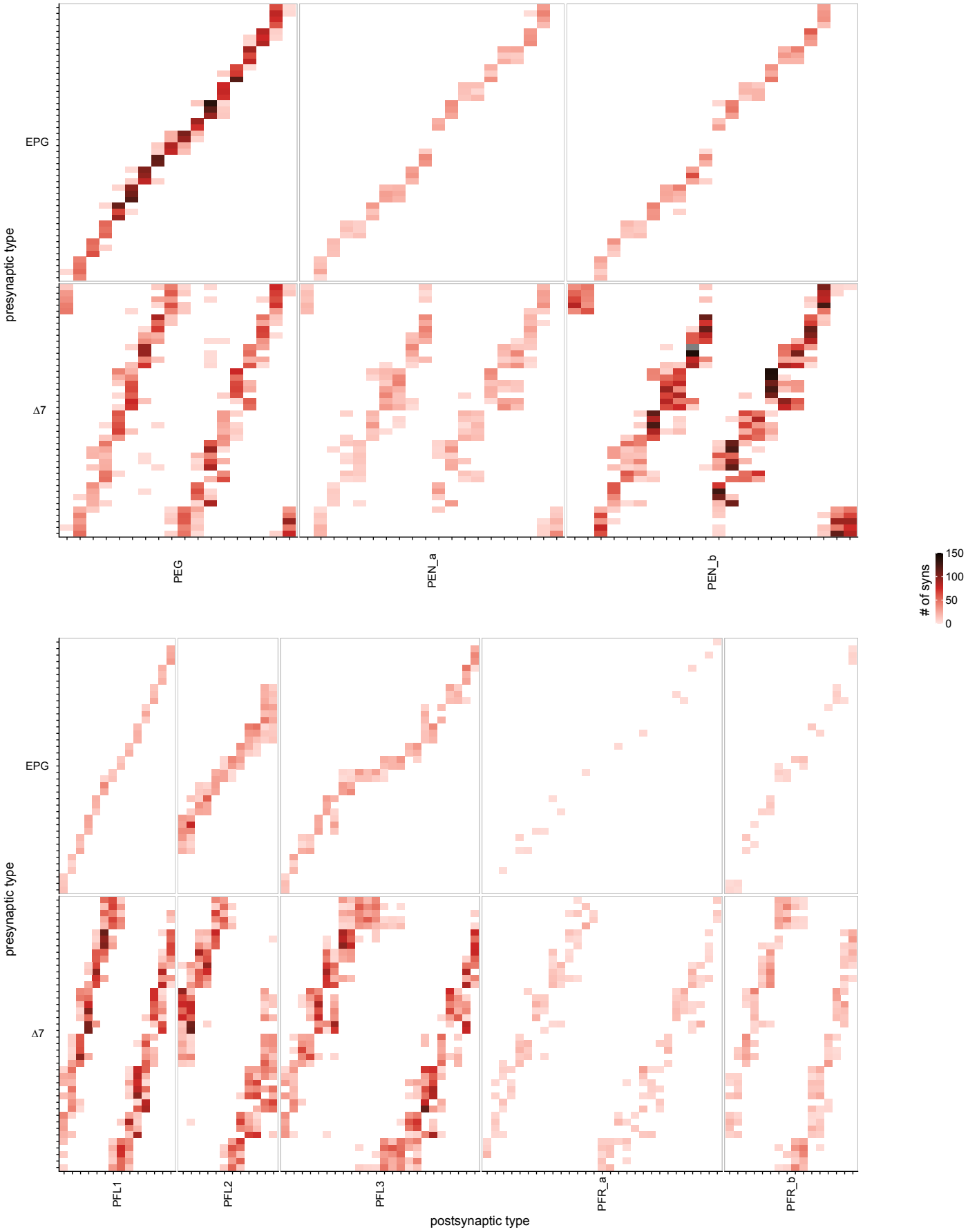


Figure 20–figure supplement 1: EPG and Δ7 neuron-to-neuron connectivity to PEG, PEN, PFL, and PFR neurons

Neuron-to-neuron connection tables showing the columnar, postsynaptic PEG, PEN, PFL, and PFR partners of the EPG and Δ7 neurons in the PB

Figure 20—figure supplement 2: EPG and Δ7 neuron-to-neuron connectivity to PFN neurons

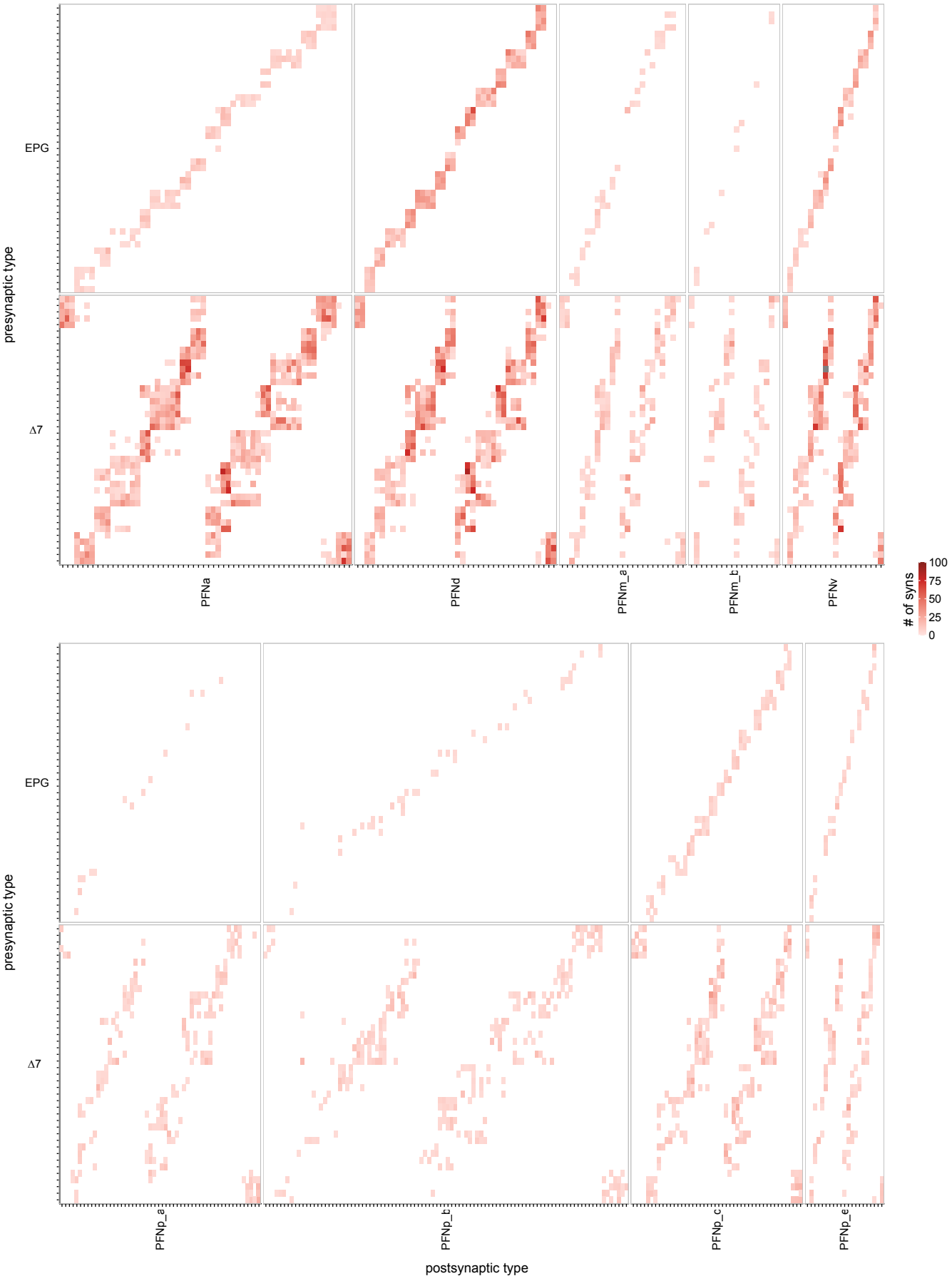


Figure 20–figure supplement 2: EPG and Δ7 neuron-to-neuron connectivity to PFN neurons

Neuron-to-neuron connection tables showing the columnar, postsynaptic PFN partners of the EPG and Δ7 neurons in the PB

Figure 21:P6-8P9 neuron morphology and connectivity is similar to that of the Δ7 neurons that arborize in the outer glomeruli

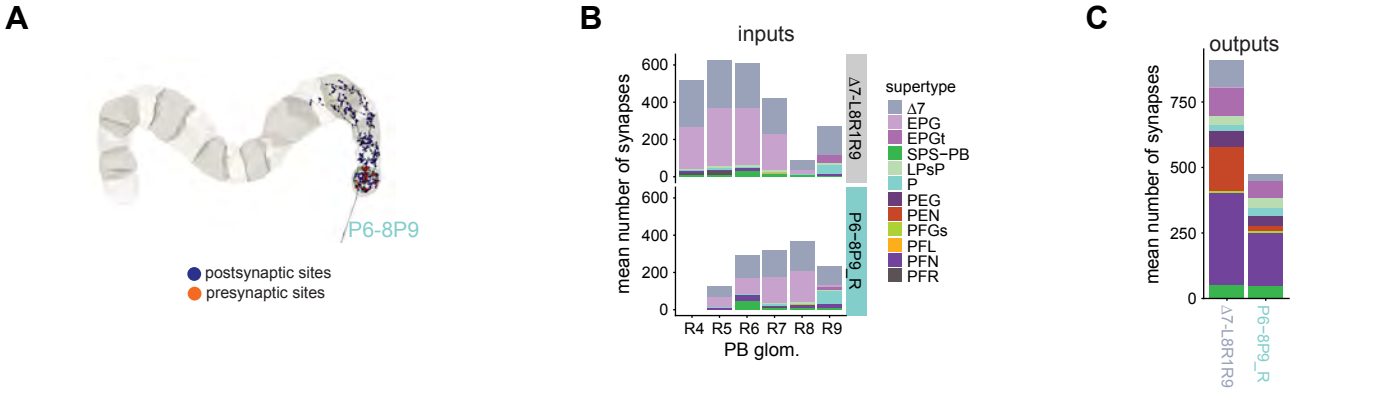


Figure 21: P6-8P9 neuron morphology and connectivity is similar to that of the Δ7 neurons that arborize in the outer glomeruli

A) A morphological rendering of a P6-8P9 neuron. There are two P6-8P9 neurons on each side of the PB, both of which are presynaptic in glomerulus 9.

B) The mean number of synapses onto each Δ7_L8R1R9 neuron (top) or P6-8P9_R neuron (bottom) for PB glomeruli R4-9. The left PB is not considered as one of the two P6-8P9_L neurons was not able to be fully connected due to a hot knife error.

C) The mean number of output synapses from each Δ7_L8R1R9 neuron (left) or P6-8P9_R neuron (right) in PB glomerulus R9. The color code is identical to that in C.

Figure 22: PB input and inner neuron connectivity to output neurons

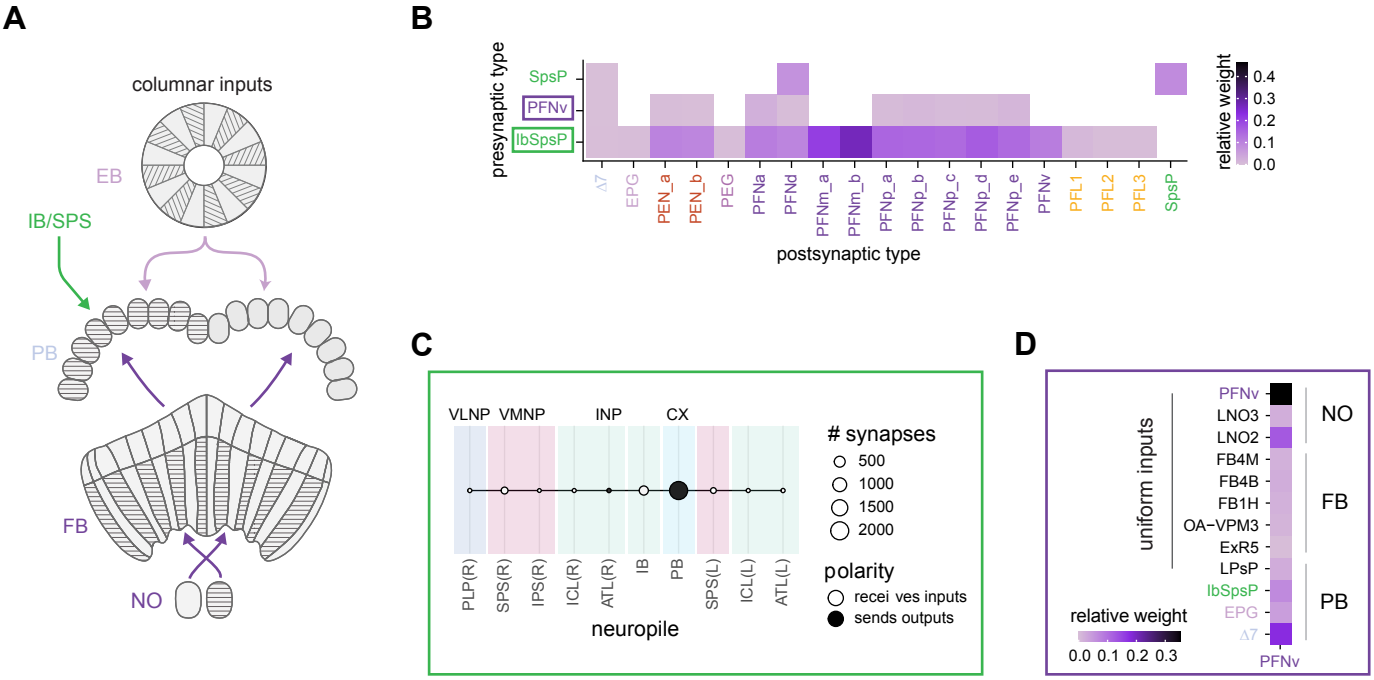


Figure 22: PB input and inner neuron connectivity to output neurons

A) Schematic depicting the neuropil that bring input to the PB via columnar neurons that target single PB glomeruli.

B) Type-to-type connectivity table from select PB inputs (IbSpsPs, PFNvs, and SpsPs) to PB output neurons. The SpsP neurons also connect to themselves.

C) Region arborization plot for the right IbSpsP neurons. The left IbSpsP neurons were not fully contained in the imaged volume.

D) Type-to-type connectivity onto the PFNv neurons by neuropil region.

Figure 22—figure supplement 1: Presynaptic partners of the IbSpsP neurons, outside of the PB

Figure 22–figure supplement 1: Presynaptic partners of the IbSpsP neurons, outside of the PB

Neuron-to-neuron connection table showing the presynaptic partners of the IbSpsP neurons in all regions outside of the PB

Figure 23: Neuromodulatory neurons in the PB output broadly across types

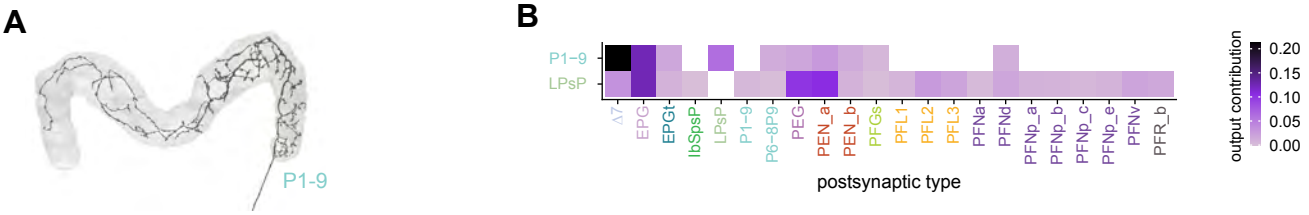


Figure 23: Neuromodulatory neurons in the PB output broadly across types.

A) A morphological rendering of a putative octapaminergic P1-9 neuron.

B) Type-to-type connectivity table for the outputs of the P1-9 neurons and the putative dopaminergic LPsP neurons in the PB.

Figure 24: The number of neurons per glomerulus varies for each columnar neuron type

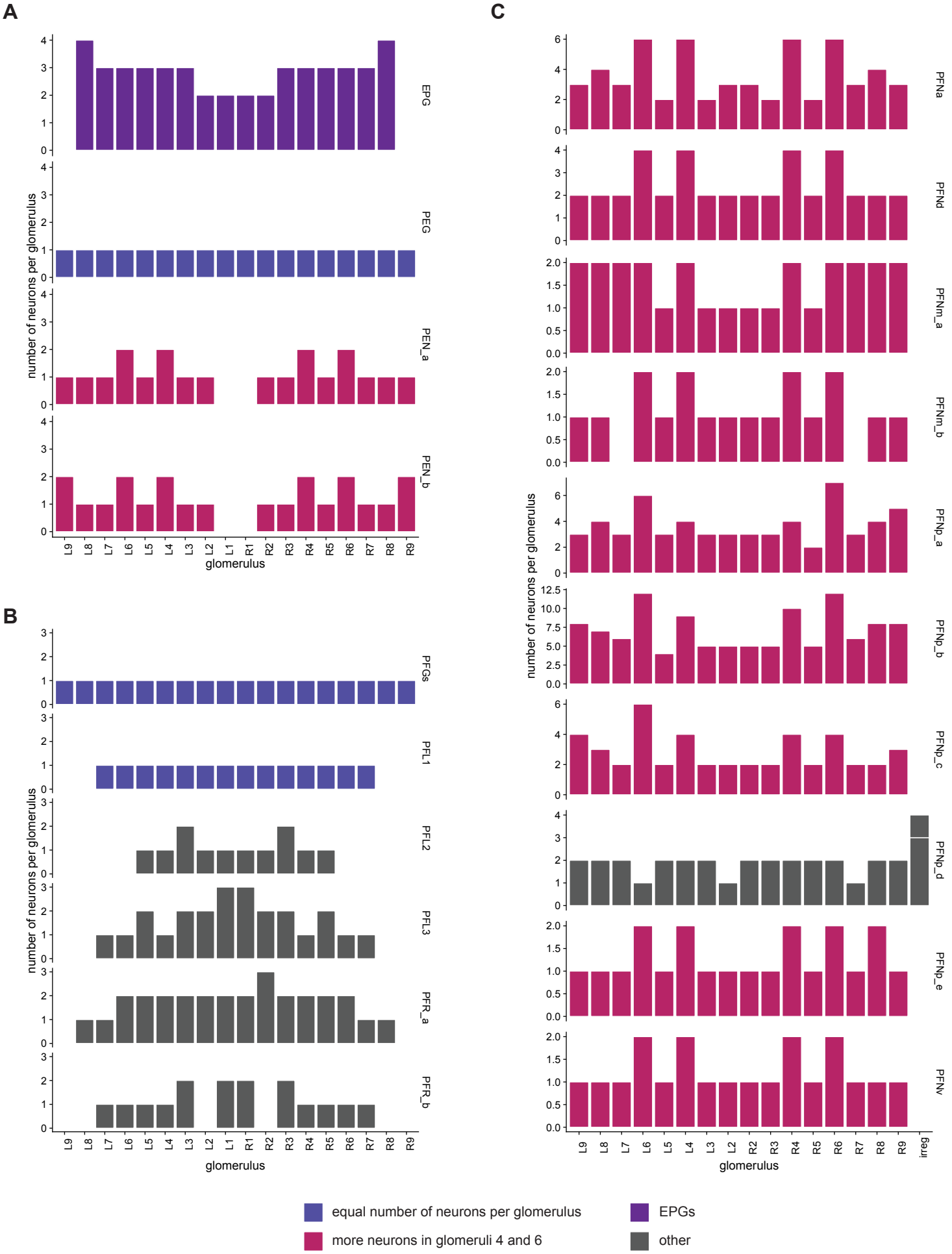


Figure 24: The number of neurons per glomerulus varies for each columnar neuron type

A) Number of neurons per PB glomerulus for each of the PB-EB neuron types.

B) As in **A**, for the PFGs, PFLs, and PFRs.

C) As in **A**, for the PFNs.

Figure 25: Overview of the NO

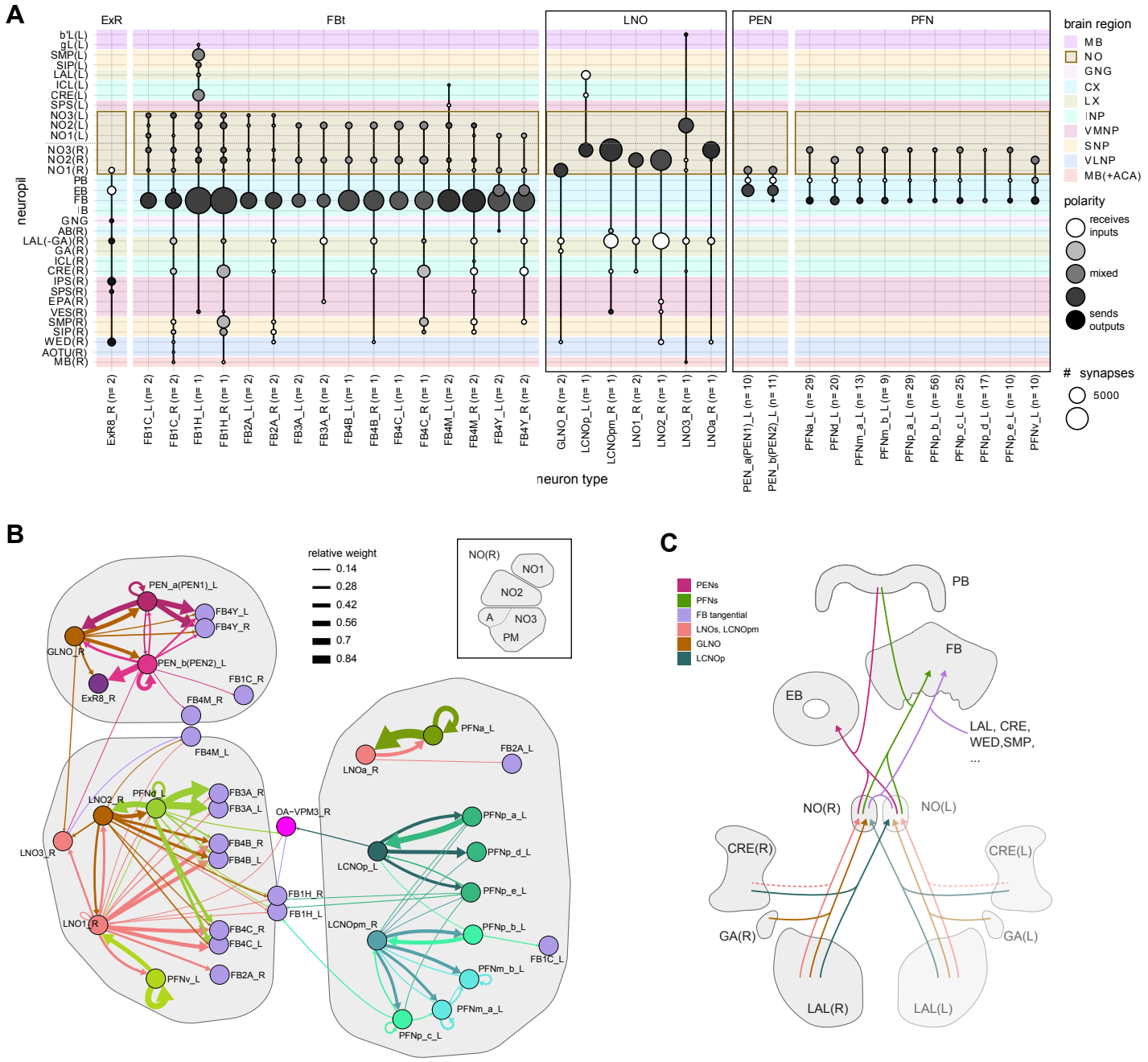


Figure 25: Overview of the noduli and illustration of separate compartments

A) Region arborization plot summarizing all cell types that innervate the noduli (NO), showing average pre- and postsynaptic counts by region. Boxes mark groups of neuron types that will be described in more detail in this section.

B) Connectivity graph of all neuron types in the right NO, highlighting clusters that approximately correspond to anatomically defined sub-compartments (see inset). The line thickness corresponds to the relative weight of a given type-to-type connection. Only connections with a relative weight of at least 0.05 are shown.

C) Schematic of how the NO connect to other brain regions.

Figure 26: Columnar neurons in the NO

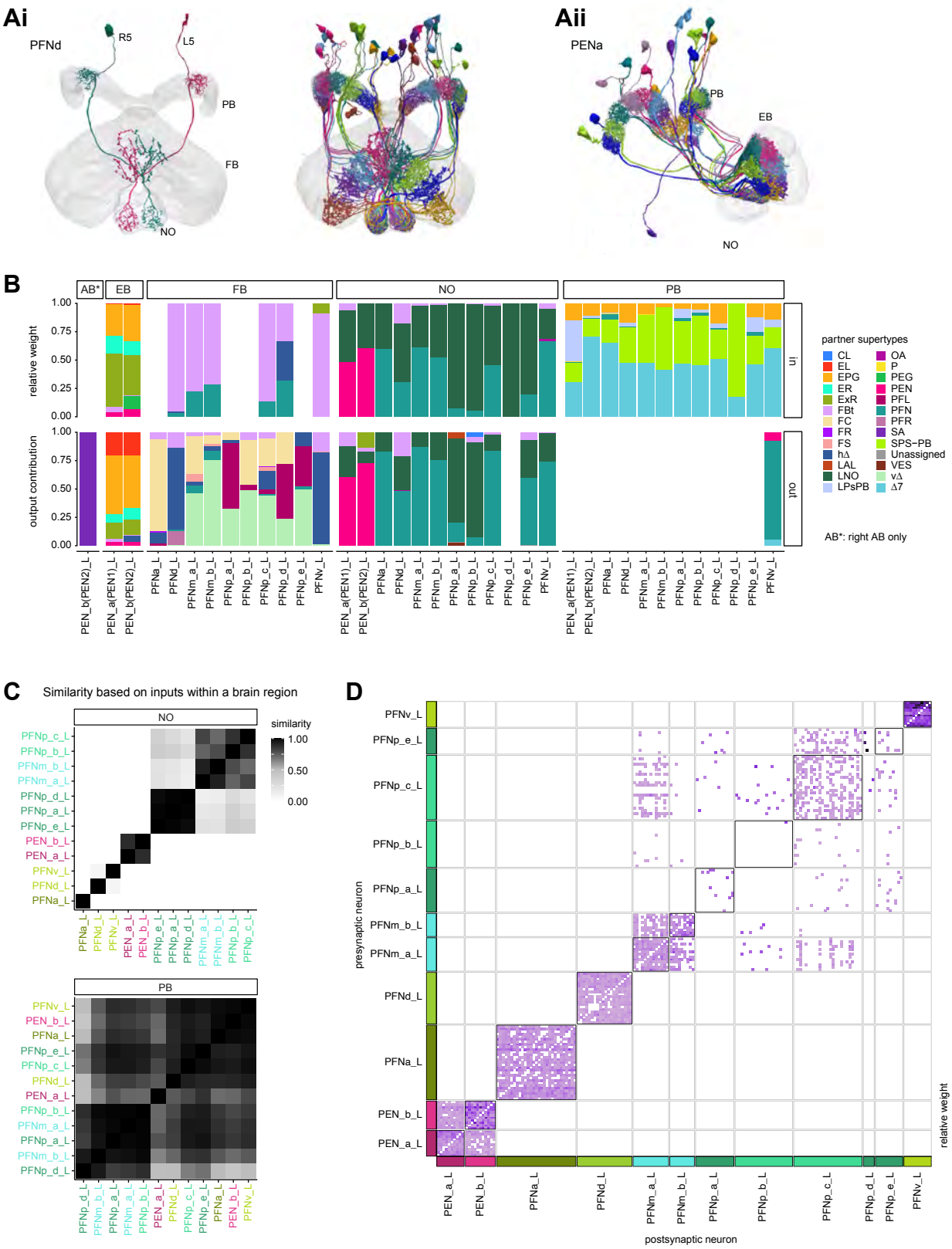


Figure 26: Columnar neurons in the noduli

A) Morphological rendering of columnar neurons. **Ai**: PFNd neurons. *Left*: two example neurons from the left and right PFNd population. *right*: Complete population of PFNd neurons. **Aii**: PENa neurons.

A) Stacked bar graph illustrating the fraction of inputs and outputs to PFN and PEN partners grouped into supertypes and separated by brain region. Inputs and outputs are normalized per neuron type and brain region. The connectivity strength for inputs and outputs is measured by relative weight and output contribution, respectively.

B) Similarity matrices (see Methods) for columnar NO neurons based on their inputs in the NO (*top*) and PB (*bottom*).

C) Neuron-to-neuron connectivity matrix for columnar neurons in the right NO. Connections between neurons of the same type are highlighted with black boxes.

Figure 26—figure supplement 1

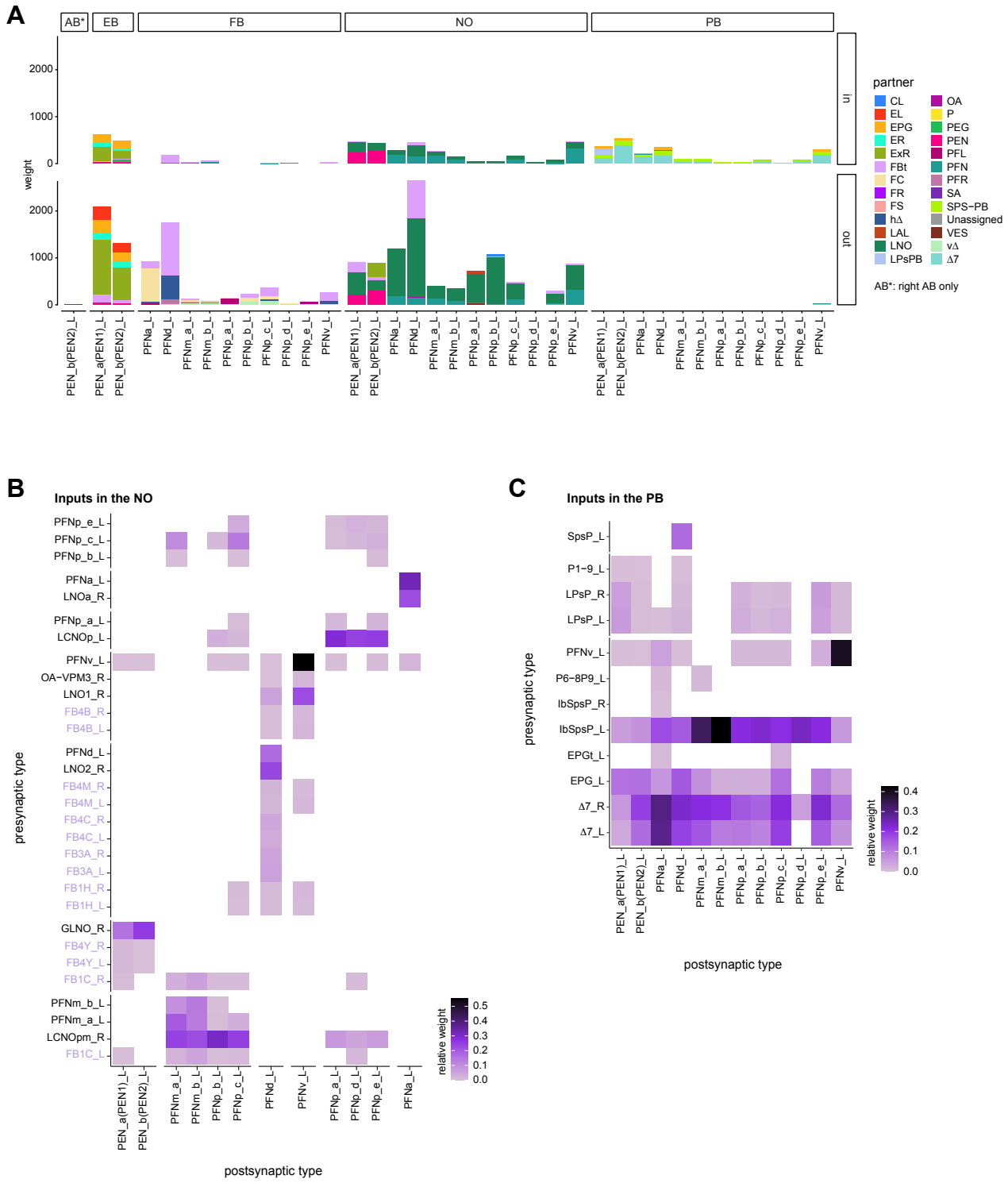


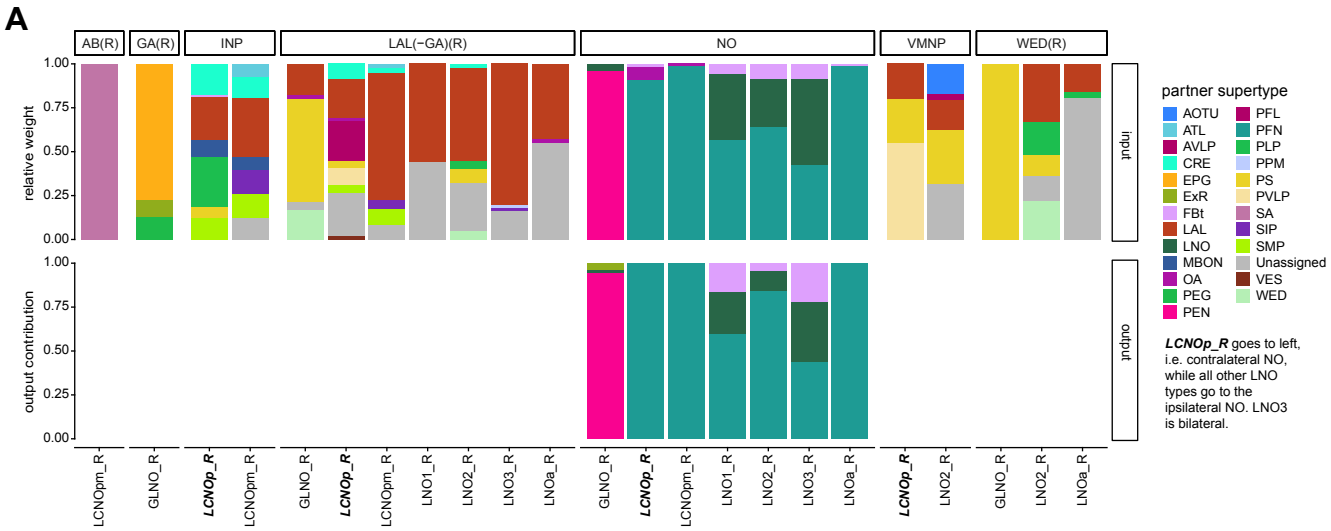
Figure 26—figure supplement 1

A) Stacked bar graph illustrating the weight of inputs and outputs to partners grouped into supertypes and separated by brain region.

B) Connectivity matrix showing inputs to PEN and PFN types in the NO. Connectivity is measured on a type-to-type level.

C) Same as (**B**), but for inputs in the PB.

Figure 27: Non-CX inputs to the NO



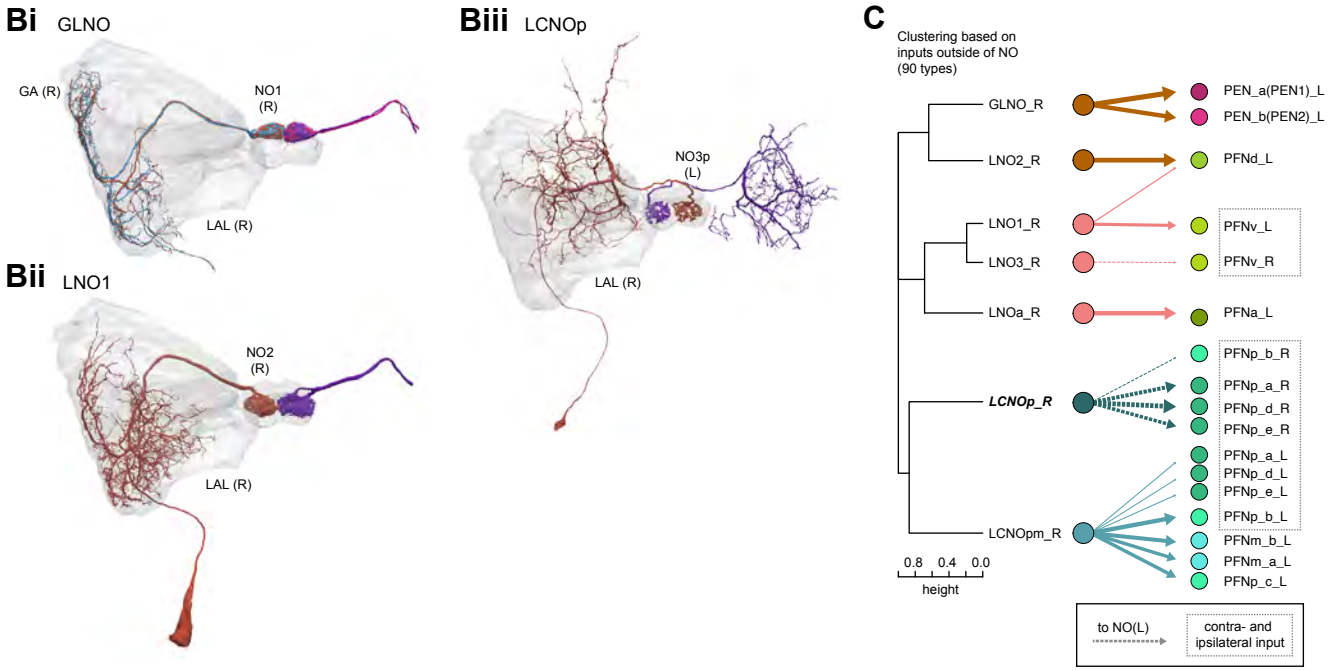


Figure 27: Comparison of LNO neurons, which provide input to columnar neurons

A) Stacked bar graph illustrating the fraction of inputs and outputs of LNO to partners grouped into supertypes and separated by brain region. Inputs and outputs are normalized per neuron type and brain region. The connectivity strength for inputs and outputs is measured by relative weight and output contribution, respectively.

B) Rendering of LNO neurons. **Bi**: GLNO, **Bii**: LNO, **Biii**: LCNOp.Note that LCNOp crosses the midline and arborizes in the contralateral NO.

C) Illustration of how similarity between LNO neuron types relates to their connectivity to columnar NO neurons. *Left*: Dendrogram depicting the similarity between GLNO, LNO and LCNO neuron types based on their inputs outside of the NO (that is, excluding feedback connections from PFN or PEN neurons). The branch height in the dendrogram indicates the normalized distance between types within the similarity space. *Right*: Connectivity from GLNO, LNO and LCNO neurons onto columnar NO neurons, visualized as in the connectivity graph in **Figure 25B**. Note that LCNOp and LNO3 neurons project to the ipsilateral NO and therefore target the right-side population of certain PFN types. PFN types that receive inputs from ipsi- and contralateral LNO and LCNO types are highlighted with dashed boxes.

Figure 27—figure supplement 1

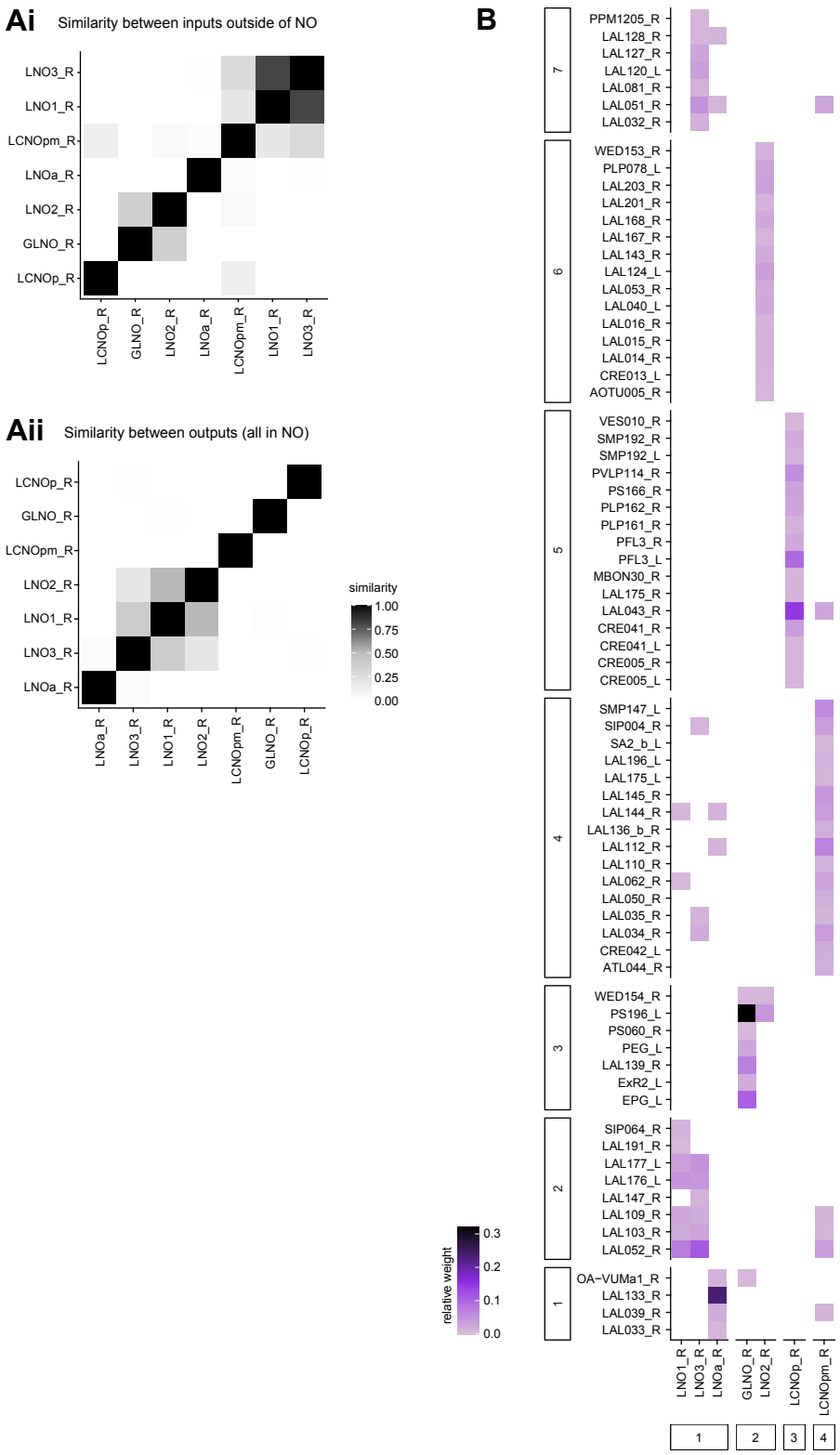


Figure 27—figure supplement 1

A) Similarity matrices (see Methods) for LNO neurons based on their inputs outside of the NO (**Ai**) and within the NO (**Aii**).

B) Connectivity matrix showing all inputs (including those in the NO) to GLNO, LNO and LCNO neurons. The matrix columns and row were rearranged based on clustering GLNO, LNO and LCNO neurons on the basis of their inputs and the input types based on their outputs to GLNO, LNO and LCNO neurons.

Figure 28: Fan-shaped body overview

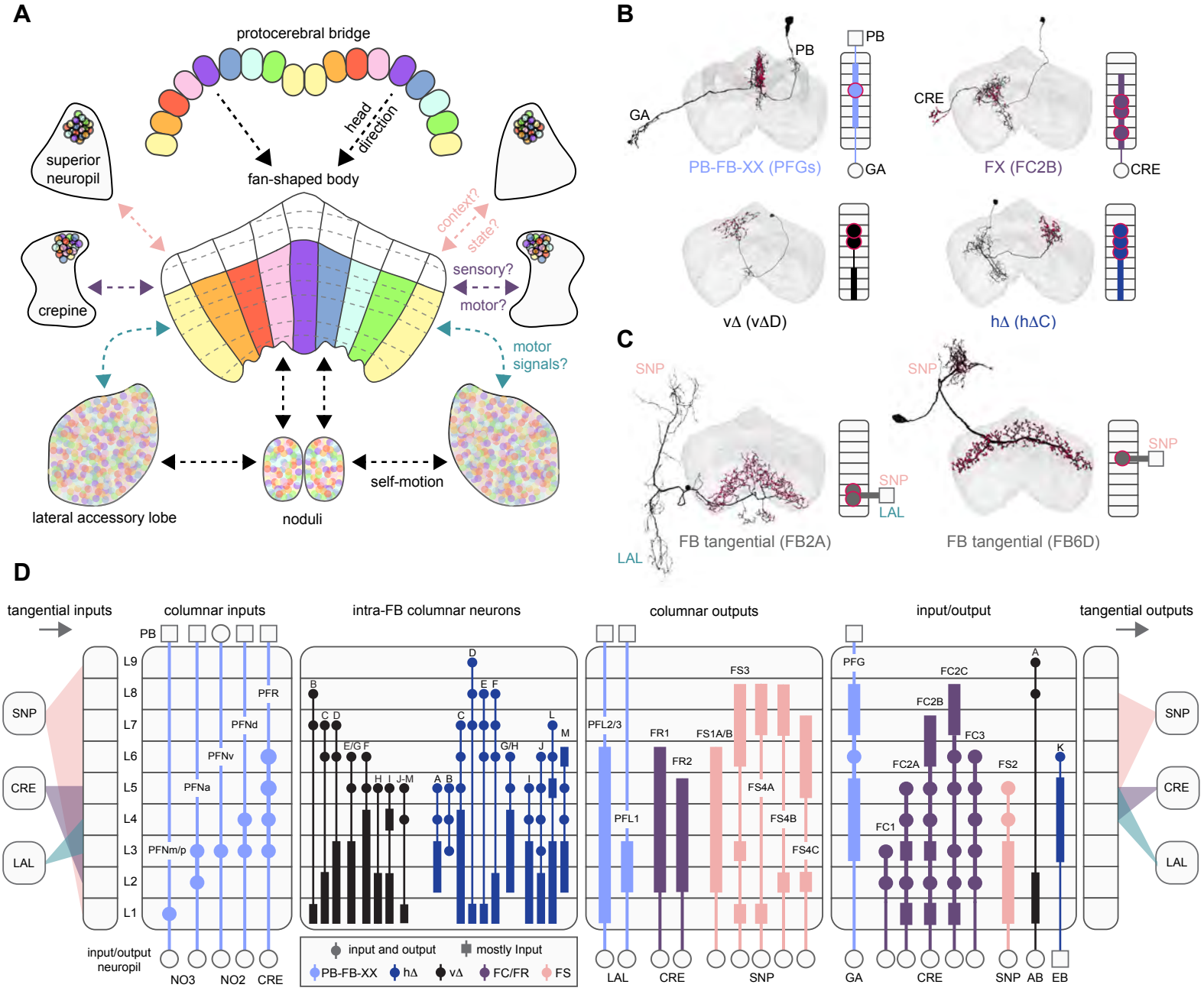


Figure 28: Fan-shaped body overview

A) Schematic showing the fan-shaped body, its main associated input and output neuropil, and the general types of information thought to be conveyed. The FB is divided into nine vertical columns defined by PB-FB neurons, which map the nine glomeruli in the left and right PB to corresponding columns in the FB. Here, FB columns and PB glomeruli are colored by anatomical phase in a way that indicates matching bump locations. In addition to columns, FB tangential cells divide the structure into nine horizontal layers. The ventral FB (layers ~1-6) receives columnar input from the PB while the dorsal FB (layers ~7-9) does not.

B) Morphological renderings of individual columnar neurons (shown in black; red circles are presynaptic sites) from each of the four broad columnar neuron classes: PB-FB-X, FX, vΔ, and hΔ (where X stands for an additional, neuron type-specific neuropil). Each class contains many distinct neuron types. The population of neurons comprising each neuron type innervates all columns of the FB, but in a layer-restricted manner. As shown next to each anatomical rendering, a neuron type's morphology can be summarized by illustrating the location of dendritic (rectangle) and axonal (circles) compartments for the 9 FB layers and any associated neuropil. Here, each neuron type is colored according to its class (see legend in **D**).

C) Same as in **B**, but for two of the 145 types of FB tangential cells.

D) Schematic showing the innervation pattern of every FB columnar neuron type, each illustrated as in (B). Columnar neurons can be roughly grouped into four putative functional groups: those that convey information from outside the fan-shaped body to specific fan-shaped body layers (Columnar inputs; subset of PB-FB-X neurons), those that convey information between layers of the fan-shaped body (Intra-FB columnar neurons; vΔ and hΔ neurons), those that convey information out of the fan-shaped body (Columnar outputs; PB-FB-X and FX), and those that could perform a mixture of these functions (Input/Output). Columnar inputs have axons in every FB layer they innervate, intra-FB columnar neurons have processes confined to the FB, and columnar outputs have dendrites (and very few axons) in every FB layer they innervate. In addition, tangential cells innervating the SNP, CRE, and/or LAL (and additional structures) provide input to (left panel) and output from (right panel) specific FB layers. Tangential cells in many different layers send processes to the SNP, CRE, and/or LAL, but only consistently target these regions in most cell types in the layers that are shown. See **Figure 28—figure supplement 1** for average pre- and postsynaptic counts by region and columnar neuron type.

Figure 28—figure supplement 1: FB columnar neuron region arborization plots

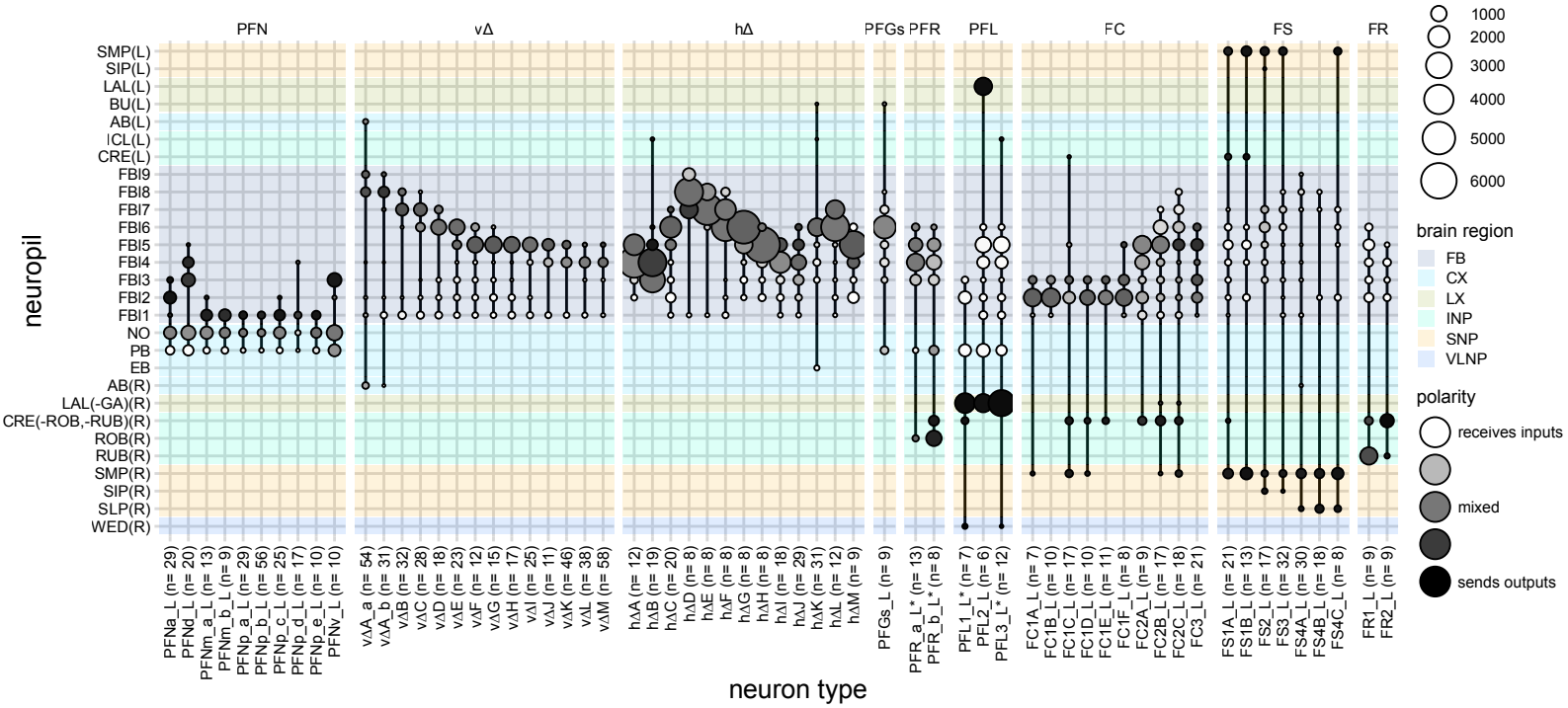


Figure 28—figure supplement 1: FB columnar neuron region arborization plots

Figure 29: PB-FB-X neurons form 9 structural columns in the FB

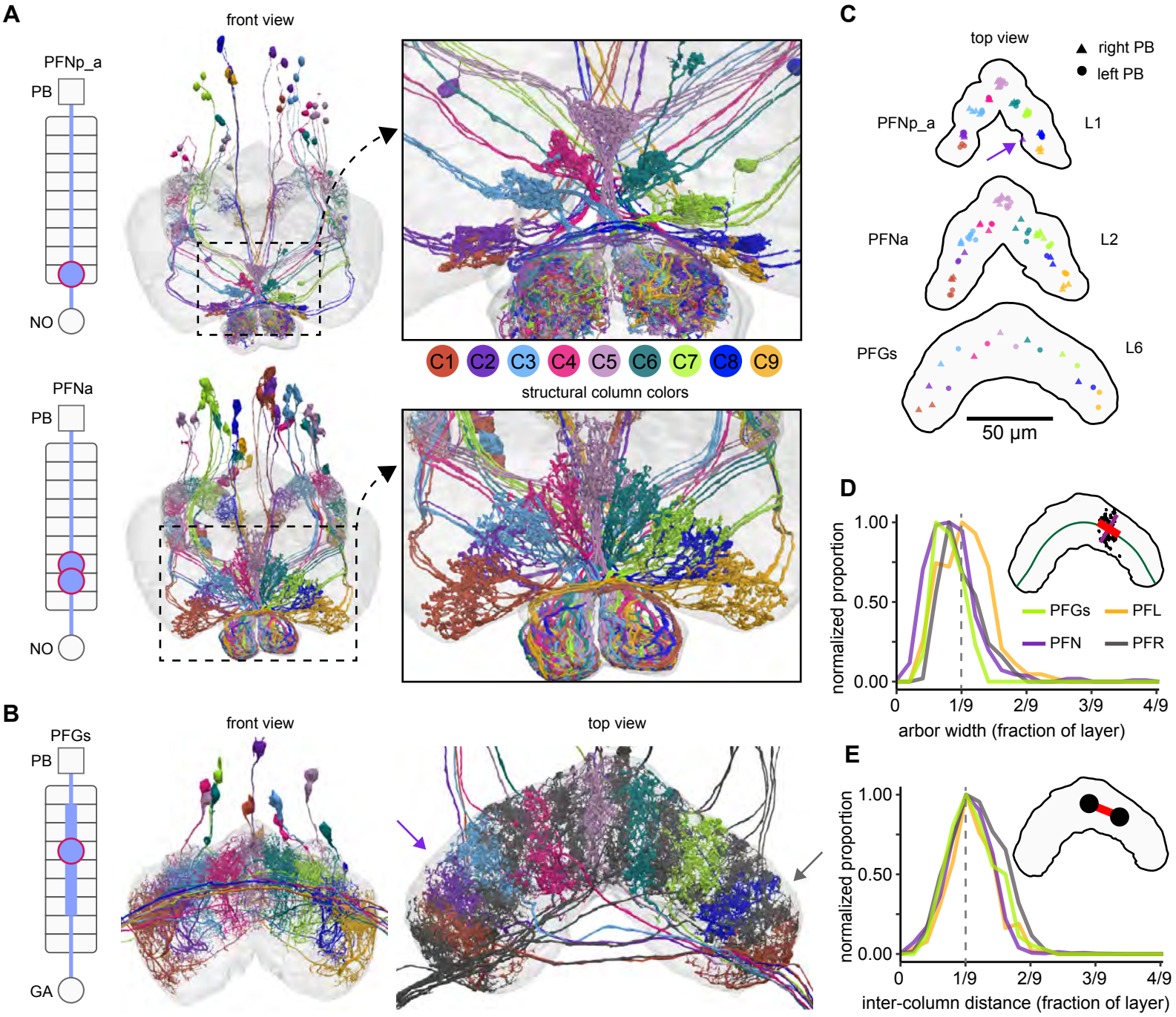


Figure 29: PB-FB-X neurons form 9 structural columns in the FB

A) Morphological renderings of PFNp_a and PFNa populations, colored by structural column (C1-C9). Right panels show zoomed in views of the FB, revealing a 9-column structure. Notice that PFNp_a columns are clustered while PFNa columns tile the FB more evenly. Note also that columns can be partially identified by fiber tracts.

B) Morphological rendering of the 18 neurons composing the PFGs population. Middle panel grays the 9 neurons that project to the right GAL, revealing that each column has left/right halves, which breaks the 9 columns into ~18 "demi-columns", one neuron per demi-column, with two exceptions (purple and gray arrows). The purple arrow marks a demi-column which lacks separation from adjacent demi-columns. Similarly, the gray arrow marks a demi-column containing two neurons, whereas all other demi-columns contain 1 neuron. Whether these are the result of wiring errors requires further investigation.

C) Top down view showing every neuron's mean location for all individual neurons in the PFNp_a, PFNa, and PFGs populations. Notice that while PFNp_a forms 9 clear clusters, PFGs tile space more evenly. The arrow in the PFNp_a panel points to a neuron that innervates both C1/C2 (assigned to C2) and C9, which is why its synapse location ended up outside of either cluster.

D) Distribution of neuronal arbor widths for PFGs, PFN, PFL, and PFR neurons. As shown in the inset, the width (red line) of synaptic point clouds (black dots) from individual neurons was measured along a direction locally tangent to a line bisecting the FB layer (green). To account for differences in layer size, the raw width (red line) was normalized by dividing the length of the layer (green line). Each distribution was normalized to have a peak of 1. The vertical dashed line marks $1/9^{th}$ of the layer width, the arbor width that would result from 9 evenly spaced columns that have minimal overlap and collectively tile the layer. Notice that most neurons take up slightly less than $1/9^{th}$ of the layer. Importantly, this measure is independent of the neuron's column assignment.

E) Distribution of inter-column distance, expressed as a fraction of the layer width, as in (**D**). Inter-column distance was measured by calculating the distance between pairs of neurons in adjacent columns (as shown in inset), normalized to the length of the layer.

Figure 29—figure supplement 1: Columnar structure of PB-FB-X neuron types

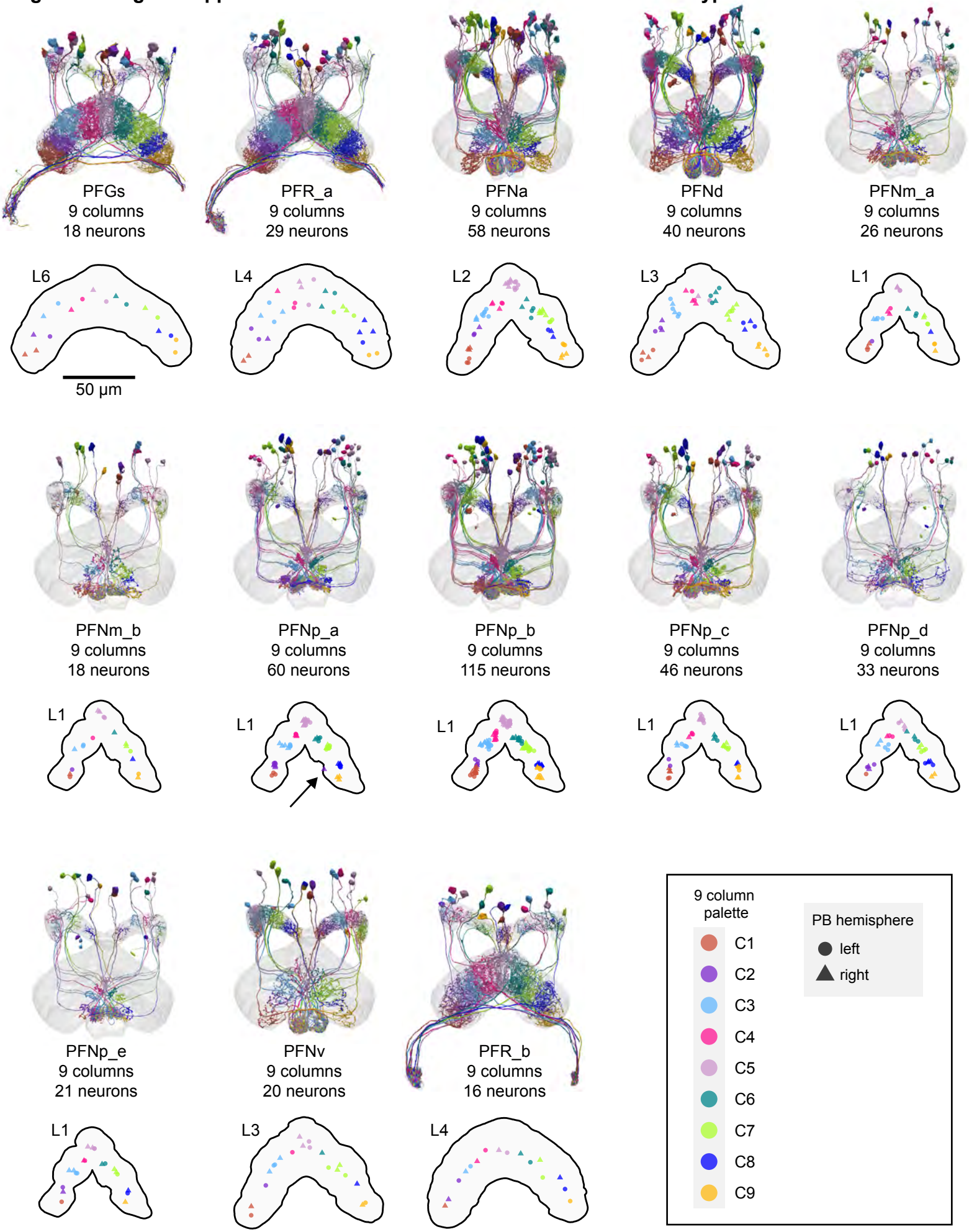


Figure 29—figure supplement 1: Columnar structure of PB-FB-X neuron types

Population morphological renderings (top panels) and median neuron locations (bottom panels) for every PB-FB-XX neuron type, with the exception of PFL1-3, which are shown later. Median neuron locations are shown for the FB layer with the most synapses for each neuron type. Neurons are colored by structural column (see legend). Note that most neuron types that innervate L1 most heavily show evidence for 9 clustered structural columns. Neuron types that innervate more ventral layers tend to show less clear clustering. Unlike all other PFN neurons, PFNd neurons form 8 structural columns. At present, the PFNd neuron names reflect the 9 column scheme, but will be changed to 8 columns in future database versions. The arrow in the PFNp_a panel points to a neuron that innervates both C1/C2 (assigned to C2) and C9, which is why its synapse location ended up outside of either cluster.

Figure 30: PB-FB-X neurons have type-specific phase shifts in PB-to-FB projections

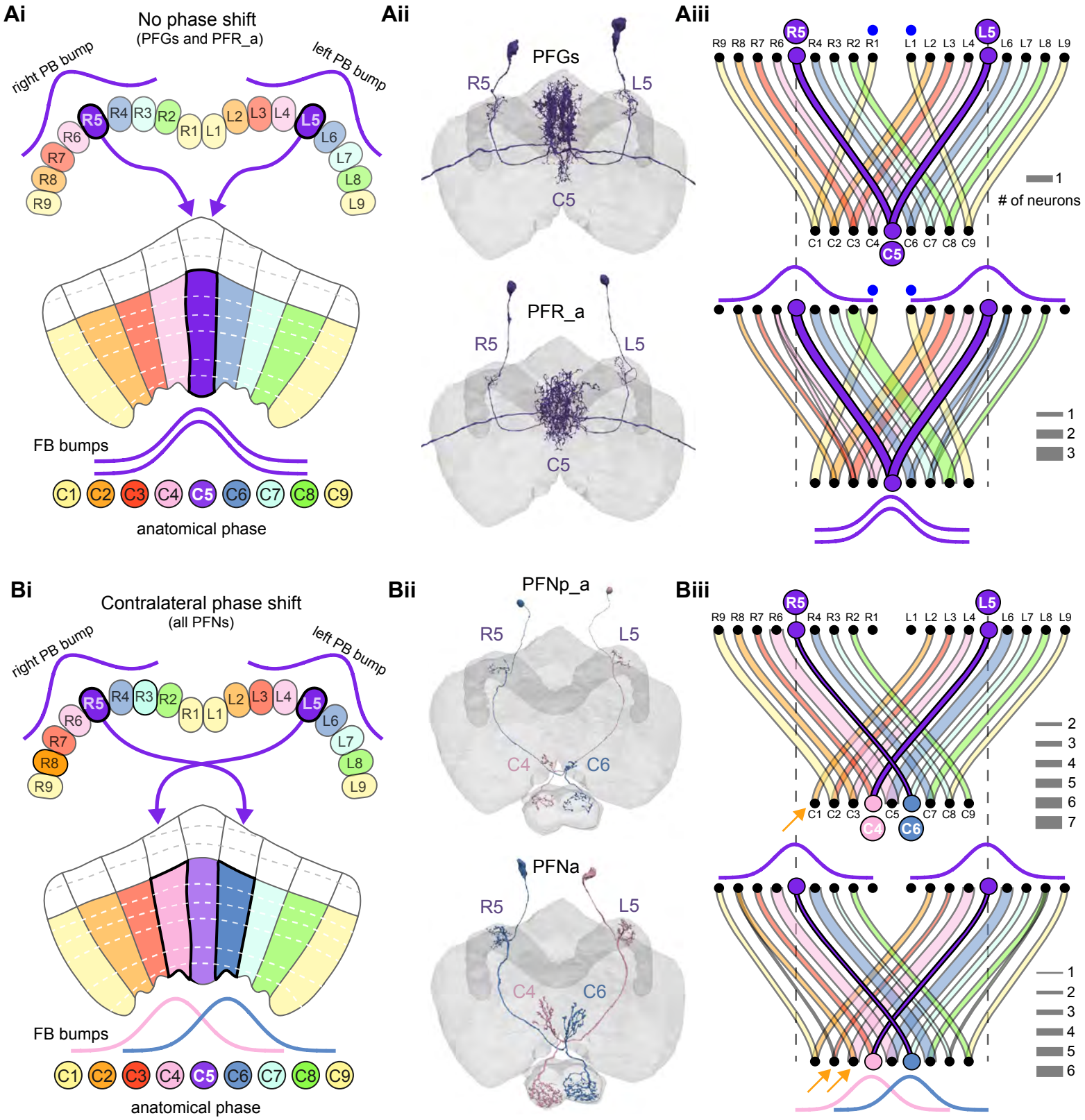


Figure 30: PB-FB-X neurons have type-specific phase shifts in PB-to-FB projections

A) PFGs and PFRa neurons connect PB glomeruli to FB columns with no phase shift.

i) Schematic of a PB-to-FB projection pattern with no phase shift. PB glomeruli and FB columns are colored according to anatomical phase, which indicates matching bump locations. Based on EB-to-PB columnar neuron projection patterns (EPG neurons, see **Figure 16-19**), when a bump is centered at R5 in the right PB, a second bump will be centered at L5 in the left PB (both marked in purple). With no phase shift in their projection pattern, neurons innervating R5/L5 both project to C5 in the FB. This pattern, repeated across glomeruli/columns (see **Aiii**), would bring the two bumps in the PB to the same FB location.

ii) Morphological renderings of single neurons innervating R5 and L5, from the PFGs (top panel) and PFR_a (bottom panel) populations. Neurons are colored according to their FB column. Notice that the R5/L5 neurons end up at mastching locations (C5) in the FB.

iii) Graphs showing the projection pattern from PB glomeruli to FB columns for all neurons in the PFGs (top panel) and PFR_a (bottom panel) populations. R5 and L5 projections have been highlighted as in **Ai**, and edges are colored according to PB glomerulus. As this full mapping shows, corresponding bump locations in the left and right PB get mapped to a corresponding FB column, regardless of bump position. Blue dots mark glomeruli R1 and L1, whose neurons project to the opposite hemisphere (GAL for PFGs; ROB for PFR_a) than the other neurons in their half of the PB, the functional significance of which is unknown.

B) PFN types have 1-column contralateral phase shifts in their PB-to-FB projection pattern.

i) Schematic of a PB-to-FB projection pattern, as in **Ai**, but now showing a 1-column contralateral phase shift. Notice that R5 projects to C6, and L5 projects to C4. This pattern, repeated across glomeruli/columns (see Biii), would cause PB bumps centered at R5 and L5 to end up at different locations in the FB.

ii) Morphological renderings of single neurons innervating R5 and L5, as in **Aii**, but now for PFNp_a and PFNa. Notice that the R5 neurons project to C6 and the L5 neurons project to C4.

iii) Graphs showing the projection pattern from PB glomeruli to FB columns, as in Aiii, but for PFNp_a and PFNa. Edges beginning at R5 and L5 have been highlighted, as in **Bi**. Edges are colored according to PB glomeruli. As this full mapping shows, corresponding bump locations in the left and right PB get mapped to spatially-shifted locations in the FB. Orange arrow in top panel marks PFNp_a C1, which receive input from a single PB glomerulus (L2). Orange arrows in bottom panel mark PFNa C2 and C3 columns, which receive input from the same glomerulus (R8).

Figure 30—figure supplement 1: Continuous phase shifts of PB-FB-X columnar neurons with inputs to the FB

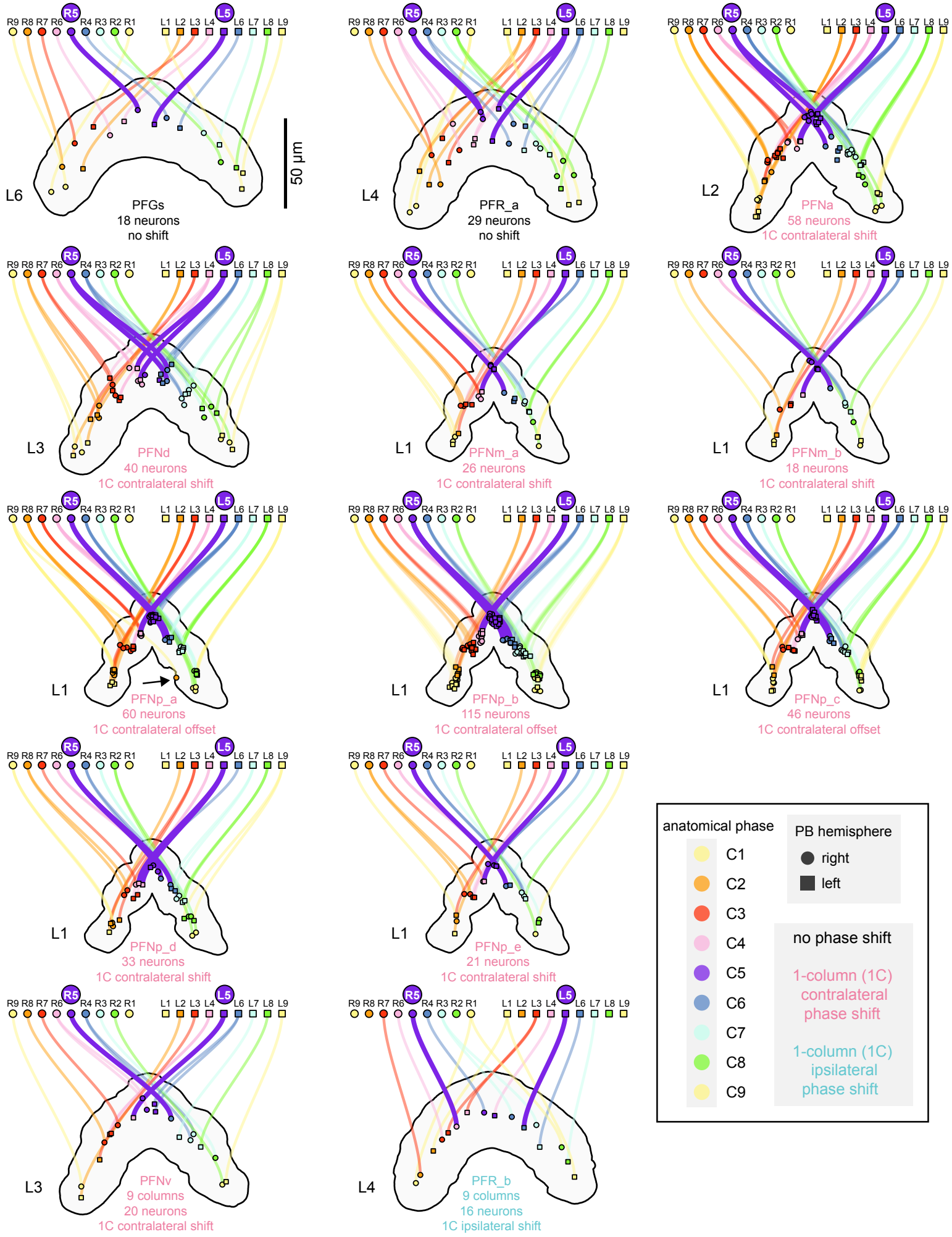


Figure 30—figure supplement 1: Continuous phase shifts of PB-FB-X columnar neurons with inputs to the FB

Phase shift maps showing the PB-to-FB projection pattern for each PB-FB-X columnar neuron type that provides input to the FB (PFL1-3 phase shifts shown later). Each panel shows median neuron locations of a PB-FB-X type, as in previous plots, but with neurons colored according to functional column (see legend). To illustrate the continuous nature of the PB-FB phase shifts, each FB neuron location has a line connecting it to the PB glomerulus it innervates. PB glomeruli and connecting lines are colored by functional column as well. Lines that connect nodes (PB glomeruli and FB columns) of two different colors indicate a phase shift connectivity pattern. Glomeruli R5 and L5 are highlighted to aid in identifying phase shifts. Note that PFGs and PFR_a neuron types connect R5 and L5, which are both purple nodes, to C5, which is also a purple node, indicating no phase shift. In contrast, every PFN neuron type connects R5 (purple) to C6 (blue) and L5 (purple) to C4 (pink), indicating a 1-column contralateral phase shift. Similarly, PFR_b has a 1-column ipsilateral phase shift in its PB-to-FB projection pattern.

Figure 31: Overview of vΔ and hΔ columnar structure

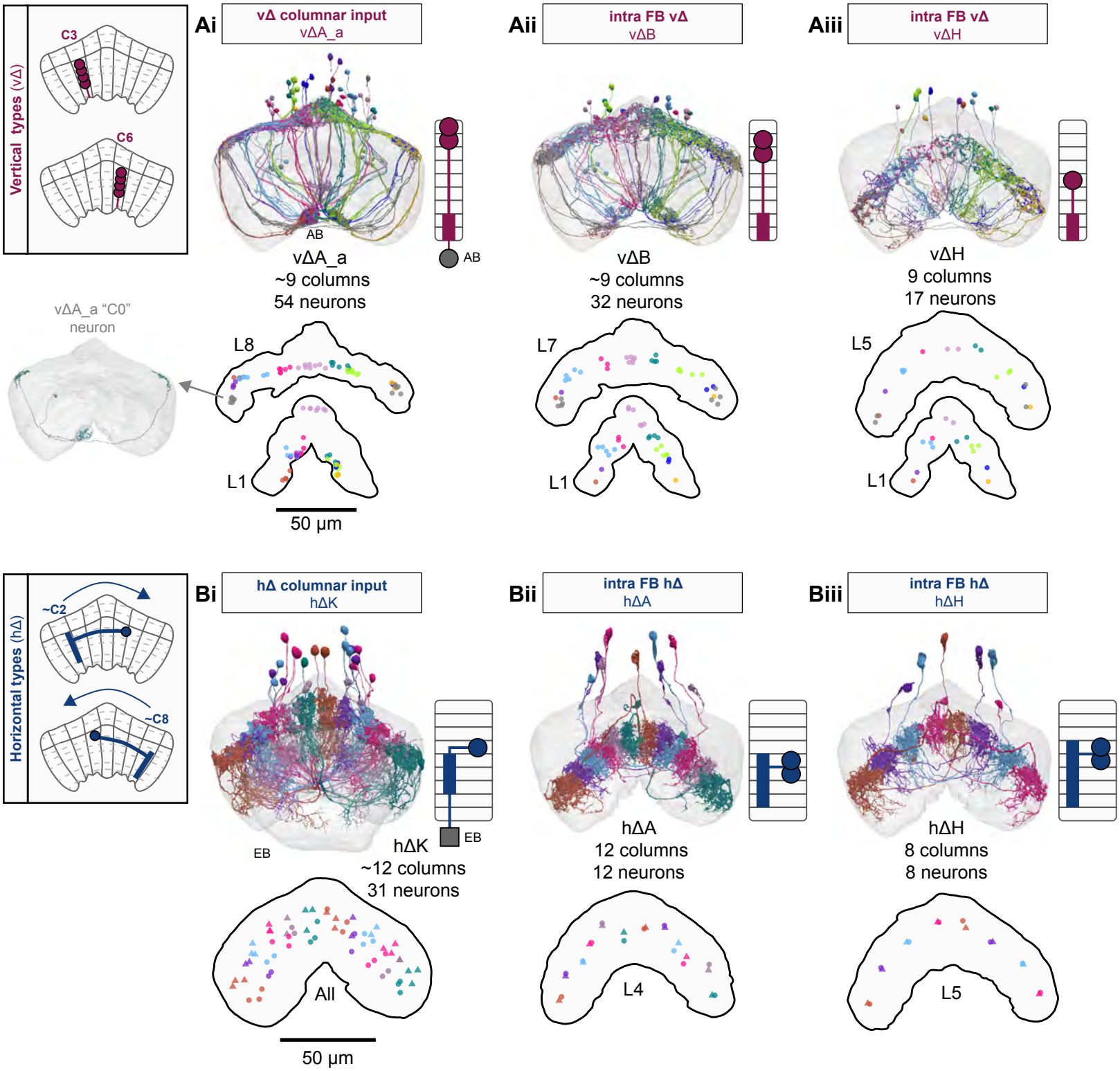


Figure 31: Overview of vΔ and hΔ columnar structure

A) Vertical columnar interneurons—the vΔ neuron types— have individual neurons with processes centered around one FB column. Schematic on left shows two schematized neurons with arbors centered on C3 and C6.

- **i)** Morphological rendering of the vΔA_a population, along with their schematized innervation pattern. Individual neurons are colored by FB structural column (from C1 to C9). In addition to innervating the FB, vΔA_a neurons (and some vΔA_b) are unique among vΔ neurons in that they innervate an extra-FB area, the asymmetric body. Also notice the high degree of overlap of processes in the dorsal FB and the messy columnar structure of the population. Inset to the left shows a "C0" neuron, which have arbors in both C1 and C9.
- **ii)** Same as in **Ai**, but for the vΔB population. As with all other vΔ types, these neurons have processes restricted to the FB and receive most of their input in ventral layers while sending most of their output to more dorsal layers.
- **iii)** Same as in **Ai**, but for the vΔH population.

B) Horizontal columnar interneurons —the hΔ types— have individual neurons with processes centered on two distant FB columns, as shown in the illustration for two generic hΔ neurons. In particular, each hΔ neuron has a dendritic compartment that is ~180 degrees away from its axonal compartment. Half of the population has dendrites in right FB columns and project to left FB columns, while other the half of the population does the opposite. Individual hΔ neurons are assigned to columns based on the location of their dendritic compartment.

- **i)** Morphological rendering of the hΔK population, along with their schematized innervation pattern. Individual neurons are colored according to FB column, with matching columns given matching colors. To achieve the ~180 degree phase shift, all hΔ types form an even number of columns. In this case, 12 columns (marked with 6 colors). In addition to innervating the FB, hΔK neurons are unique among hΔ neurons in that they innervate an extra-FB area, the EB.
- **ii)** Same as in **Bi** but for the hΔA population, which also forms 12 columns. Like most hΔ neurons, hΔA receives the majority of its input in ventral FB layers and provides most of its output to more dorsal FB layers.
- **iii)** Same as in **Bi** but the for the hΔH population, which forms 8 columns instead of 12. Note the highly columnar structure of hΔ neuron types compared to the vΔ neuron types from **Ai-Aiii**.

Figure 31—figure supplement 1: Columnar structure of vΔ neuron types

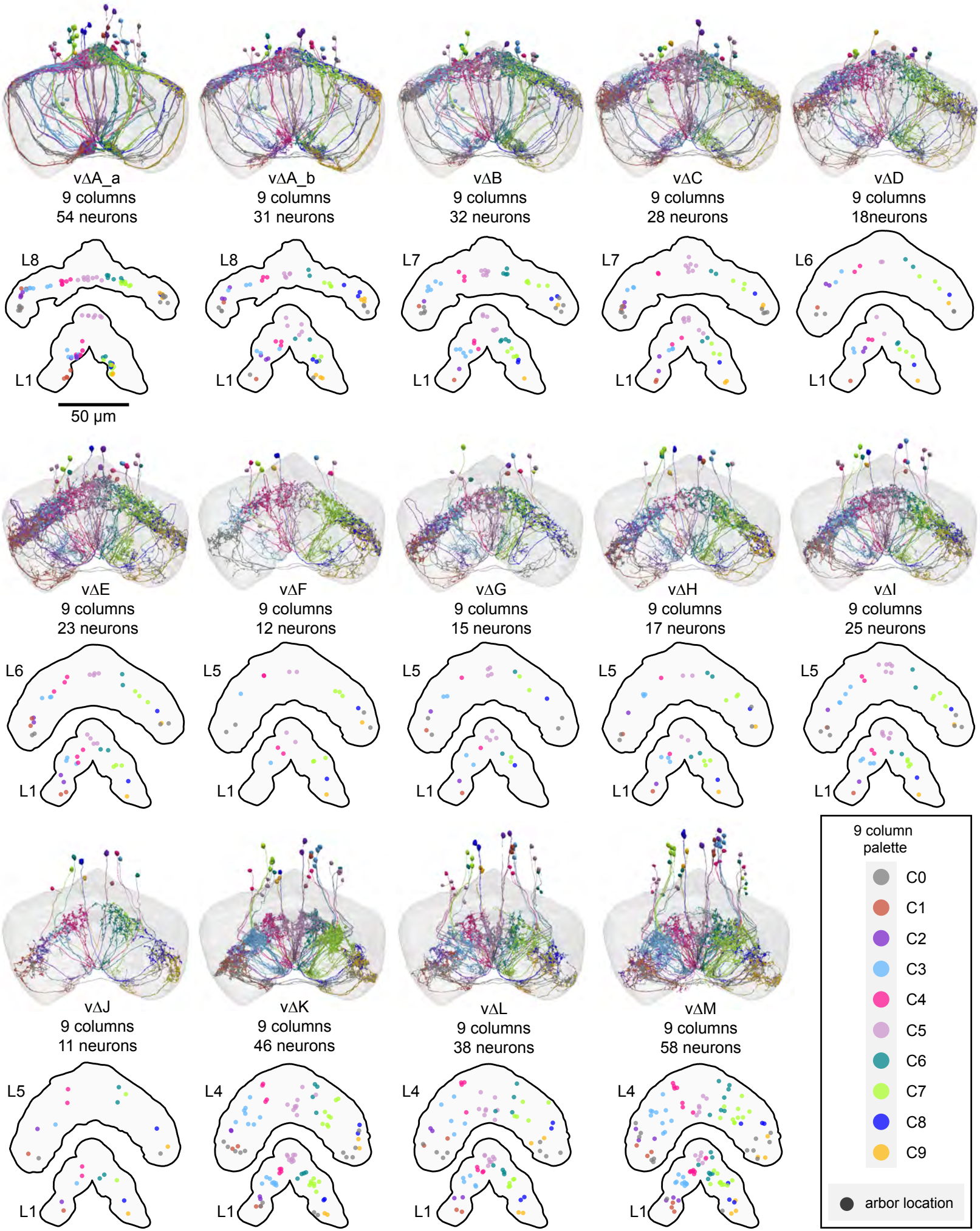


Figure 31—figure supplement 1: Columnar structure of vΔ neuron types

Population morphological renderings (top panels) and median neuron locations (bottom panels) for every vΔ neuron type. Median neuron locations are shown for layer 1, where most vΔ types have primarily dendritic arbors, as well as the dorsal layer containing the most synapses, where vΔ types have axonal arbors. Neurons are colored by structural column (see legend). Gray neurons indicate those vΔ neurons that project to both C1 and C9, which we refer to as C0. Instead of marking median neuron location in the bottom panels, the gray dots mark the median location of the two arbors. Note that most vΔ neuron types show a highly variable columnar structure.

Figure 31—figure supplement 2: Columnar structure of hΔ neuron types

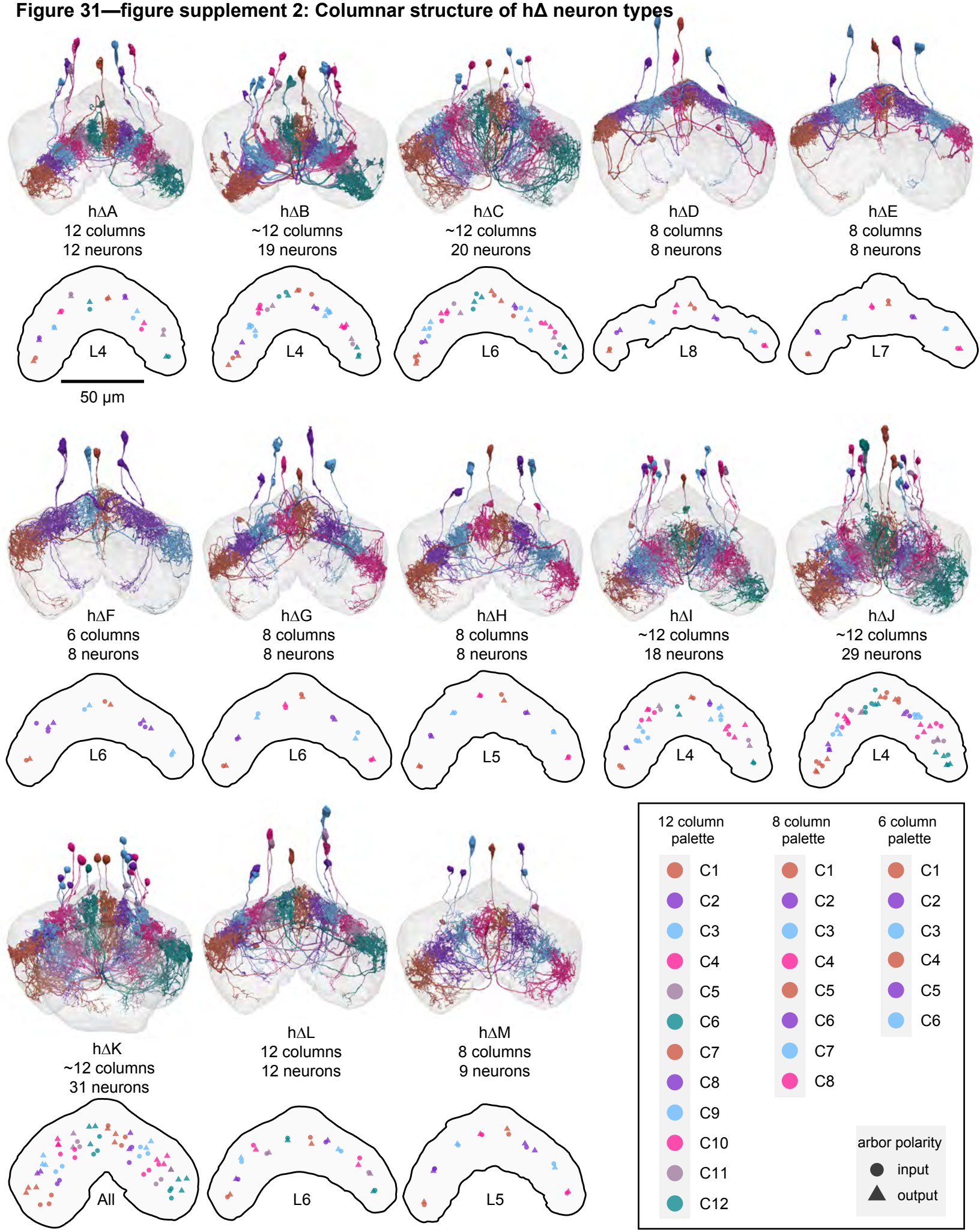


Figure 31—figure supplement 2: Columnar structure of hΔ neuron types

Population morphological renderings (top panels) and median input and output arbor locations (bottom panels) for every hΔ neuron type. Median input (circles) and output (triangles) arbor locations are shown for the FB layer with the most synapses for each neuron type, except for hΔ which has axons and dendrites in separate layers so all layers were used. Neurons are colored by structural column in a way that preserves left- and right-projecting pairs (see legend). Note that hΔ neuron types make a variable number of columns and that some types show a tighter columnar structure than others.

Figure 32: Overview of FX columnar structure

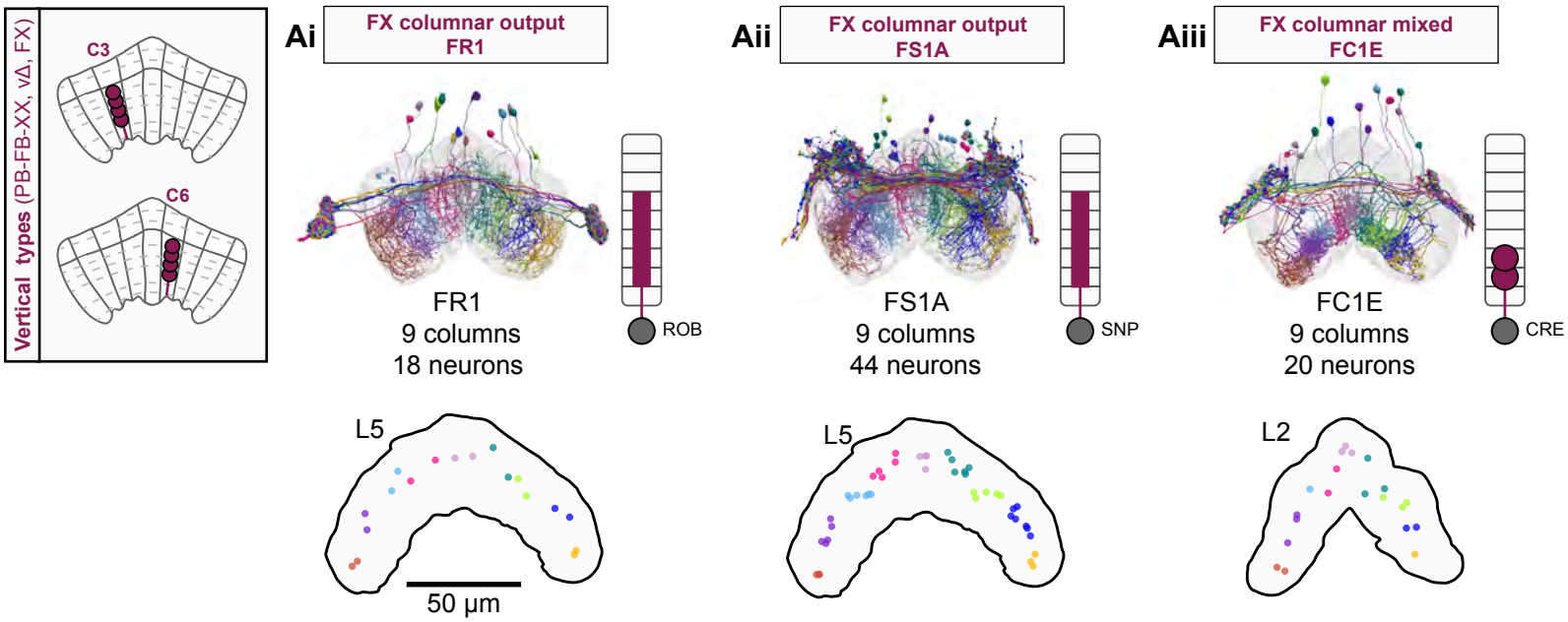


Figure 32: Overview of FX columnar structure

A) FX neurons types all have a vertical morphology, with processes centered around one FB column. Schematic on left shows two schematized neurons with arbors centered on C3 and C6.

i) Morphological rendering of the FR1 population, along with their schematized innervation pattern. Individual neurons are colored by FB structural column (from C1 to C9). In addition to innervating the FB, FR types innervate the ROB.

ii) Same as in **Ai**, but for the FS1A population. FS types innervate both the FB and the SNP.

iii) Same as in **Ai**, but for the FC1E population. FC types innervate both the FB and the CRE.

Figure 32—figure supplement 1: Columnar structure of FR and FS neuron types

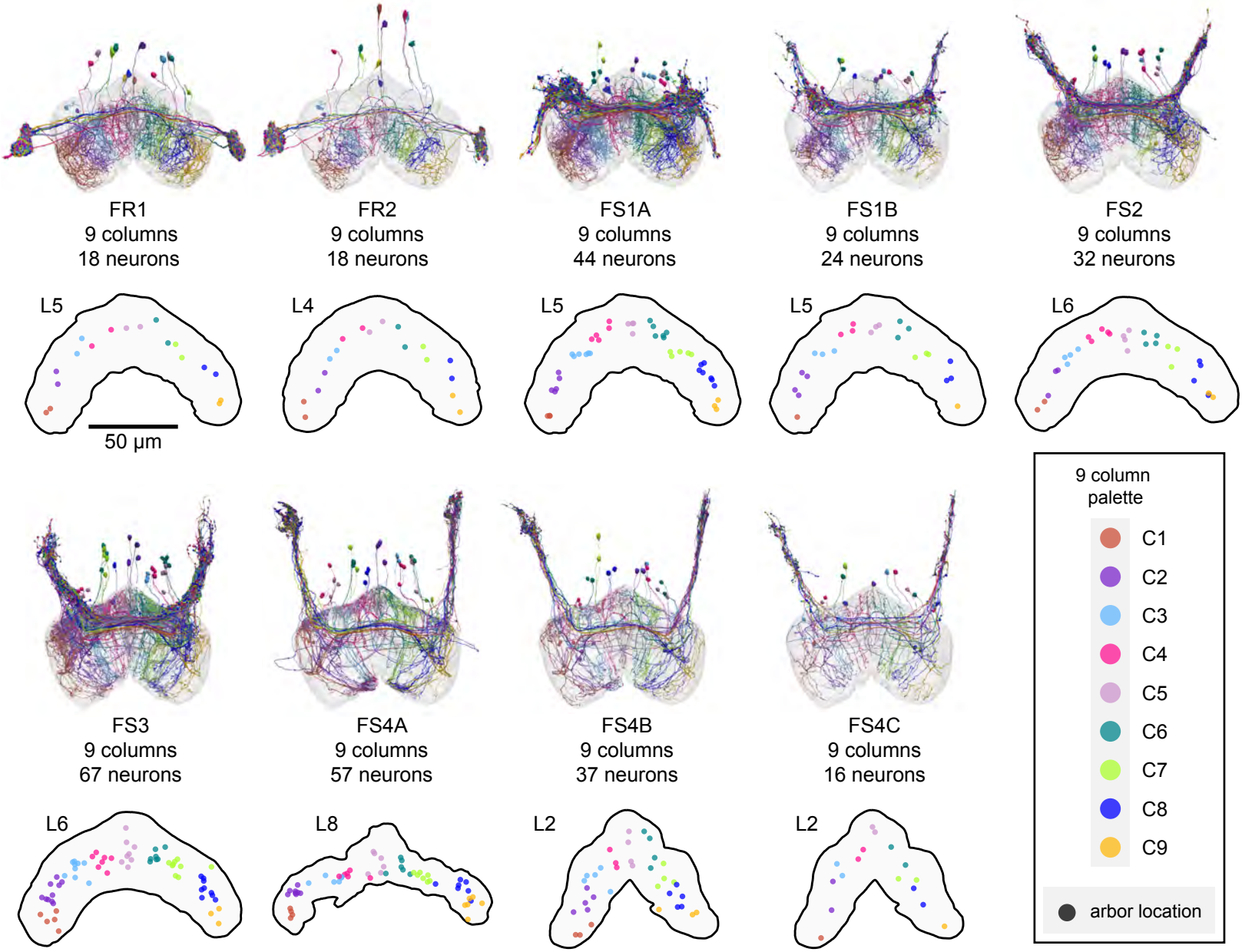


Figure 32—figure supplement 1: Columnar structure of FR and FS neuron types

Population morphological renderings (top panels) and median neuron locations (bottom panels) for every FR and FS neuron type. Median neuron locations are shown for the FB layer containing the most synapses for each neuron type. Neurons are colored by structural column (see legend). Note that FR1 and FR2 are each composed of 18 neurons, with 2 neurons per column. Note also that some FS types, such as FS1A, show evidence for 9 clustered structural columns.

Figure 32—figure supplement 2: Columnar structure of FC neuron types

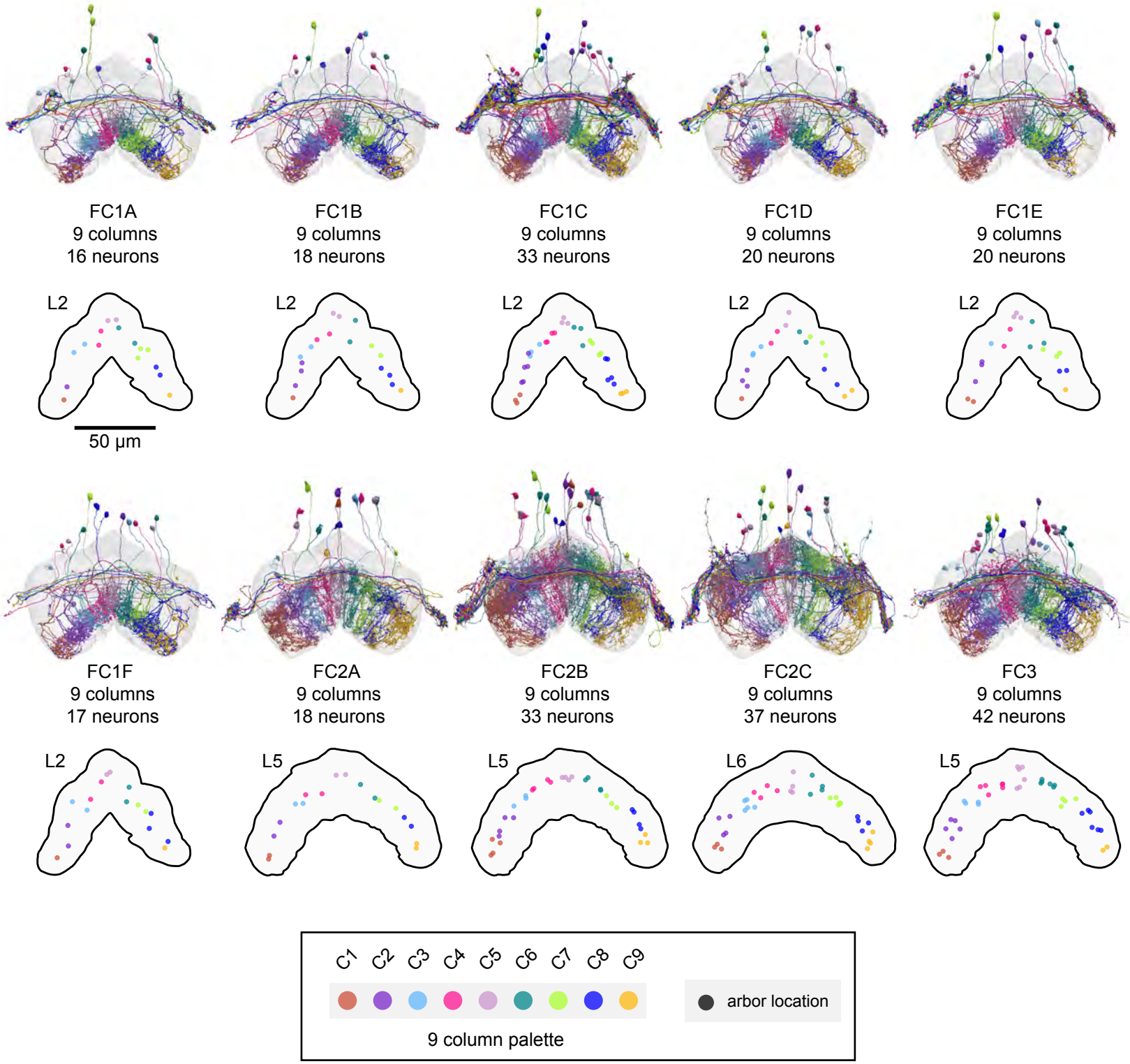


Figure 32—figure supplement 2: Columnar structure of FC neuron types

Population morphological renderings (top panels) and median neuron locations (bottom panels) for every FC neuron type. Median neuron locations are shown for the FB layer containing the most synapses for each neuron type. Neurons are colored by structural column (see legend).

Figure 33: FB columnar type to columnar type connectivity

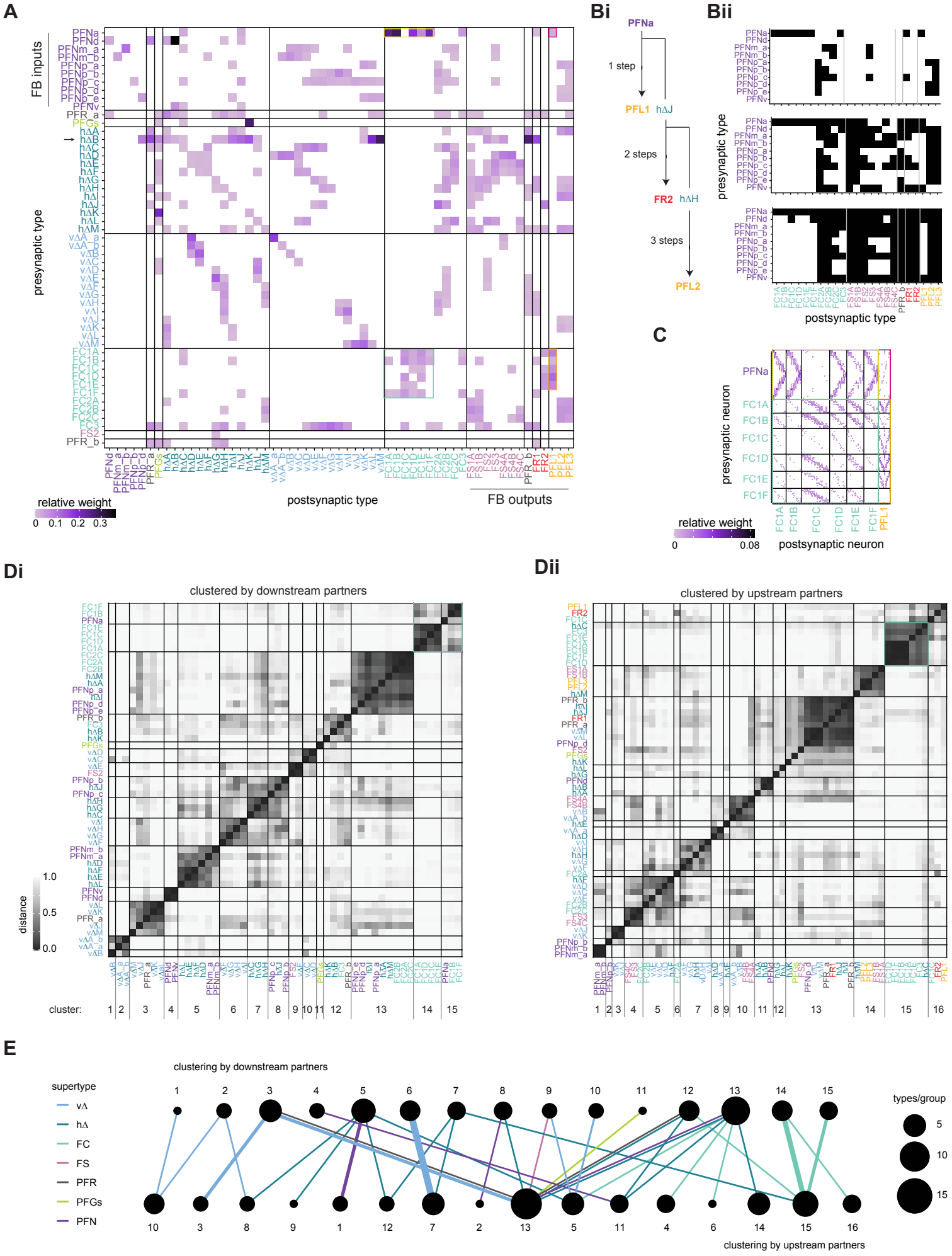


Figure 33: FB columnar type to columnar type connectivity

A) Type-to-type connectivity table for the FB columnar neurons. Neuron type labels are color coded by that neuron's class. FB inputs are noted on the y axis, while FB outputs are noted on the x axis. Only connections where most of the presynaptic neurons connect to a postsynaptic neuron of the given type are shown (more than 2/3 of the columns must connect across types).

B) The number of steps between columnar FB inputs and columnar FB outputs through other columnar FB neurons

- **i)** While PFNs directly connect to a few of the FB columnar output neurons in the FB (top), the pathways between PFNs and columnar outputs are often longer, traveling through one (middle), two (bottom), or more intermediate columnar neurons.
- **ii)** Direct (top), 2 step (middle), and 3 step (bottom) connections between PFNs and FB columnar output neurons are shown in black.

C) Neuron-to-neuron connectivity table for the PFNa, FC1, and PFL1 neurons. Each quadrant is surrounded by a colored box that corresponds to a similar colored box around the type-to-type connectivity for these neurons in **A**.

D) Cosine distance similarity matrix for columnar FB neuron downstream and upstream partners

- **i)** Similarity matrix for the vectors of relative weights from downstream partners. The types correspond to the presynaptic types in **A**.
- **ii)** Similarity matrix for the vectors of relative weights from upstream partners. The types correspond to the postsynaptic types in **A**.

E) Each neuron type is linked to its downstream and upstream cluster. The thickness of each edge denotes the number of types within the given connection. The color denotes the supertype.

Figure 33—figure supplement 1: Pathways between FB columnar inputs and outputs

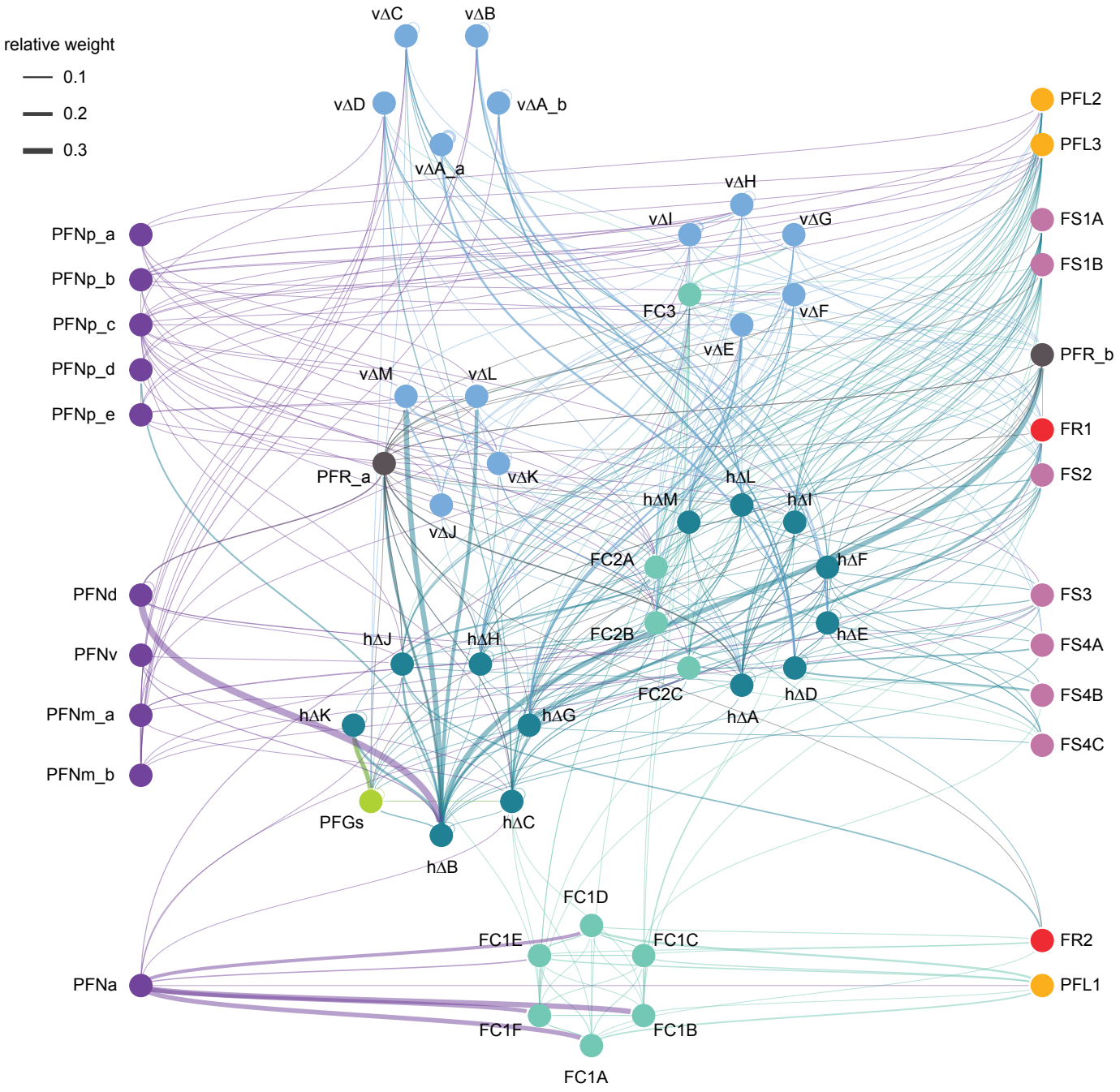
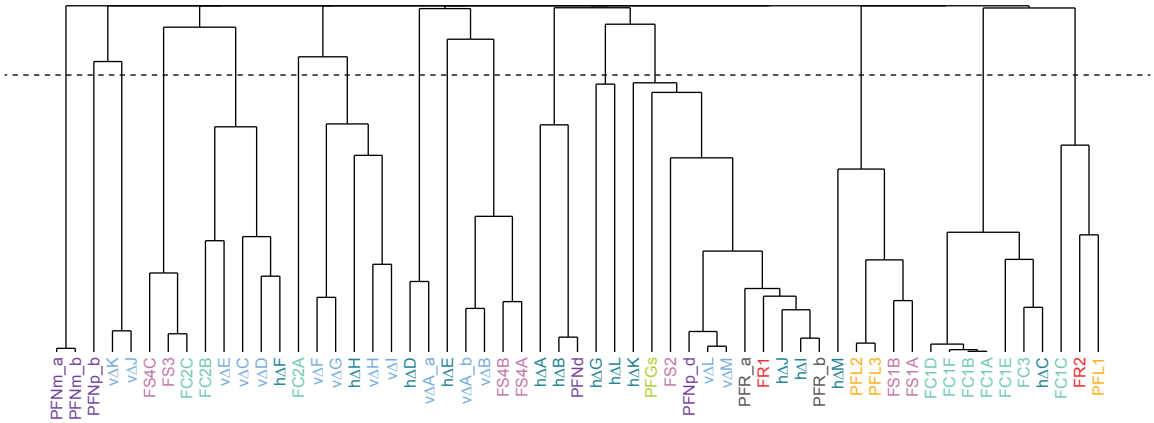


Figure 33—figure supplement 1: Pathways between FB columnar inputs and outputs

The type-to-type connectivity from **Figure 33** in a 3-layer network diagram. FB inputs are shown at far left while FB outputs are shown at far right. The subnetwork from **Figure 33C** can be seen at bottom.

Figure 33—figure supplement 2: Clustering by upstream and downstream partners

A



B

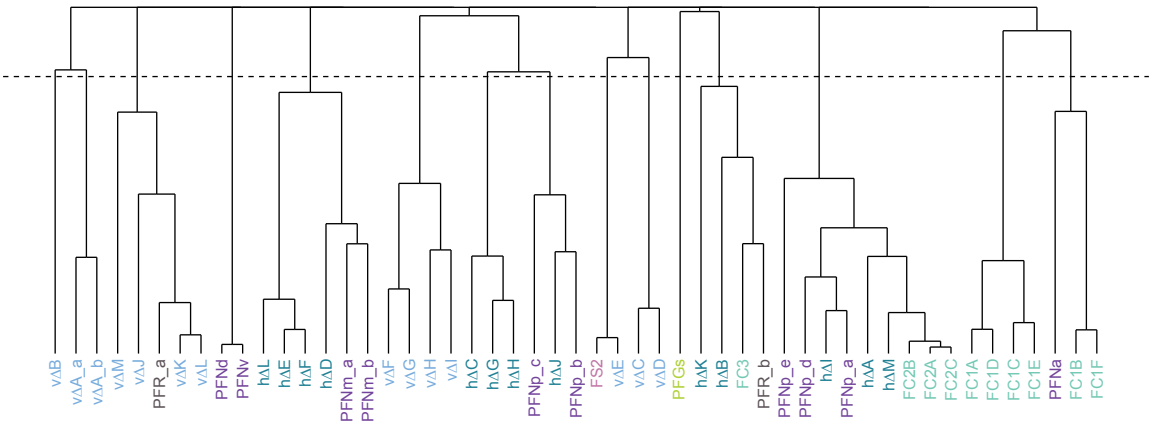


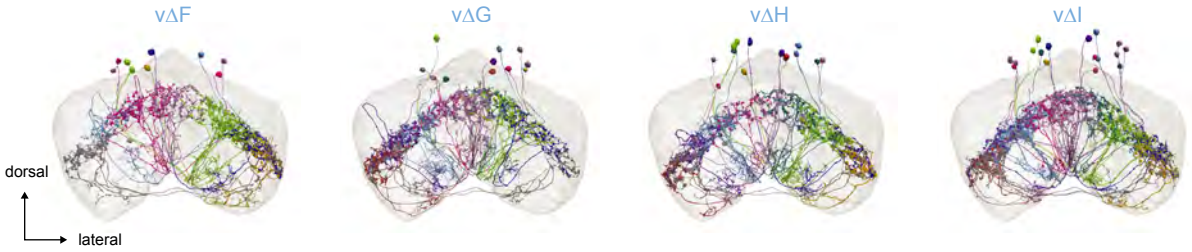
Figure 33—figure supplement 2: Clustering by upstream and downstream partners

A) Hierarchical tree showing the similarity of different neuron types based on their upstream partners. The dotted line shows the cutoff of 0.8 that was used to form the clusters shown in **Figure 33 Di, E**.

B) As in A, now for the downstream partner. The dotted line shows the cutoff of 0.8 that was used to form the clusters shown in **Figure 33 Dii, E**.

Figure 33—figure supplement 3: The vΔF, G, H, and I subnetwork

A



B

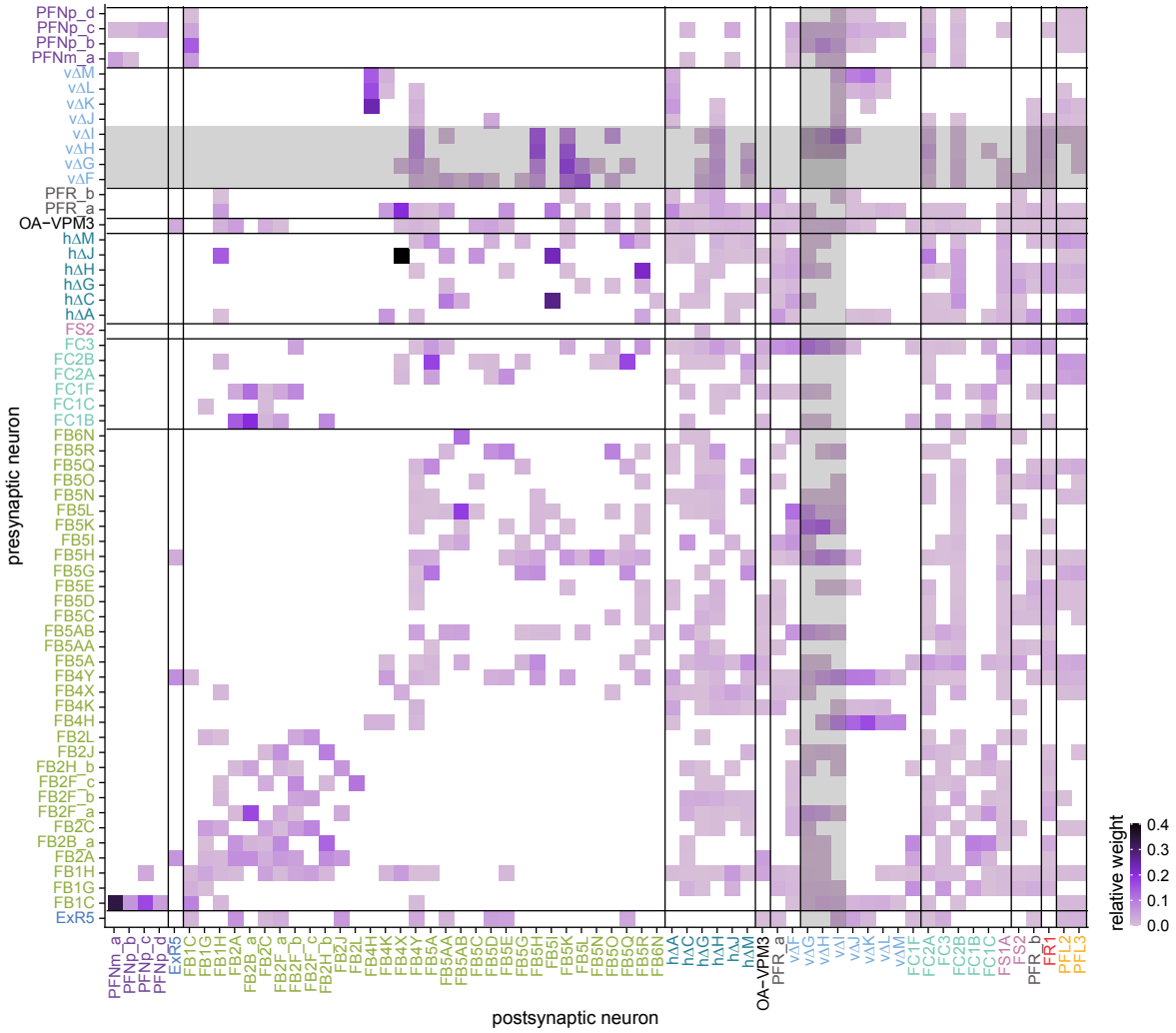


Figure 33—figure supplement 3: The vΔF, G, G, and I subnetwork

A) Morphological renderings of the vΔF, G, H, and I neurons. These neurons have common upstream and downstream connectivity, forming a subnetwork.

B) Type-to-type connectivity matrix highlighting the common input and output partners of the vΔF, G, H, and I neurons.

Figure 34: PB-FB phase shifts determine PFN connectivity within the FB

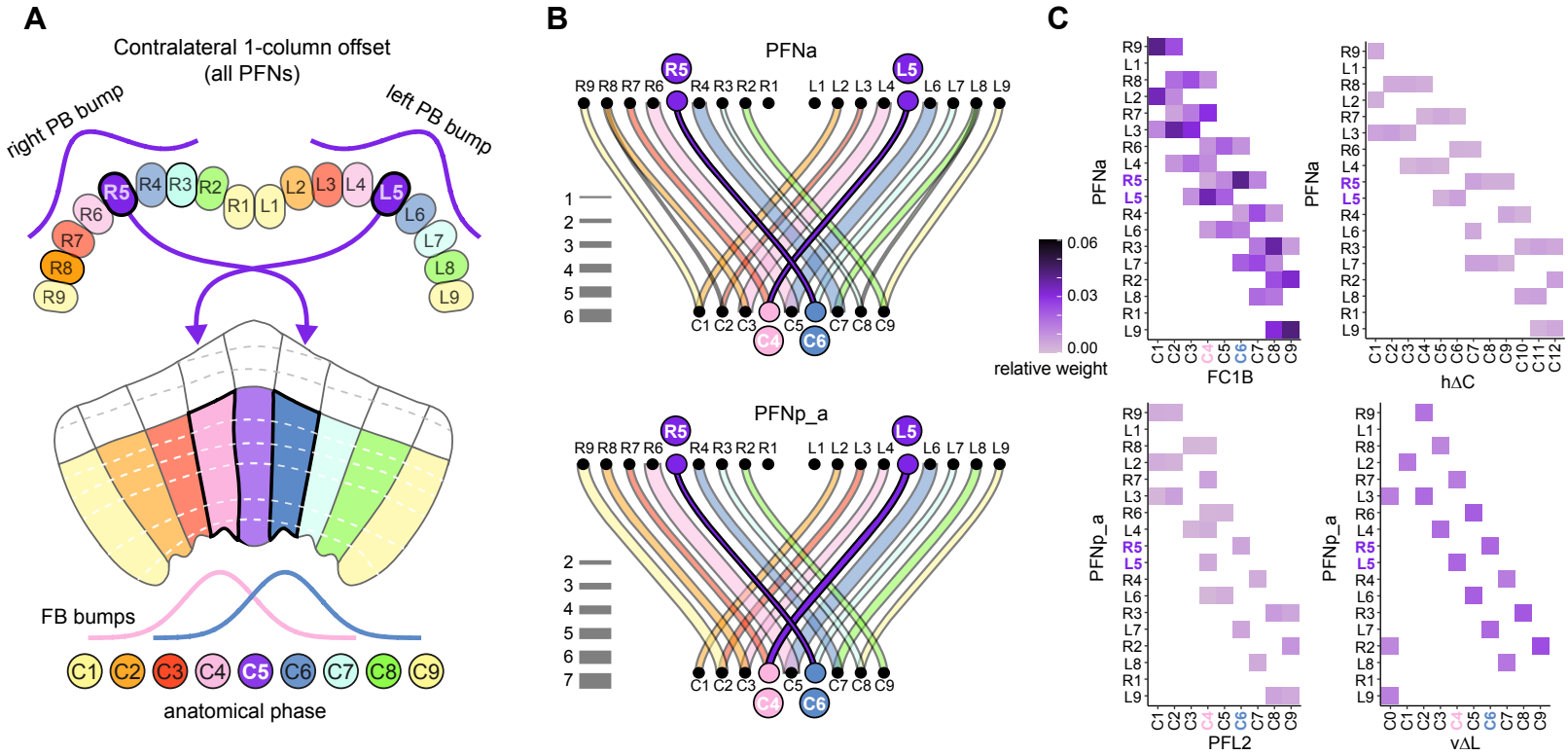


Figure 34: PB-FB phase shifts determine PFN connectivity within the FB

A) Schematic of a PB-to-FB projection pattern showing a 1-column contralateral phase shift, as in **Figure 30 B**.

B) Graphs showing the projection pattern from PB glomeruli to FB columns for all neurons in the PFNa (top panel) and PFNp_a (bottom panel) populations, as in **Figure 30 B**.

C) Connectivity between PFNa (top panel) or PFNp_a (bottom panels) neurons and two of their downstream partners within the FB. Notice that PFN neurons that arborize in glomeruli R5 or L5 connected with distinct columns in the FB, consistent with their PB-FB phase shifts.

Figure 35: Right and left PB-FB-* populations target the same FB neuron types and neurons

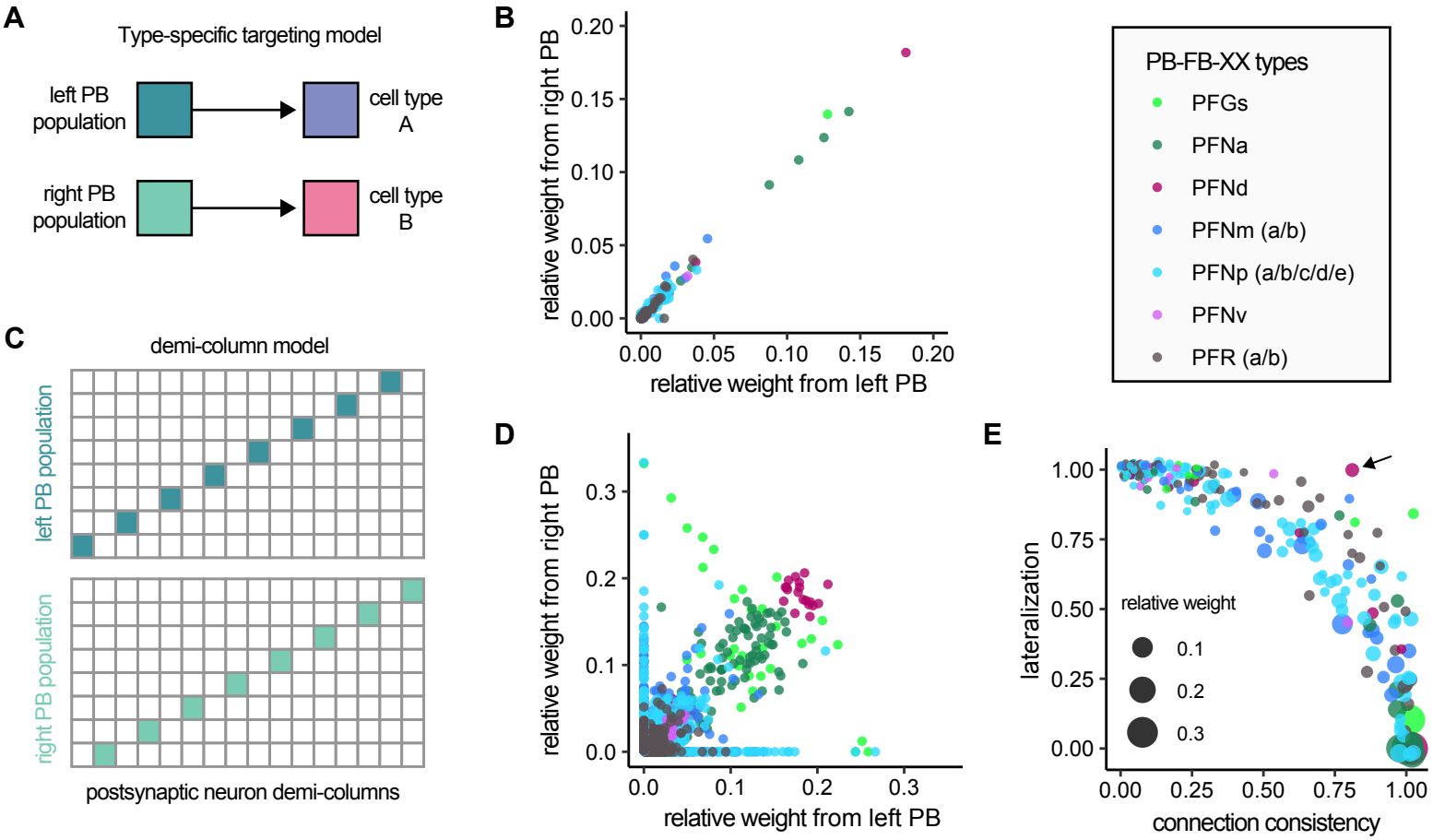


Figure 35: Right and left PB-FB-* populations target the same FB neuron types and neurons

A) Schematic showing one potential mechanism—type-specific targeting by left and right PB-FB-XX populations—by which activity from the left and right PB could propagate through separate FB channels. This model predicts that PB-FB-X neurons from the left and right PB should target distinct downstream neuron types in the FB.

B) Scatter plot showing the average input from left (x-axis) and right (y-axis) PB-FB-X neurons onto downstream neuron types. Each circle is a downstream neuron type, and circles are colored according to the upstream PB-FB-X type (see legend). If the model from A were true, some points should lie along the x and y axes, indicating specific input from the left or right PB populations. Instead, every downstream type receives approximately equal input from left and right PB populations, ruling out the model from A.

C) Schematic showing a second potential mechanism—demi-column specific targeting by left and right PB-FB-X populations—by which activity from left and right PB could propagate through separate FB channels. This model predicts that individual neurons in a downstream population should receive input from the left or the right PB population (high "lateralization"), but not both.

D) Scatter plot showing the average input from left (x-axis) and right (y-axis) PB-FB-X neurons onto individual neurons in downstream populations. Each circle is a downstream neuron, and circles are colored according to the upstream PB-FB-X type (see legend). If the model from **C** were true, all neurons (circles) in a downstream population would lie along the x or y-axis, indicating specific input from the left or right PB population. While some points do lie along the axes, the majority of circles lie along the diagonal, suggesting roughly equal input from the left and right PB populations, similar to **B**.

E) Scatter plot showing left/right lateralization (y-axis; the proportion of downstream neurons that receive input from the left or the right PB but not both) according to the connection consistency (x-axis; the proportion of neurons in a downstream neuron type targeted by a PB-FB-XX neuron type). The size of circles indicates connection strength. Each circle is a downstream neuron type targeted by an upstream PB-FB-X type. The model from **C** predicts that points should lie in the upper right portion of the plot, indicating a strong connection that is highly lateralized. instead, only weak and inconsistent connections show lateralization. With few exceptions (PFNd-to-PFNd), strong and consistent connections have downstream neurons that receive input from both left and right PB populations (low lateralization). This rules out the model from **C**. Note that a small jitter was introduced so that overlapping points could be resolved. Arrow marks PFNd to PFNd connectivity, a connection that is fairly strong and lateralized.

Figure 36: Overview of the asymmetric body (AB)

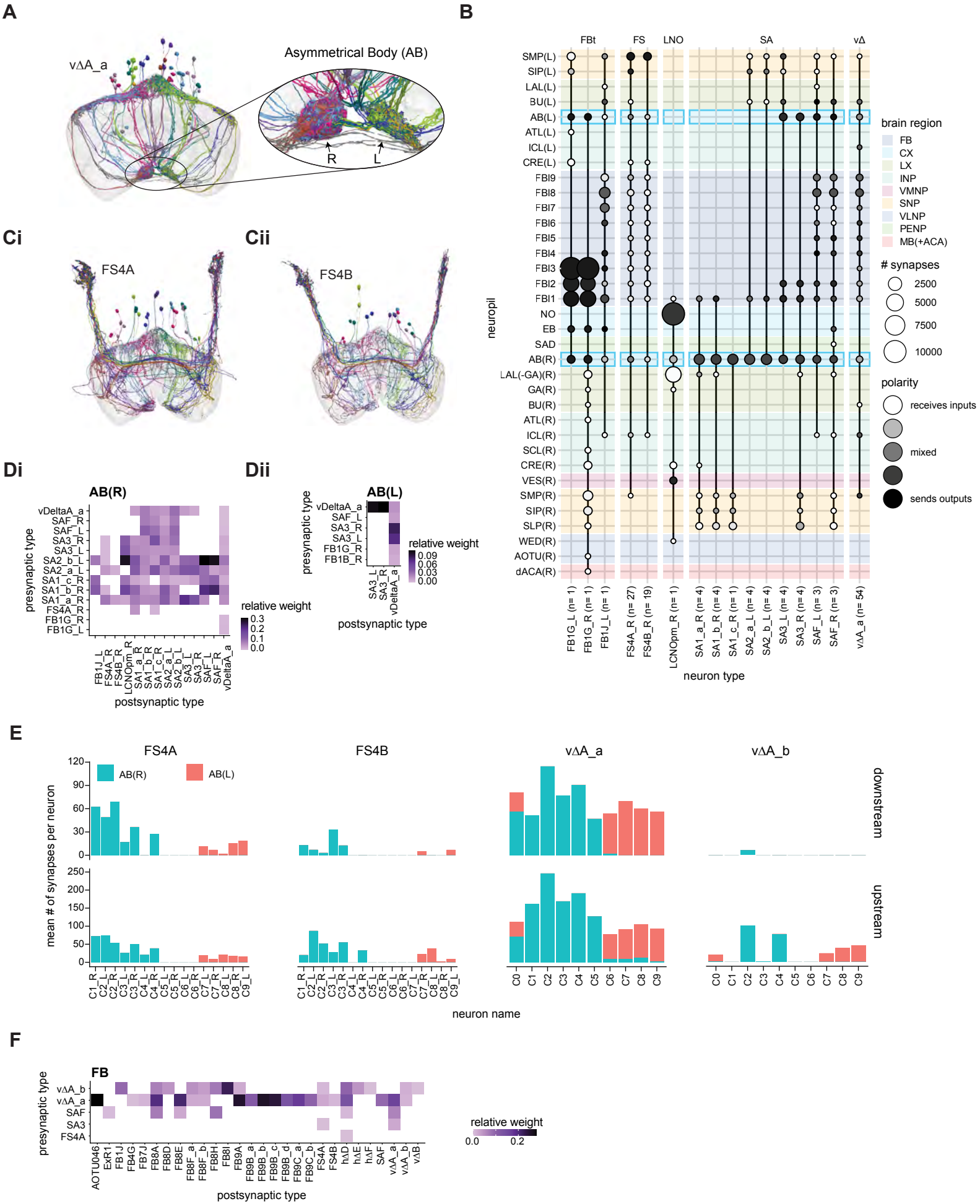


Figure 36: Overview of the asymmetric body (AB)

A) Morphological renderings of the vΔA_a neurons, which arborize in the FB and in the AB. They are columnar in the FB, with columns 1-5 projecting into the right AB and columns 6-9 projecting into the left AB. (inset at right) The right AB is noticeably larger than the left AB.

B) Region arborization plots for each neuron type that contains arbors in the AB.

C) Renderings of FS4A (**Ci**) and FS4B (**Cii**) neural populations. These neuron types are columnar and receive input in both the AB and the FB and output to the SNP.

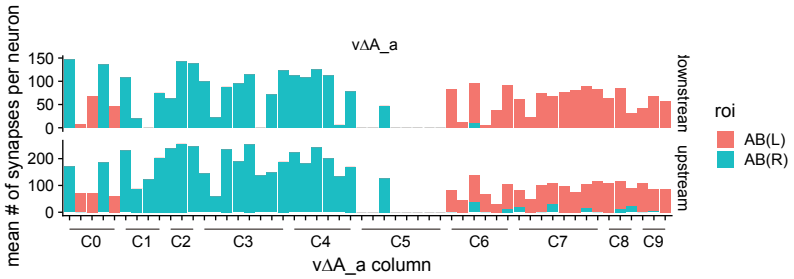
D) Type-to-type connection table for the right (**Di**) and left (**Dii**) AB. The smaller left AB has fewer types that make significant connections.

E) The mean number of downstream (top) and upstream (bottom) synapses in the right (cyan) or left (red) AB by their FB column of origin for the columnar FB-AB neurons.

F) Type-to-type connection table for the downstream partners of the columnar FB-AB neurons in the FB.

Figure 36—figure supplement 1: Additional AB connectivity

A



B

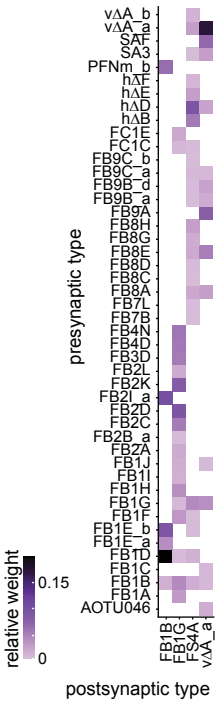


Figure 36—figure supplement 1: Additional AB connectivity

A) As in FB Figure 9E for the vΔA_a neurons, but now with individual neurons on the x-axis.
B) As in FB Figure 9F, but now for the upstream partners of the columnar FB-AB neurons.

Figure 37: The intra-FB columnar network is built from a small number of circuit motifs

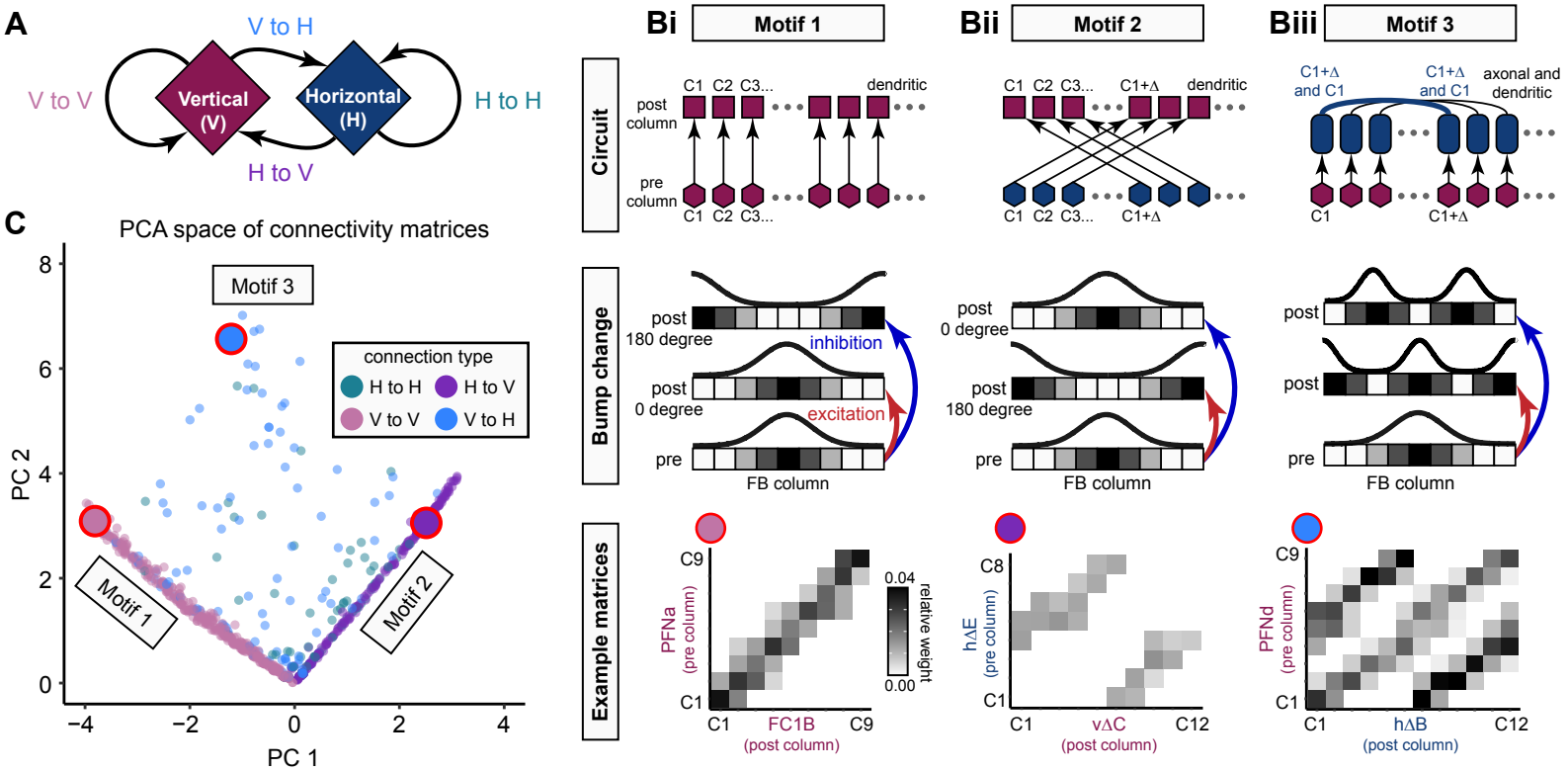


Figure 37: The intra-FB columnar network is built from a small number of circuit motifs

A) FB columnar neurons can be divided into vertical and horizontal types. Throughout the figure, vertical types are marked in maroon and horizontal types are marked in dark blue. Note that hΔ neurons are named according to the column containing their dendritic arbor, which impacts the connectivity matrix structure, as shown in the examples in **B**. Vertical and horizontal neurons give rise to four connection types: vertical-to-vertical (V to V), vertical-to-horizontal (V to H), horizontal-to-horizontal (H to H), and horizontal-to-vertical (H to V).

B) Three columnar-to-columnar connectivity motifs generated by three circuit motifs. Top panels show a circuit motif that generate the corresponding column-to-column connectivity matrix shown in the bottom panels. The middle panels show how excitatory or inhibitory connections would impact bump phase. In each circuit diagram all presynaptic columns are marked with hexagons, while postsynaptic columns can be dendritic (squares), axonal (circles), or contain multiple hΔ neurons with either dendritic or axonal arbors (rounded rectangles). See legend in **Figure 37—figure supplement 1** for details. Circuit motifs are shown with ellipsis (…) to indicate variable column numbers, while connectivity matrices and bump change diagrams are shown with the 9 column pattern typical of most FB columnar neurons.

i) Motifs which generate a diagonal column-to-column connectivity matrix. Excitatory connections could pass the bump to a second layer while maintaining its phase, while inhibitory connections could shift the bump's position by 180 degrees

ii) Motifs which generate a shifted column-to-column connectivity matrix. Excitatory connections would shift the bump by 180 degrees while inhibitory connections would maintain its phase (bottom panel).

iii) Motifs which could produce a column-to-column connectivity matrix with two diagonal bands. Excitation and inhibition could produce a double-bump pattern, as shown in the bottom panel. Alternatively, if the axonal compartment receives inhibitory input and the dendritic compartment receives excitatory input, a single bump would be preserved (not shown).

C) Scatter plot showing that FB connectivity matrices can be grouped into one of the three motifs. Each circle in the scatter plot marks the location of a single connectivity matrix in principal component space. Briefly, each column-to-column connectivity matrix was coerced to a 9 column scheme, binarized, and transformed into a vector. PCA was performed using a matrix containing all connectivity vectors (n=903 connectivity matrices), and each vector was projected onto the larges two PCs (PC1 and PC 2). Circles are colored according to pre-to-post connection type (see legend). Note that the large majority of connectivity matrices correspond to motifs 1 and 2 (diagonal point clouds), whose orthogonality is preserved in PC space. Points lying off these diagonals largely correspond to motif 3. The three large circles outlined in red correspond to the connectivity matrices in the bottom panels of **B**.

Figure 37—figure supplement 1: Detailed description of intra-FB columnar connectivity motifs

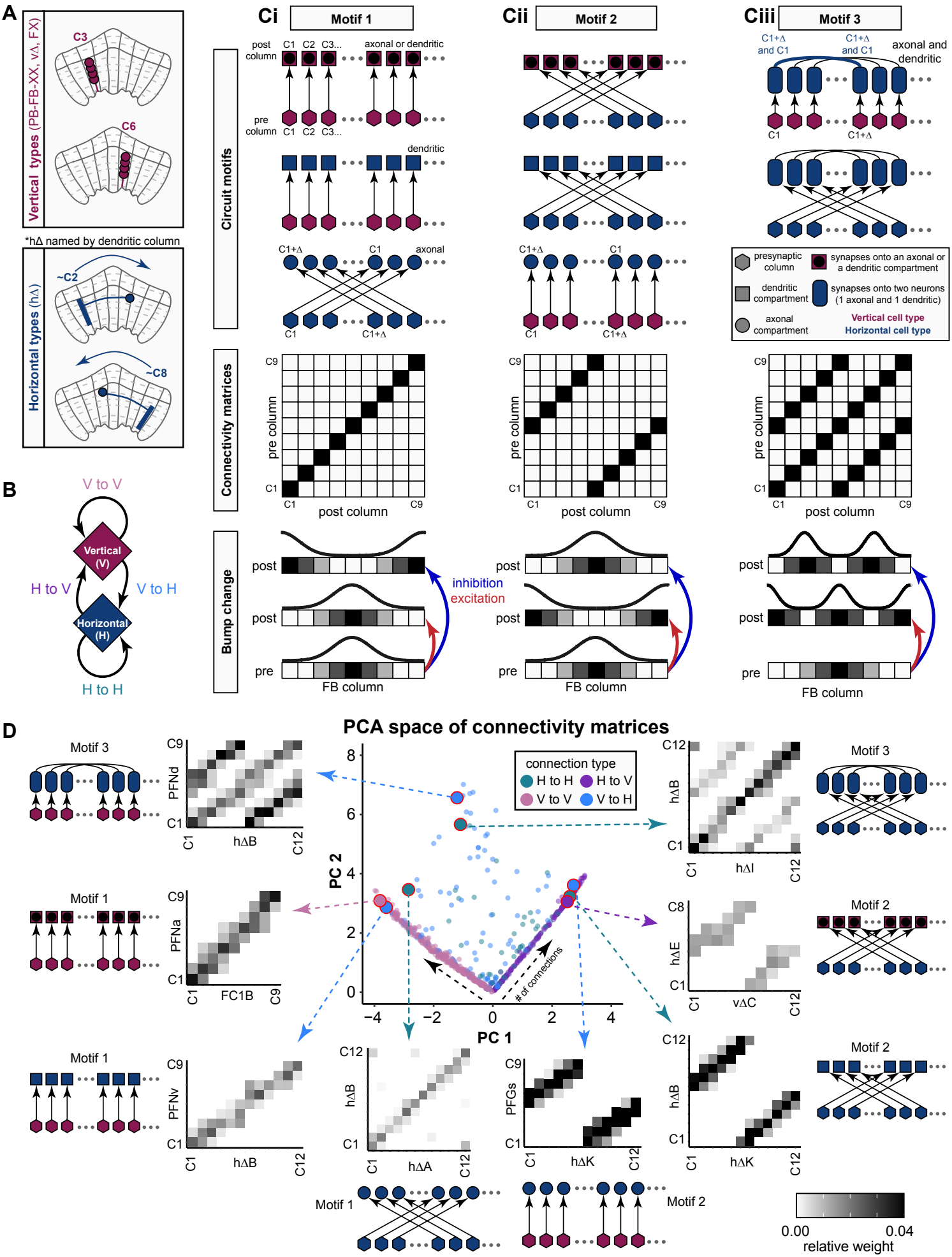


Figure 37—figure supplement 1: Detailed description of intra-FB columnar connectivity motifs

A) FB columnar neurons can be divided into vertical and horizontal types. Throughout the figure, vertical types are marked in maroon and horizontal types are marked in dark blue. hΔ neurons are named according to the column containing their dendritic arbor, which impacts the connectivity matrix structure, as shown in **B**.

B) Same as in **Figure 37 A.**

C) Same as in **Figure 37 B**, but showing eight circuit motifs that could generate the corresponding connectivity matrices. The additional circuit motifs shown here occur more rarely that those shown in **Figure 37 B**.

D) Same as in **Figure 37 C**, but now showing eight connectivity matrices, each of which corresponds to one of the eight circuit motifs from **C**.

Figure 37—figure supplement 2: Principal component analysis of FB columnar connectivity

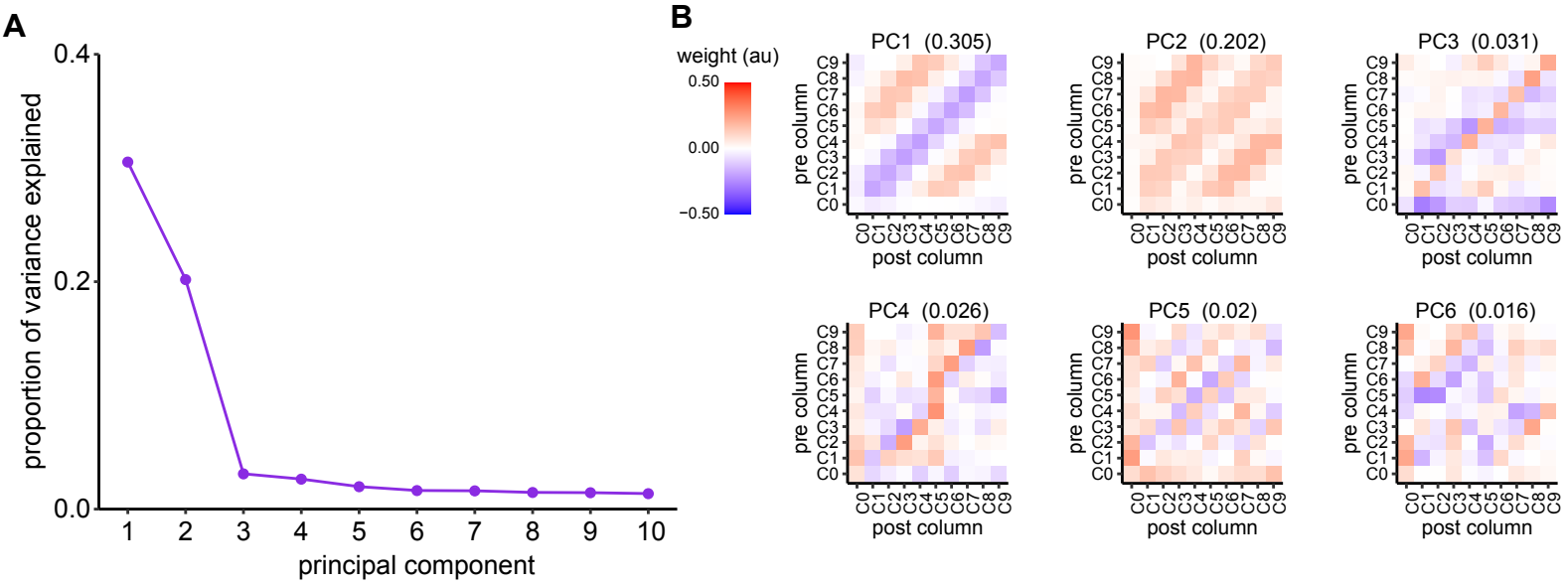


Figure 37—figure supplement 2: Principal component analysis of FB columnar connectivity

A) Plot showing the proportion of variance accounted for by the first 10 principal components, ordered from highest to lowest. Note that the first two components account for greater than 50% of the variance and subsequent components much less.

B) Matrices showing the first 6 principal components. The proportion of variance explained by each is listed above each matrix. Note that the first two principal components do not correspond to motif 1 or motif 2 (**Figure 37 Bi, Bii**). Instead, the first component is composed to two diagonal bands with positive and negative values. The second component is a rectified version of the first principal component. Linear combinations of PC1 and PC2, with appropriate weighting, produce column-to-column connection matrices that correspond to motif 1 and motif 2.

Figure 38: Delta neurons in the FB preferentially take input in lower FB layers and output to upper FB layers

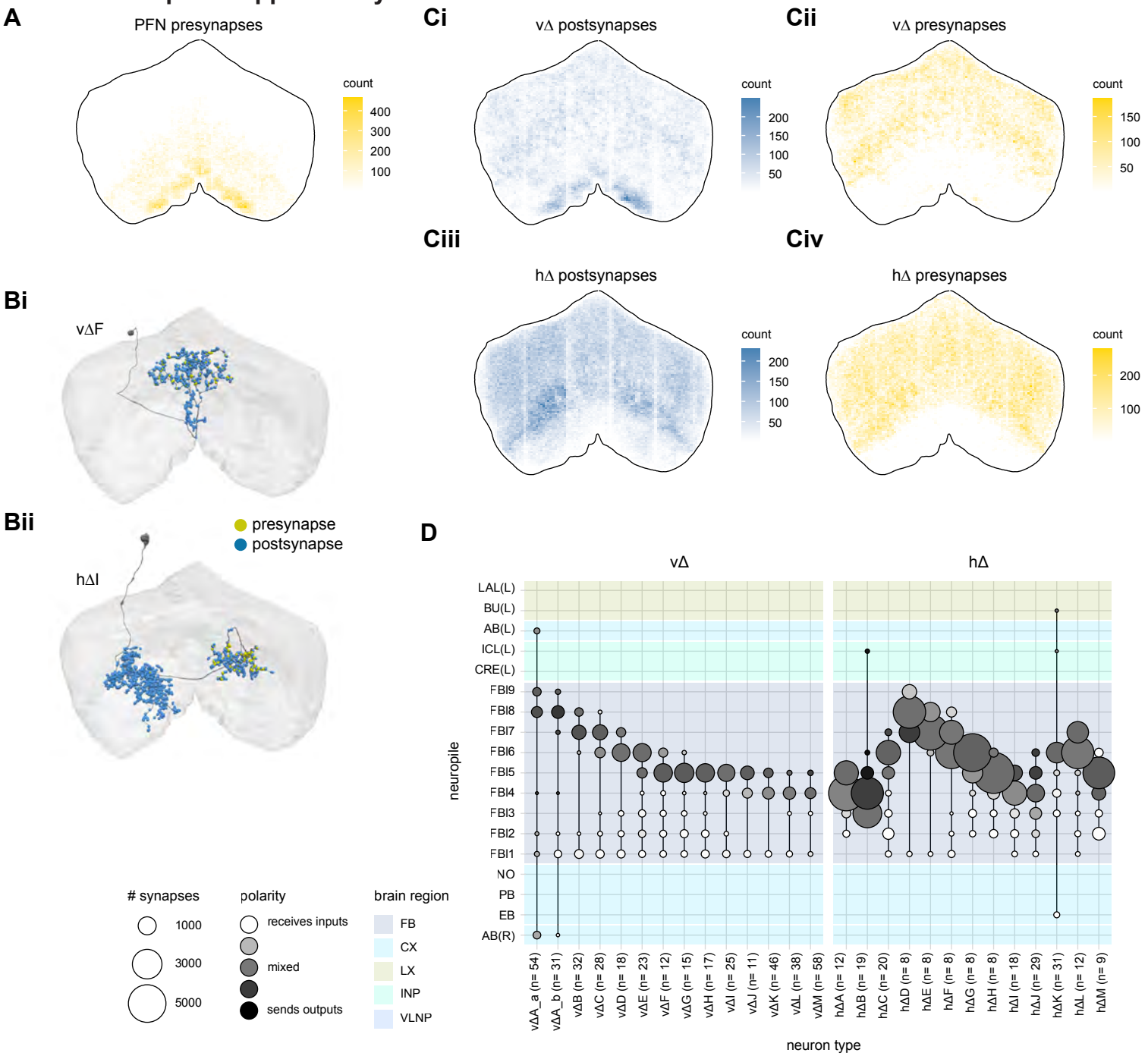


Figure 38: Delta neurons in the FB preferentially take input in lower FB layers and output to upper FB layers

A) All presynaptic sites for all of the PFN neuron types.

B) Morphological renderings of vΔF (**Ai**) and hΔI (**Aii**) neurons. Presynaptic sites are shown in yellow while postsynaptic sites are shown in blue. Both types have postsynaptic sites throughout their arbors, but their presynaptic sites output in their upper layer FB arborizations.

C) All postsynaptic (left) and presynaptic (right) sites for all of the vΔ (top) or hΔ (bottom) neurons. Postsynaptic sites are visible in the lower FB layers while presynaptic sites are restricted to the upper layers of the FB

D) Region arborization plots for each vΔ and hΔ type.

Figure 39: PFL neurons, a major FB output, have type-specific phase shifts in PB-to-FB projections

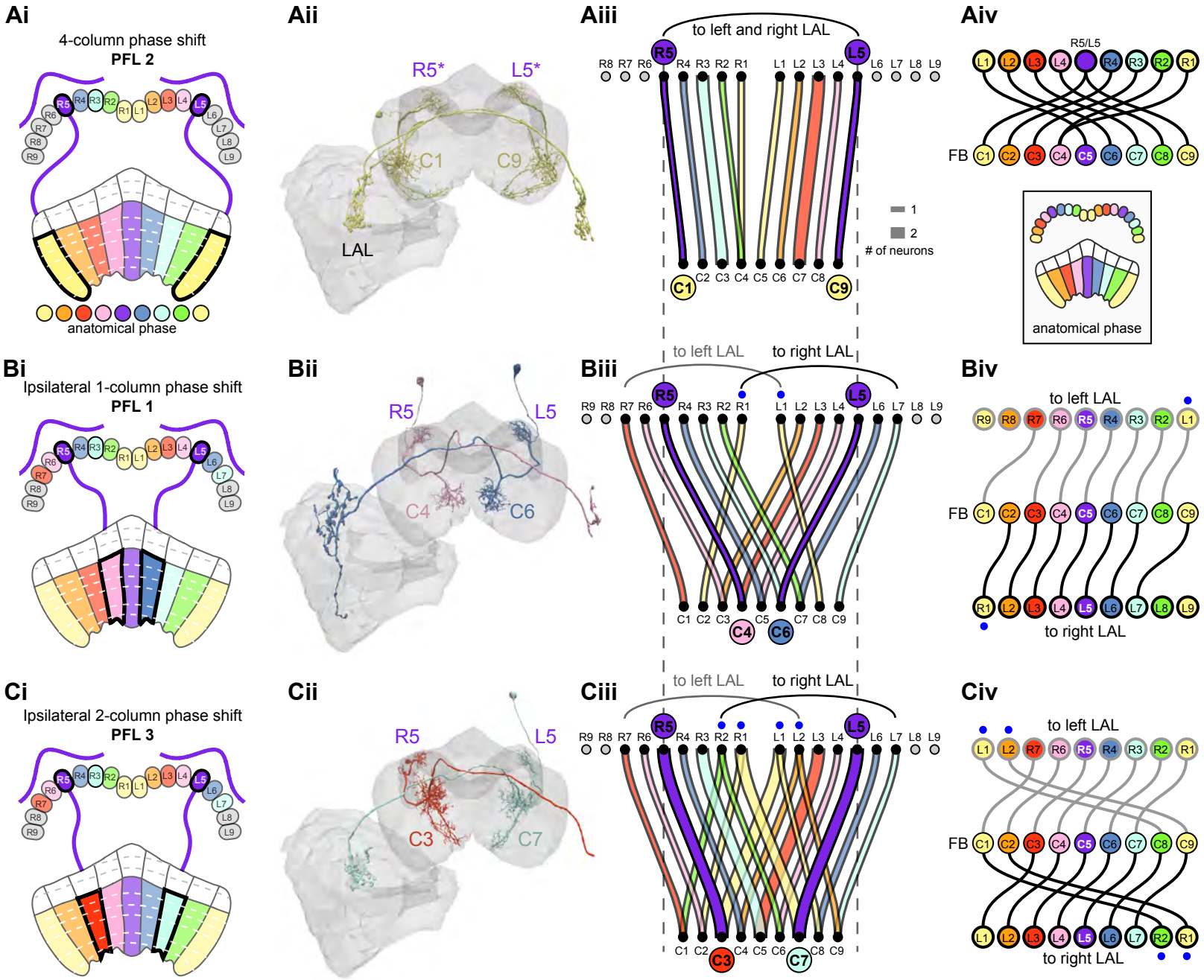


Figure 39: PFL neurons, a major FB output, have type-specific phase shifts in PB-to-FB projections.

A) PFL2 neurons have a 4-column (180-degree) PB-FB phase shift and bilateral LAL projections.
- **i)** Schematic of a PB-to-FB projection pattern with a 4-column phase shift, as shown for the R5 and L5 glomeruli. PB glomeruli and FB columns are colored according to anatomical phase, which indicates matching bump locations.
- **ii)** Morphological renderings of PFL2 neurons innervating R5 and L5. Neurons are colored according to their FB column. Notice that R5 and L5 neurons end up at C1 and C9, respectively. R5/L5 have been given asterisks because individual PFL neurons can innervate multiple PB glomeruli (in this case, R4/R5 and L4/R5).
- **iii)** Graph showing the projection pattern from PB glomeruli to FB columns for all neurons in the PFL2 population. R5 and L5 projections have been highlighted as in Ai, and edges are colored according to PB glomerulus. Unlike all other PB-FB-XX neurons, the PFL2 population should only inherit one bump in the PB, since the neurons samples from a ~360-degree region of PB space, split between left and right halves (R5-R1 and L1-L5).
- **iv)** Functional graph showing the mapping between phases in the PB (top row) and phases in the FB (bottom row). Circles are colored by anatomical phase (legend). With one exception (R1), every PB glomerulus connects to a FB column with a 180-degree phase shift.

B) PFL1 neurons have a 1-column (45-degree) ipsilateral phase shift and project to the contralateral LAL.
- **i)** Same as in **Ai**, but schematizing the 1-column ipsilateral phase shift of PFL1 neurons.
- **ii)** Same as in **Aii**, but for two PFL1 neurons. Notice that the R5 neuron projects to C4, and the L5 neuron projects to C6.
- **iii)** Similar to **Aiii**, but for PFL1. Black and gray lines indicate groups of glomeruli that project to the right or left LAL, respectively. R1 and L1 are marked with blue dots because they project to the ipsilateral LAL, unlike the other neurons in the population.
- **iv)** Similar to **Aiv**, but for PFL1. Here, instead of dividing glomeruli by their left vs right PB innervation (as in **Biii**), glomeruli are grouped by whether they project to the left LAL (top row, gray outlined circles) or the right LAL (bottom row, black outlined circles), and sorted by anatomical phase. With the exception of R7 and L7, each glomerulus projects to FB columns with a 1-column (45-degree) ipsilateral phase shift.

C) PFL3 neurons have a 2-column (90-degree) ipsilateral phase shift and project to the contralateral LAL.
- **i)** Same as in **Bi**, but schematizing the 2-column ipsilateral phase shift of PFL3 neurons.
- **ii)** Same as in **Bii**, but for two PFL3 neurons. Notice that the R5 neuron projects to C3, and the L5 neuron projects to C7.
- **iii)** Same as in **Biii**, but for PFL3. R1/R2 and L1/L2 are marked with blue dots because these glomeruli contain neurons that either project to the contralateral LAL (like most of the population) or project to the ipsilateral LAL (unlike most of the population).

iv) Same as in **Biv**, but for PFL3. Notice that every neuron that projects to the left LAL (top row) and right LAL (bottom row) samples FB columns with a 2-column (90-degree) phase shift.

Figure 39—figure supplement 1: Continuous phase shifts of PFL neuron types

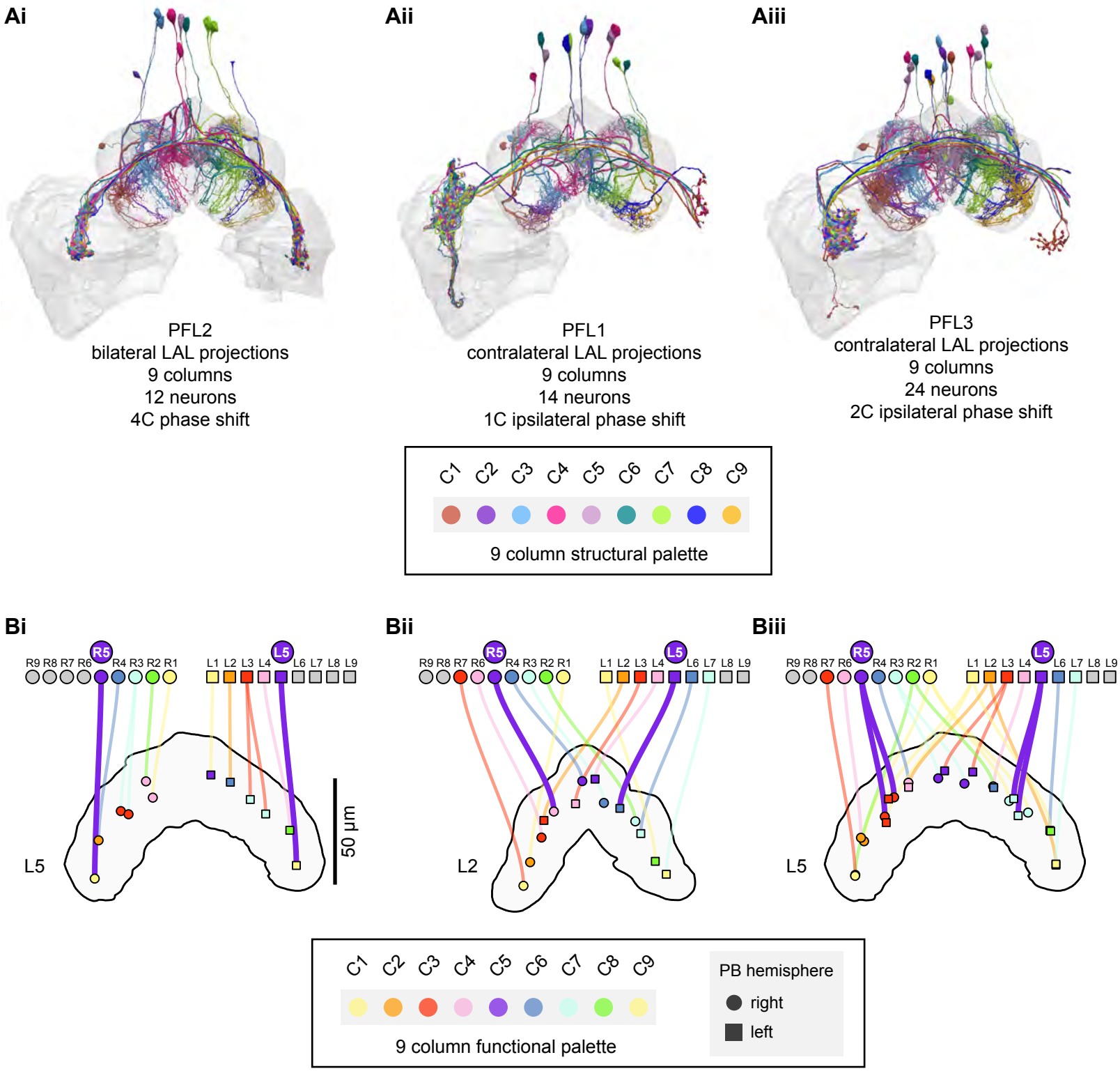


Figure 39—figure supplement 1: Continuous phase shifts of PFL neuron types

A) Population morphological renderings for the PFL2 (**Ai**), PFL1 (**Aii**), and PFL3 (**Aiii**) neuron types. As in previous figure, each neuron is colored according to its structural column (see legend).

B) Phase shift maps showing PB-to-FB projection pattern for the PFL2 (**Bi**), PFL1 (**Bii**), and PFL3 (**Biii**) neuron types. Each panel shows median neuron locations of a PFL type, as in previous plots, but with neurons colored according to functional column (see legend). To illustrate the continuous nature of the PB-FB phase shifts, each FB neuron location has a line connecting it to the PB glomerulus it innervates. PB glomeruli and connecting lines are colored by functional column as well. Lines connecting nodes (PB glomeruli and FB columns) of two different colors indicate a phase shifted connectivity pattern. Glomeruli R5 and L5 are highlighted to aid in identifying phase shifts.

Figure 40: FB tangential overview

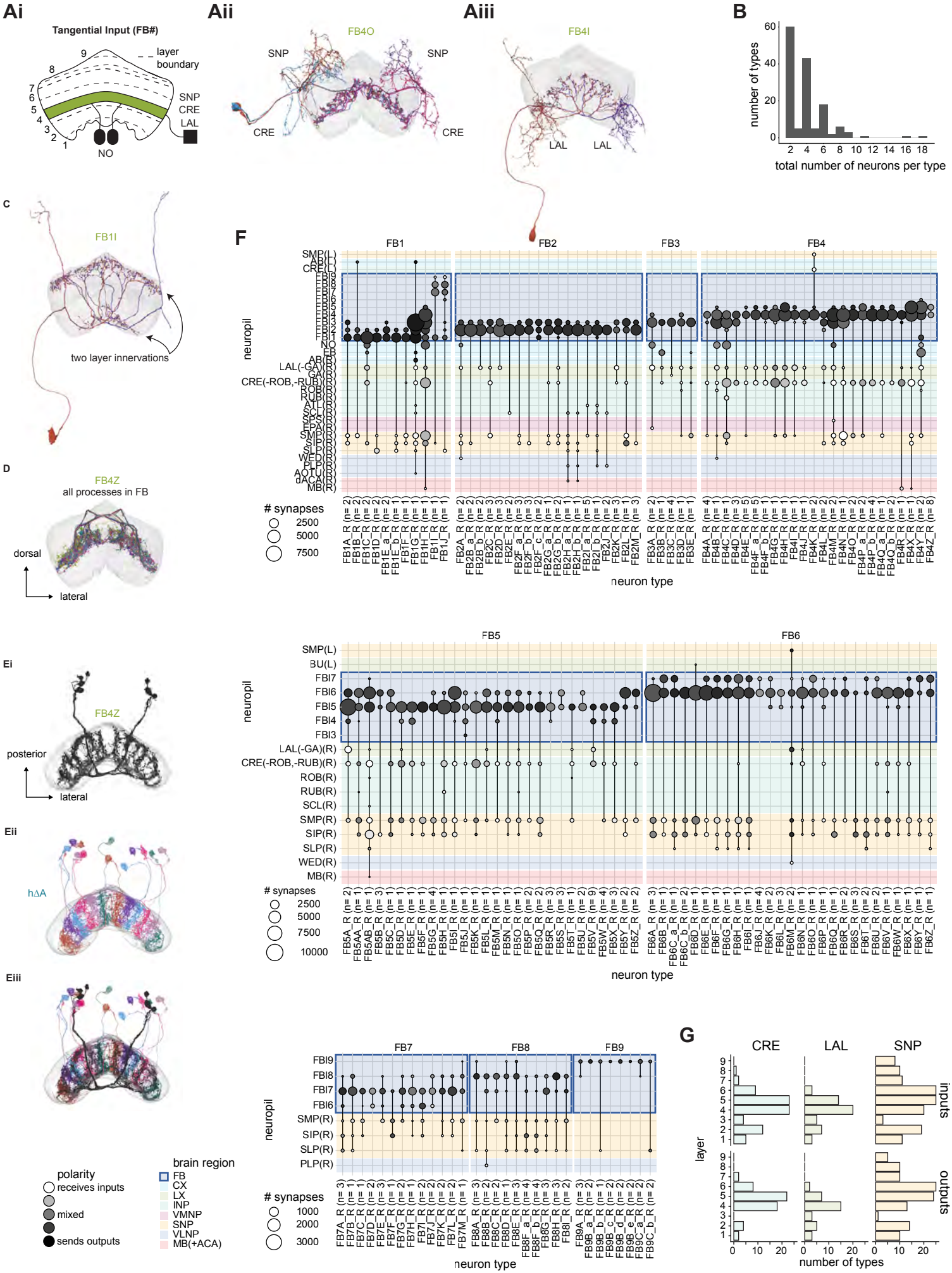


Figure 40: FB tangential overview

A) Overview of FB tangential neurons.
- **i)** FB tangential neurons output in single or multiple layers of the FB (e.g. in layer 4, shown in green) and may have mixed arbors in the NO or SNP, LAL, CRE, or other brain regions outside of the central complex.
- **ii)** A morphological render of FB4O neurons, which get input in the SNP and CRE and output in layer 4.
- **iii)** A morphological render of FB4I neurons, which get input in the LAL and output in layer 4.

B) The number of neurons per FB tangential type. With a few rare exceptions, both the right and left FB contribute an equal number of neurons to each type.

C) A morphological rendering of FB1I neurons, one example of FB tangential neurons that arborize in multiple FB layers.

D) A morphological rendering of FB4Z neurons, one example of FB tangential neurons that are both pre- and postsynaptic in the FB alone.

E) Some FB tangential neurons' arborizations are structured to target only specific neurons within a layer.
- **i)** A morphological render of the FB4Z neurons shown in D, now viewed along the dorsal-ventral axis. The FB4Z neurons do not fill the entire FB layer. This phenomenon can also be observed in other FB tangential types.
- **ii)** A render of one of the downstream targets of the FB4Z neurons, the hΔA neurons, whose processes also don't fill the entire layer.
- **iii)** The FB4Z and hΔA arborizations directly overlap.

F) Region arborization plots for each FB tangential neuron type.

G) The number of FB tangential types that receive input from (top) or give output to (bottom) the CRE, SNP, or LAL. The FB layer refers to the layer where a given type has the most expansive processes. For this analysis, only the right neurons of the type are considered, and each right neuron of that type must have, on average, at least 3 synapses in the given region.

Figure 40—figure supplement 1: FB tangential synaptic sites that are outside of the CX

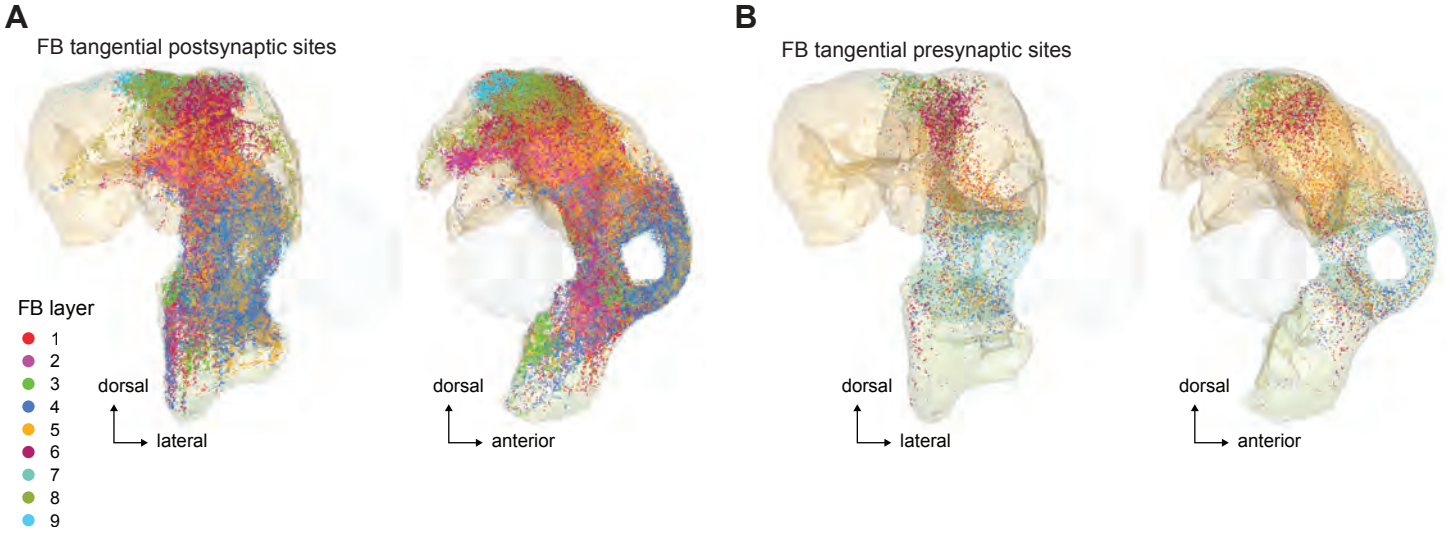


Figure 40—figure supplement 1: FB tangential synaptic sites that are outside of the CX

A) Postsynaptic sites in ROIs external to the CX for the FB tangential cells. The sites are color-coded by the FB layer where the given neuron has the most synapses.

B) As in **A**, now for presynaptic sites.

Figure 41: FB2B_a connectivity

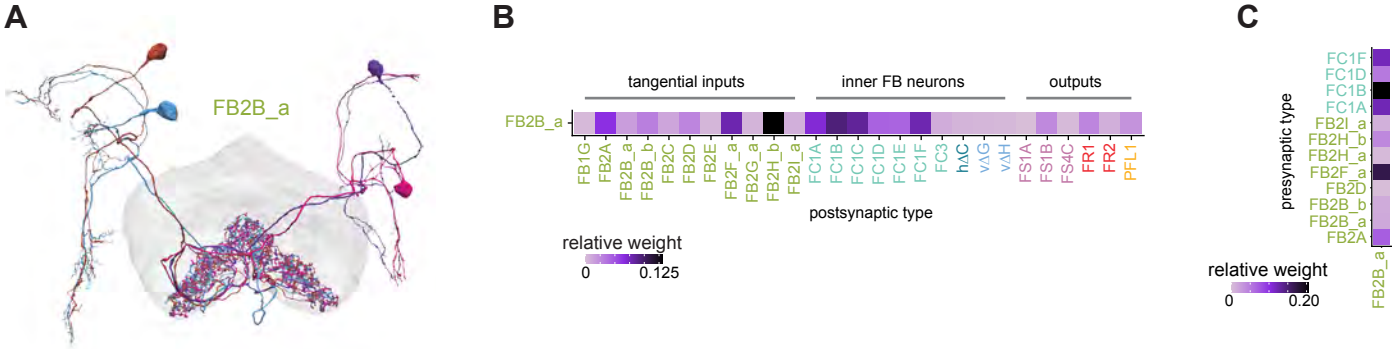


Figure 41: FB2B_a connectivity

A) FB2B_a neurons (also referred to as ExFl neurons, REF), arborize in the SNP, the CRE, and FB layer 2.

B) Postsynaptic FB2B_a partners in the FB. Partners include other FB tangential cells, FB neurons that are both pre- and postsynaptic in the FB, and FB outputs.

C) Presynaptic FB2B_a partners in the FB.

Figure 42: FB tangential presynaptic partners in the FB.

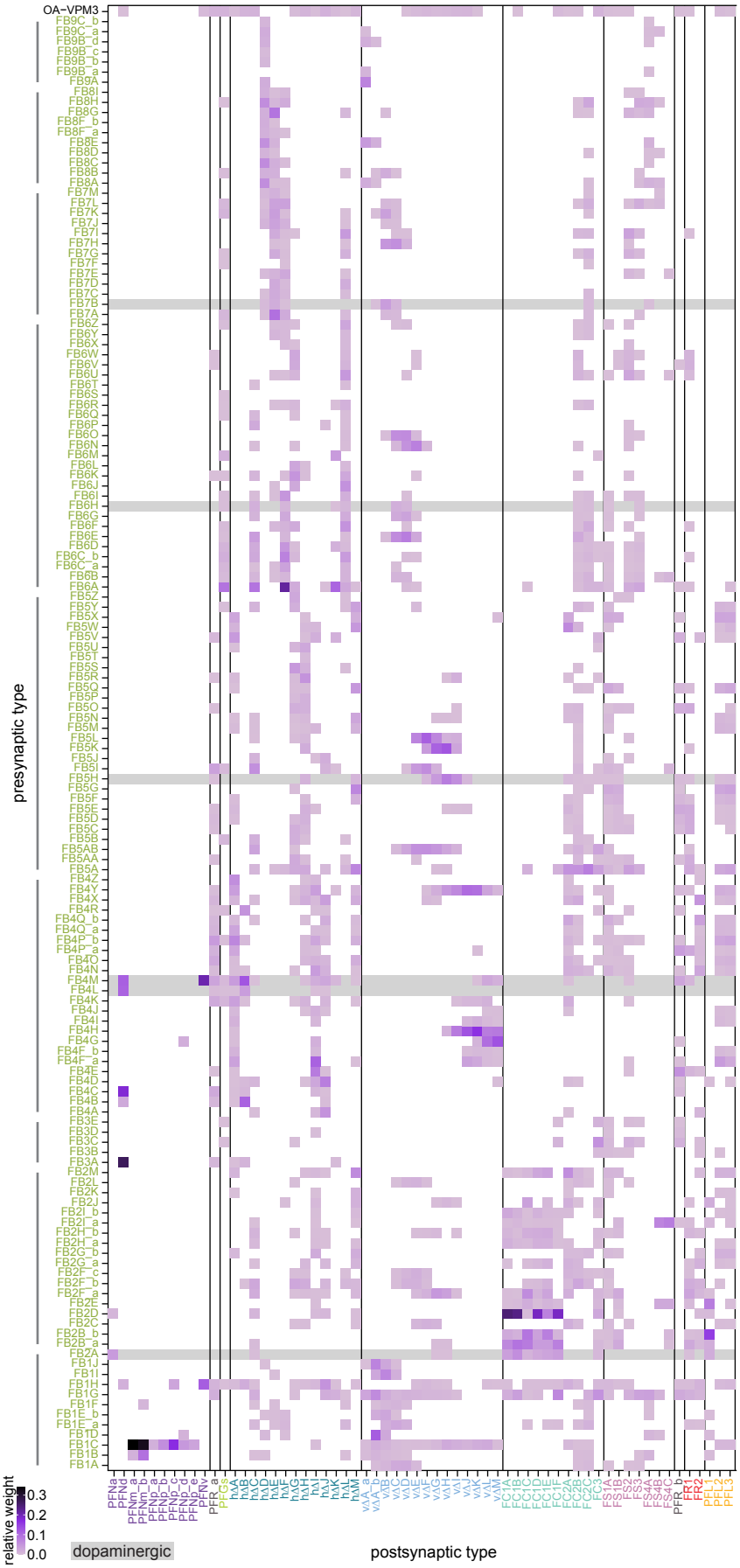


Figure 42: FB tangential presynaptic partners in the FB.

Type-to-type connectivity table for the FB tangential presynaptic partners in the FB. The connectivity of known dopaminergic neurons is highlighted in gray. Vertical lines adjacent to the y axis mark groups of FB tangential neurons that primarily arborize in the same layer.

Figure 43: FB tangential to FB tangential connections in the FB

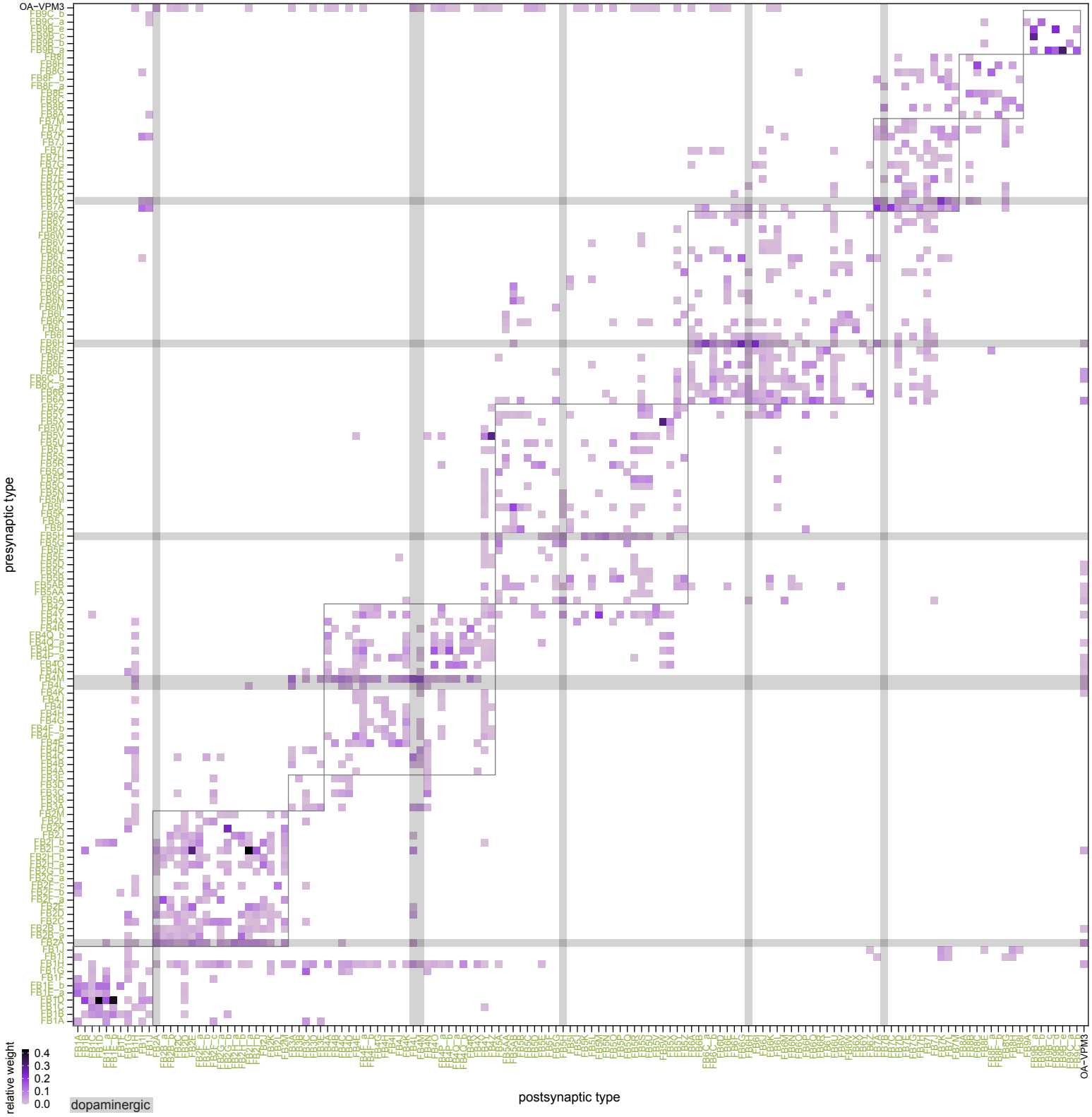
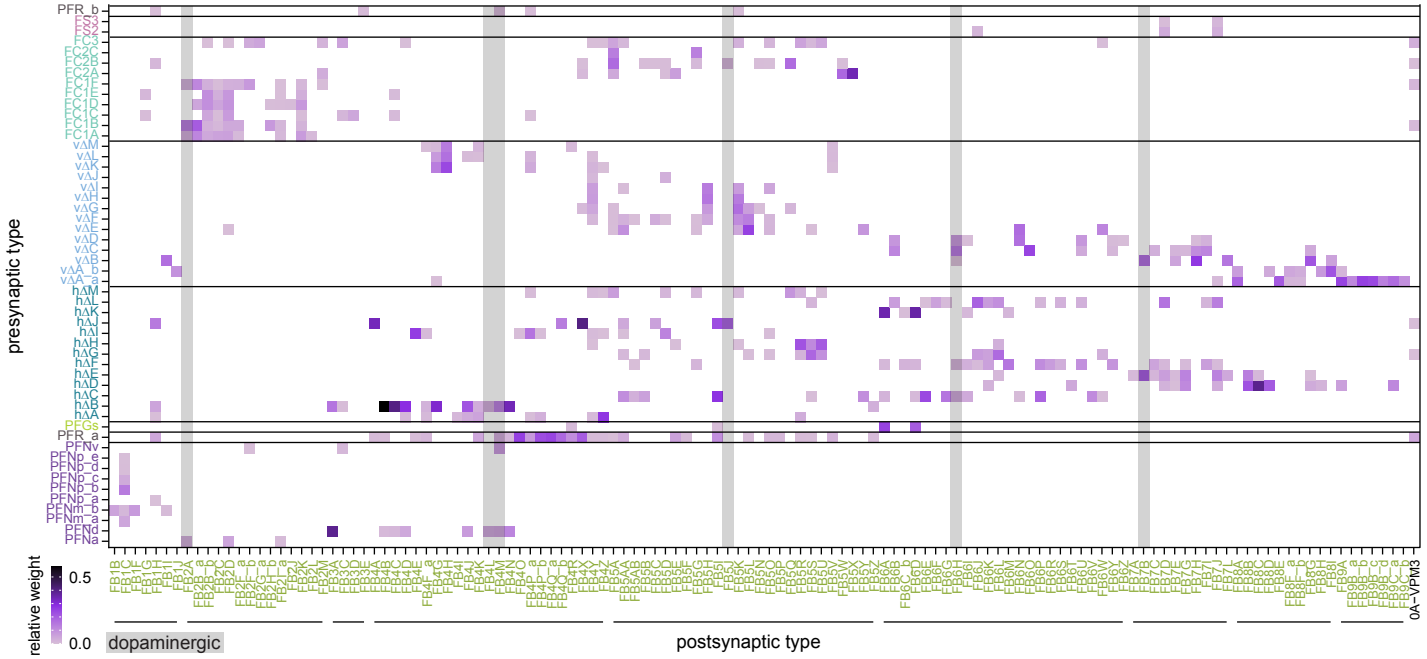


Figure 43: FB tangential to FB tangential connections in the FB

Type-to-type connectivity table between FB tangential types in the FB. The connectivity of known dopaminergic neurons is highlighted in gray. Boxes surround connections between FB tangential neurons that have their primary arborizations in the same FB layer.

Figure 44: FB tangential postsynaptic partner in the FB

A



B

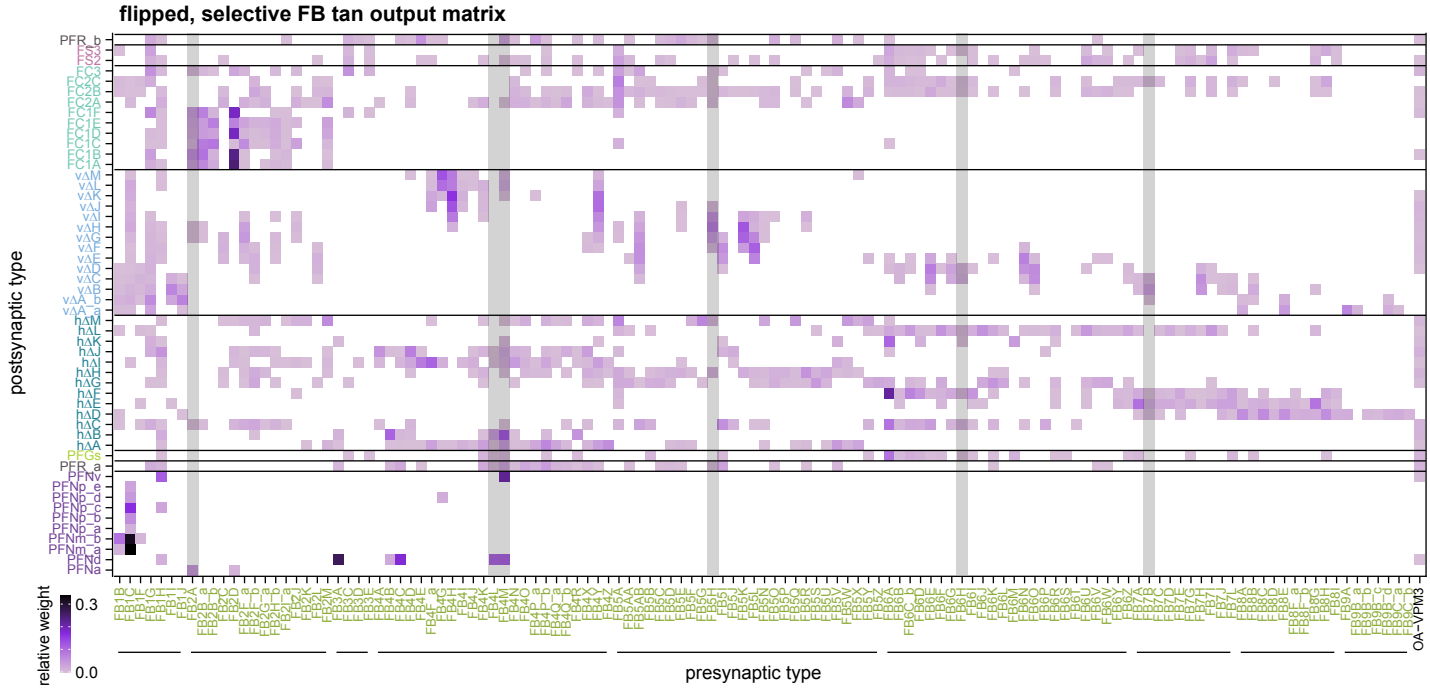


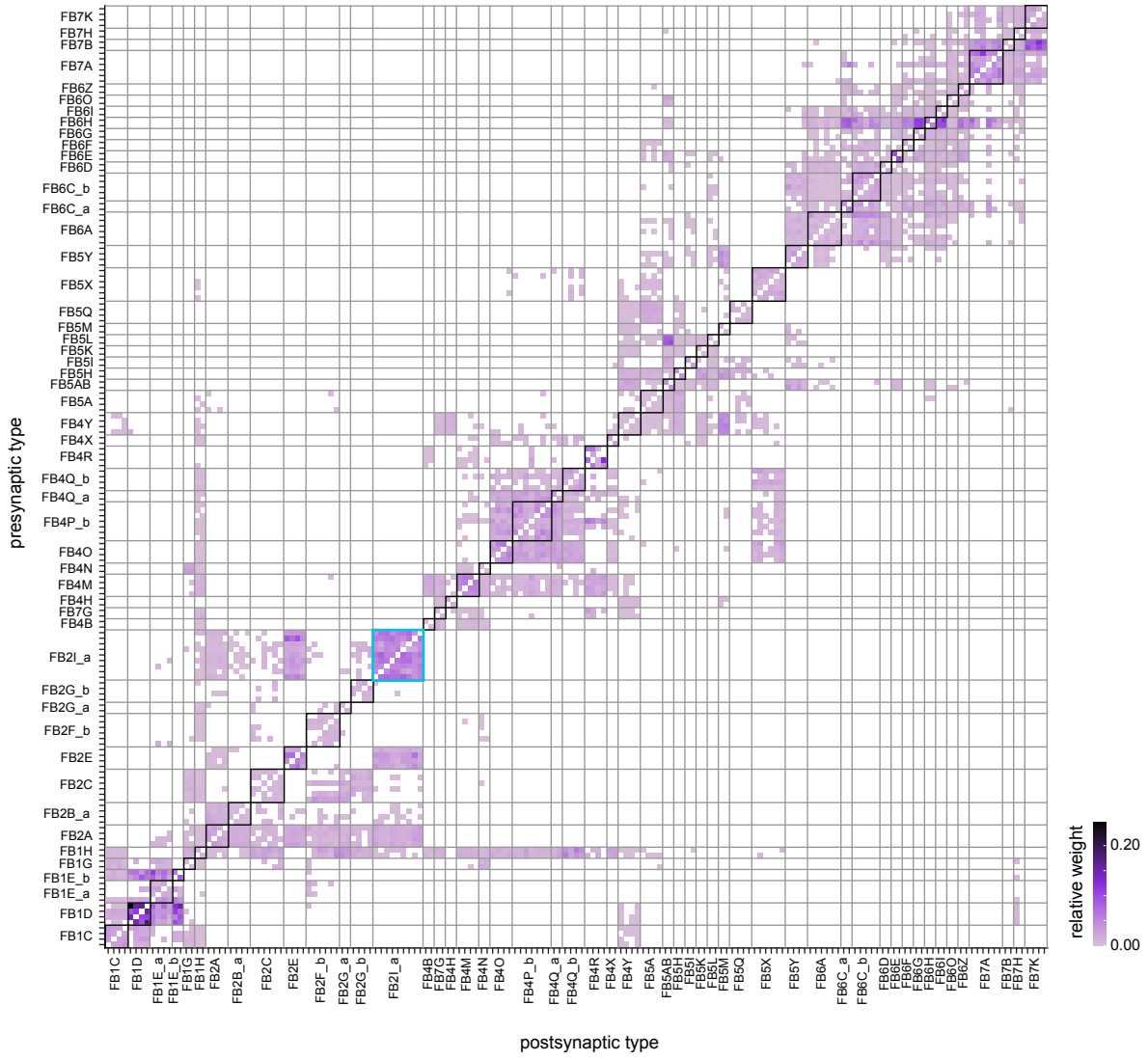
Figure 44: FB tangential postsynaptic partner in the FB

A) Type-to-type connectivity table for the FB tangential postsynaptic partners in the FB. The connectivity of known dopaminergic neurons is highlighted in gray. Horizontal lines adjacent to the x axis mark groups of FB tangential neurons that primarily arborize in the same layer.

B) Type-to-type connectivity table for the FB tangential presynaptic partners in the FB, as seen in **Figure 42A**, where the axes are now flipped and only the partners from **A** are plotted.

Figure 45: Several FB tangential neuron types show all-to-all connections that resemble connectivity patterns within and between ER neuron types

A



B

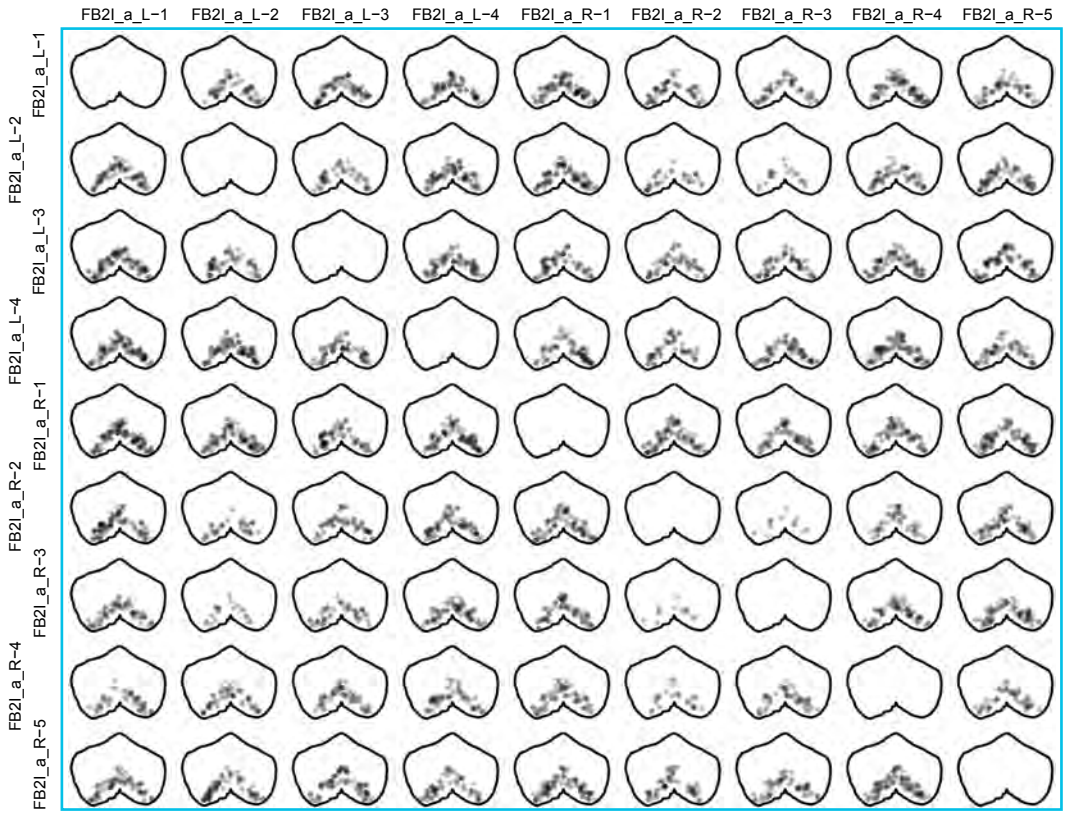


Figure 45: Several FB tangential neuron types show all-to-all connections that resemble connectivity patterns within and between ER neuron types.

A) Neuron-to-neuron connectivity matrix for the FB tangential types.

B) Location of synapse between individual FB2I neurons (box in blue in A). FB2I neurons synapse onto other neurons of the same type across all columns of the layers that they innervate.

Figure 46: Direct connections from MBONs to CX neurons

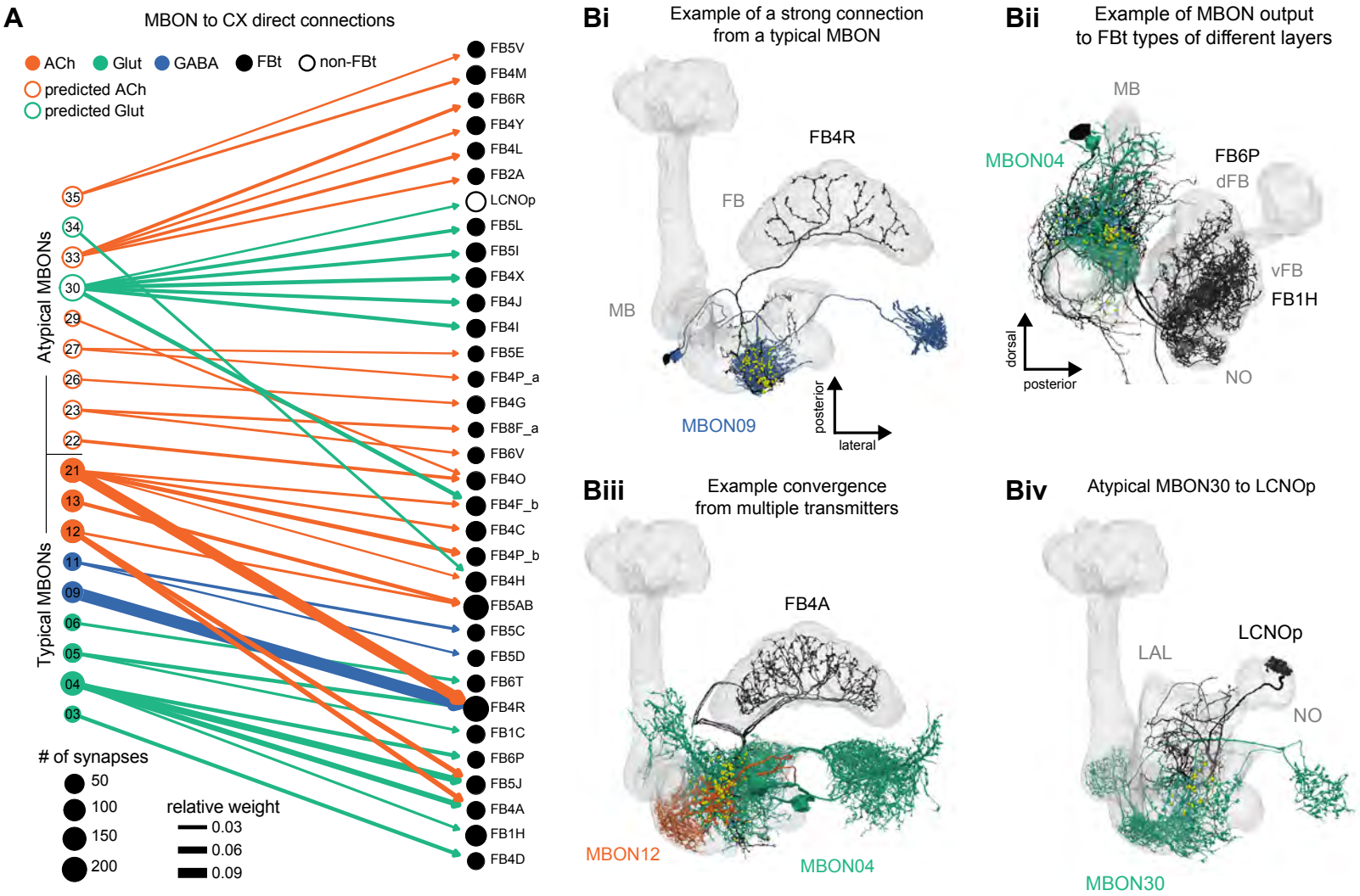


Figure 46: Direct connections from MBONs to CX neurons

A) Network graph showing direct connections from MBONs to CX neuron types, all of which are FB tangential cell types, with the exception of one connection involving LCNOp, a LAL-NO type. MBON nodes are colored according to their neurotransmitter identity as determined by RNA-seq (filled circles) (Aso et al., 2019) or as predicted by an artificial neural network trained on EM images of presynaptic boutons from MB neurons with known transmitter types (open circles) (Eckstein et al., 2020; Li et al., 2020). Both typical (01 to 23) and atypical (24 to 35) MBONS are included. The size of each node is proportional to the total number of outgoing (MBON types) or incoming (CX types) synapses, and the width of each edge is proportional to the connection's relative weight, averaged over all right hemisphere ROIs outside of the CX. Graph includes all direct connections with at least 10 synapses (as in (Li et al., 2020)) and a relative weight greater than 0.01 (see Materials and Methods and MB Figure 1—figure supplement 1 for more details).

B) Morphological renderings illustrating several aspects of MBON-to-CX connectivity. Bi shows an example of a strong direct connection, from MBON09 to FB4R. Bii shows an example of an MBON (MBON04) that contacts multiple FB tangential neurons that innervate dorsal (FB6P) or ventral (FB1H) FB layers. Biii shows an example of two MBONs that release different neurotransmitters (MBON12, acetylcholine; MBON04, glutamate) but provide convergent output to the same target (FB4A neurons). Biv highlights the one direct connection that does not involve an FB tangential type, with atypical MBON30 synapsing onto LCNOp. Yellow circles mark synapses locations.

Figure 46—figure supplement 1: Connection threshold dependence of MBON to CX connectivity.

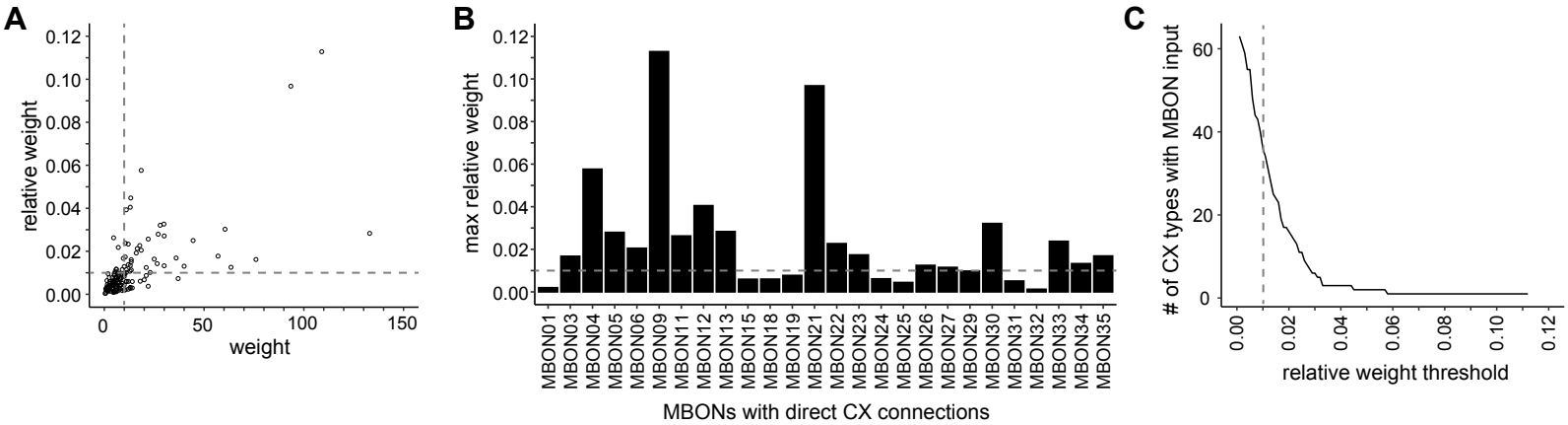


Figure 46—figure supplement 1: Connection threshold dependence of MBON to CX connectivity.

A) Scatter plot of type-to-type weight (i.e., synapse count; x-axis) versus relative weight (y-axis) for all MBON-to-CX connections. Horizontal dashed line marks relative weight threshold of 0.01. Vertical dashed line marks raw weight threshold of 10 synapses. Notice that the two thresholds mostly exclude the same cluster of connections near the origin.

B) Bar graph showing each MBON type's strongest CX connection, expressed as a relative weight. Only MBONs that made at least 3 synapses onto at least one CX type were include. Horizontal dotted line marks the 0.01 relative weight threshold.

C) Line graph showing the number of CX neuron types directly downstream of MBONs as a function of the relative weight threshold. Vertical dotted line marks the 0.01 relative weight threshold employed here.

Figure 47: Indirect MBON to CX connections

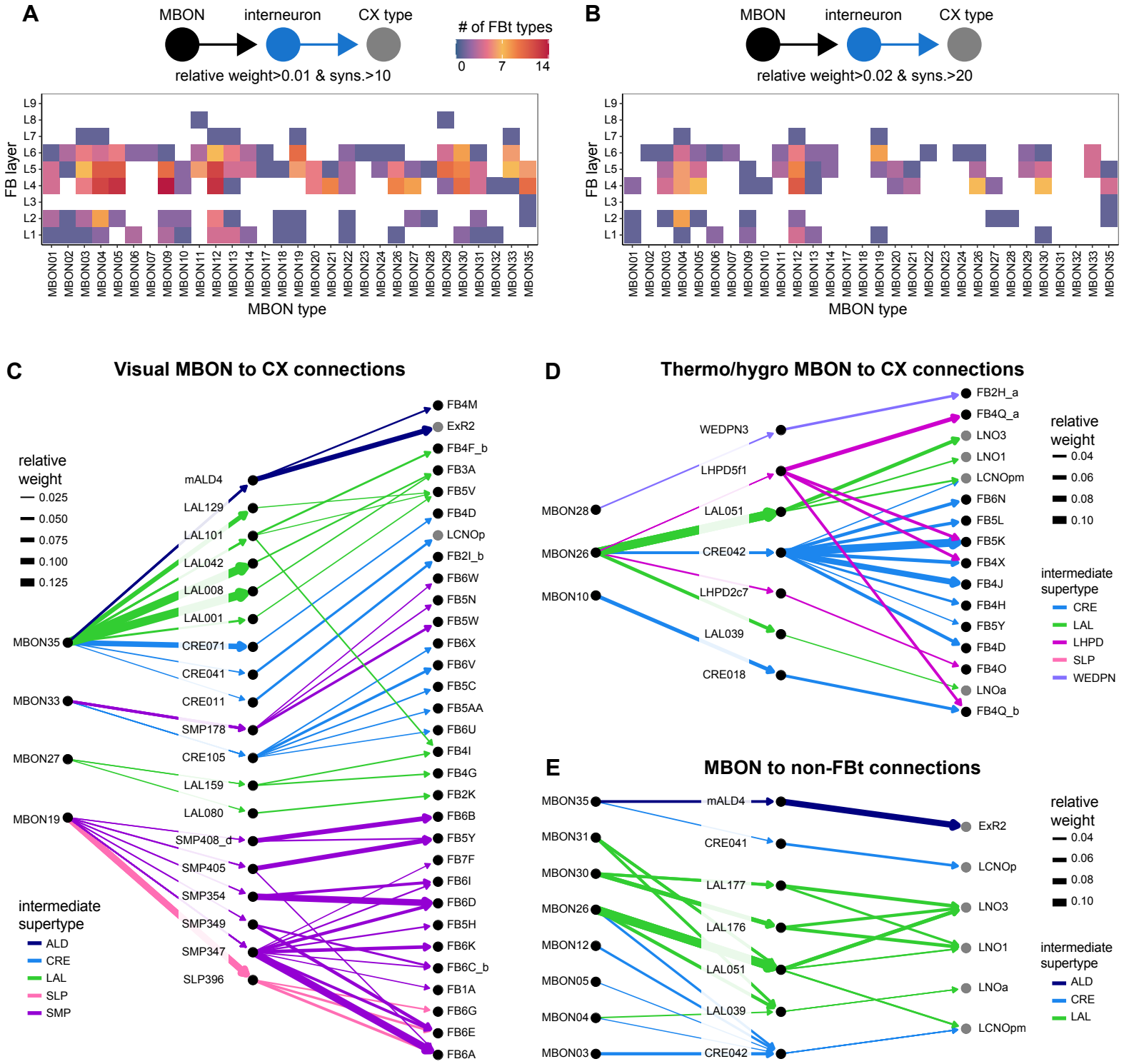


Figure 47: Indirect MBON to CX connections.

A) Plot showing the number of FB tangential types, per layer, indirectly targeted by MBONs through one intermediate neuron. Only indirect pathways where each connection involved more than 10 synapses and a relative weight greater than 0.01 were considered.

B) Same as in A, but for strong pathways, with greater than 20 synapses and relative weights greater than 0.02.

C) Network graph showing all strong (thresholds as in B), indirect connections from MBONs that receive at least 20% of their input from visual projection neurons (PNs) to CX neuron types through one intermediate layer. Edges are colored by the intermediate neuron's supertype, which largely reflects the ROI that contains its arbors. Non-FB tangential targets have gray nodes.

D) Same as in C, but for MBONs the receive at least 20% of their input from thermosensory or hygrosensory projection neurons.

E) Network graph showing all strong, indirect connections from MBONs to non-FB tangential CX neurons. Notice that, other than ExR2, the non-FB tangential targets belong to a LAL-NO type.

Figure 47—figure supplement 1: Indirect pathways from MBONs to CX neurons

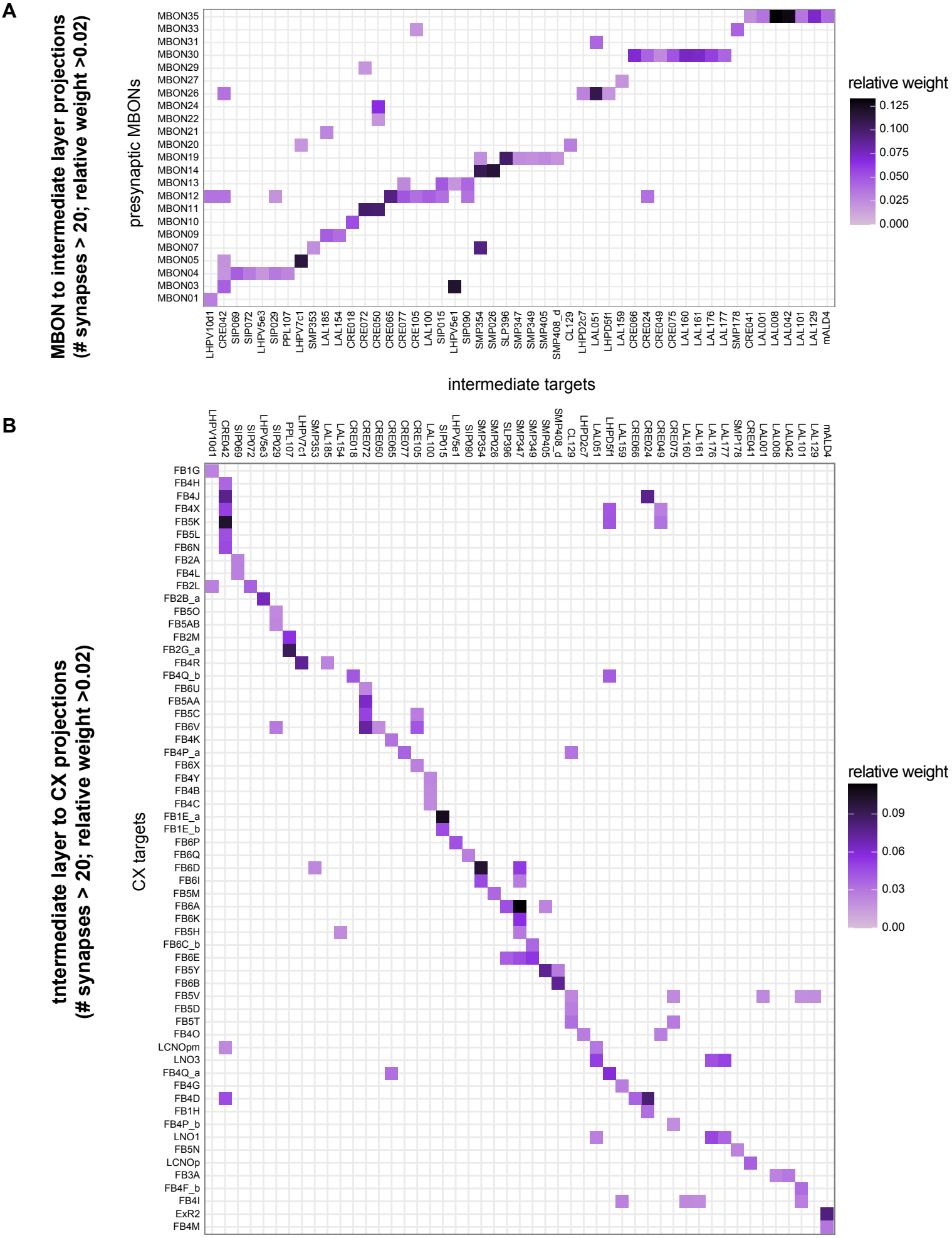


Figure 47—figure supplement 1: Indirect pathways from MBONs to CX neurons

A) Type-to-type connectivity matrix showing connections from MBON types to intermediate neuron types with projections to CX types.

B) Type-to-type connectivity matrix showing connections from the intermediate types in (A) to downstream neuron types in the CX. The connectivity matrix has been rotated 90 degrees so that presynaptic types are arrange along the x-axis, which facilitates matching neurons in A to those in B.

Figure 48: Identification of the sleep-promoting dFB tangential neuron types in the R23E10 GAL4 line

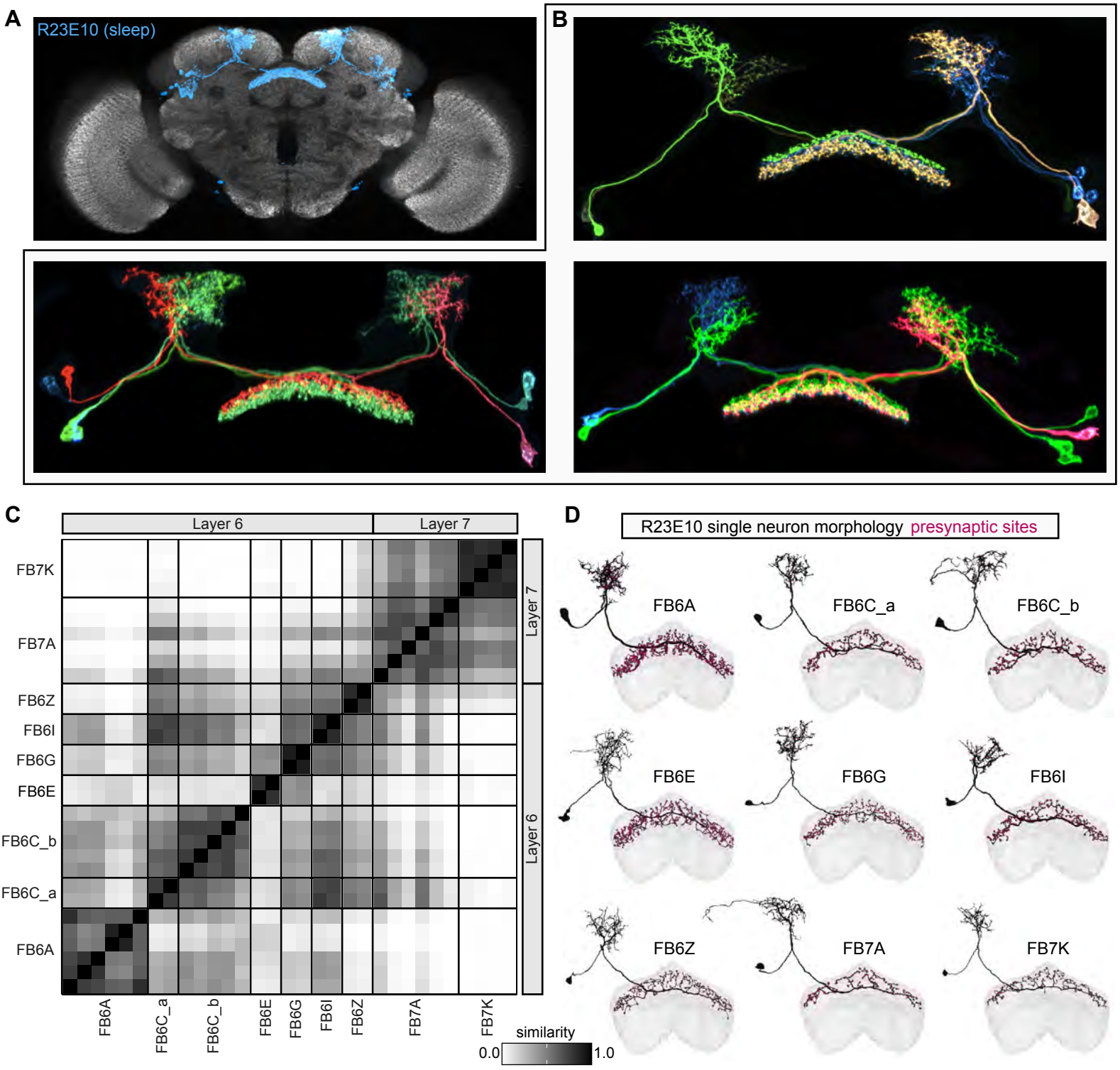


Figure 48: Identification of the sleep-promoting dFB tangential neuron types in the R23E10 GAL4 line.

A) Front view of a 3D rendering of a confocal stack showing the R23E10 expression pattern (blue) along with immunohistochemical staining against nc82 (gray). The raw confocal stack was warped to a standard reference brain and rendered in 3D using VVDviewer, which facilitates direct comparison of the R23E10 expression pattern to the EM morphologies of candidate neuron cell types (see Materials and Methods and **Figure 48—figure supplements 2-4**).

B) Stochastic labelling of subsets of R23E10 neurons made using the MCFO method (Nern et al., 2015). Note the differences in arbor morphology outside the FB and the different layers of arbors within the FB for the individual neurons in the line.

C) Matrix comparing the similarity in connectivity within the FB for the nine putative R23E10 neuron cell types (31 neurons total, see Materials and Methods).

D) Single neuron morphological renderings from the EM dataset for each of the nine neuron types that were identified in the R23E10 line. Magenta circles mark presynaptic sites. Two anatomical features of R23E10 neurons —the lateral location of their soma and a fiber track that enters the FB slightly medial to the lateral border— unambiguously identified 14 candidate tangential neuron types with processes in layers 6 and 7 whose general morphology matched that of the R23E10 pattern. Comparison of these neuron morphologies with candidate EM neuron types allowed us to exclude 5 of the 14 candidates based on the presence of arbors that lie well outside the R23E10 pattern (**Figure 48—figure supplements 2 -6**).

Figure 48—figure supplement 1: Region arborization plot of R23E10 and dopaminergic neuron cell types

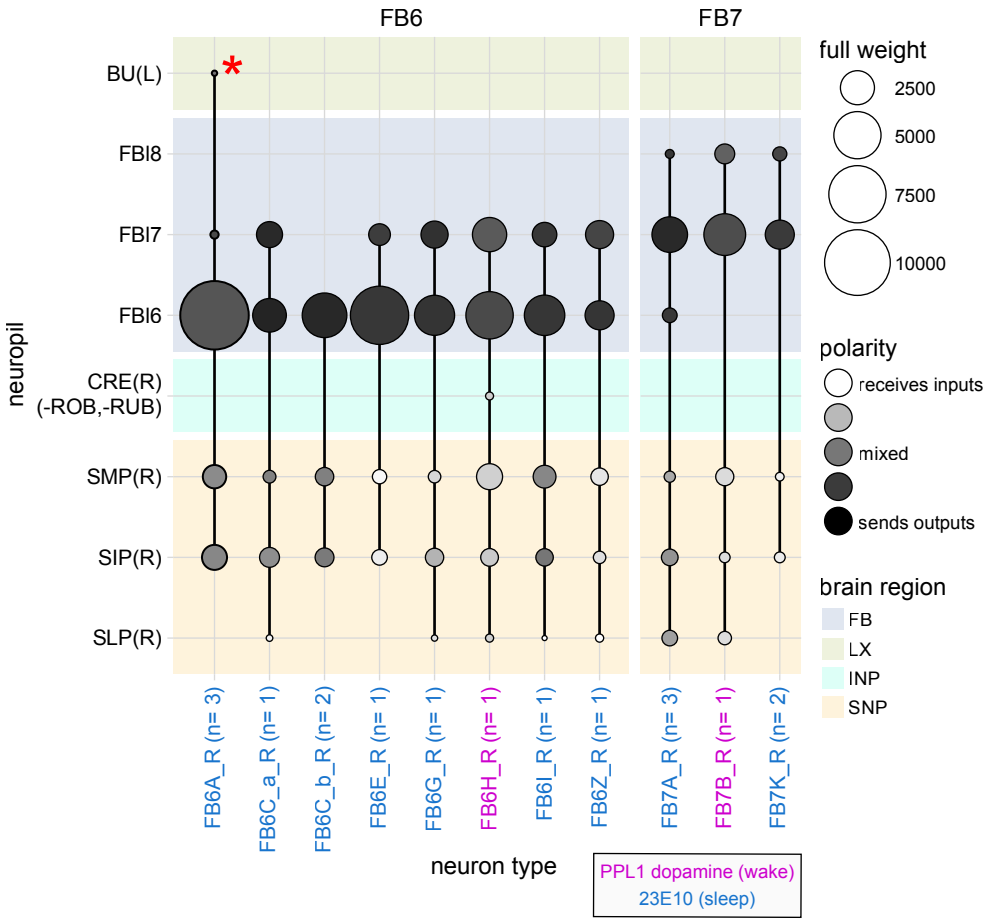


Figure 48—figure supplement 1: Region arborization plot of R23E10 and dopaminergic neuron cell types (FB6H and FB7B, see **Figure 49**).
Average pre- and postsynaptic counts by region are shown. Red asterisk marks synapses from FB6A located in the FB but that are incorrectly assigned to the left BU, whose ROI boundary requires revision.

Figure 48—figure supplement 2: Summary of sleep-promoting and PPL1 DAN neuron type identification

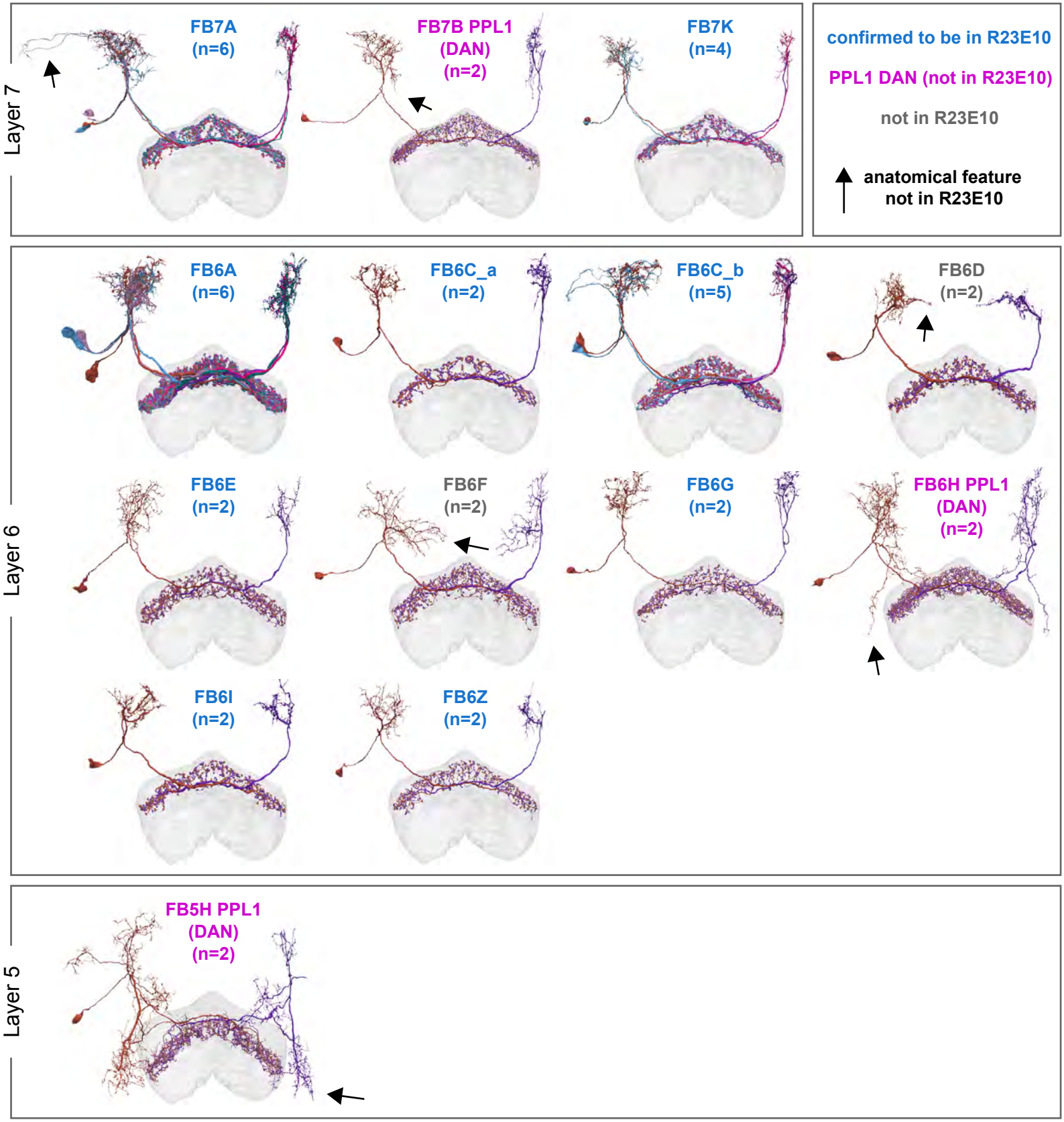


Figure 48—figure supplement 2: Summary of sleep-promoting and PPL1 DAN neuron type identification.

Morphological renderings of the 14 dFB neuron types whose general morphology matches that of the R23E10 pattern. For each neuron type, the color of text indicates whether the type is confirmed to be in 23E10 (blue text), is confirmed to be a PPL1 dopaminergic neuron (magenta), or is neither (gray text). Arrows mark anatomical features not present in R23E10, as evaluated by directly comparing R23E10 expression to EM morphologies in the same reference brain. See **Figure 48—figure supplements 3 and 4** for more details.

Figure 48—figure supplement 3: Overlap of individual R23E10 and dopamine neurons with corresponding EM neuron types

Neuron types confirmed to be in R23E10

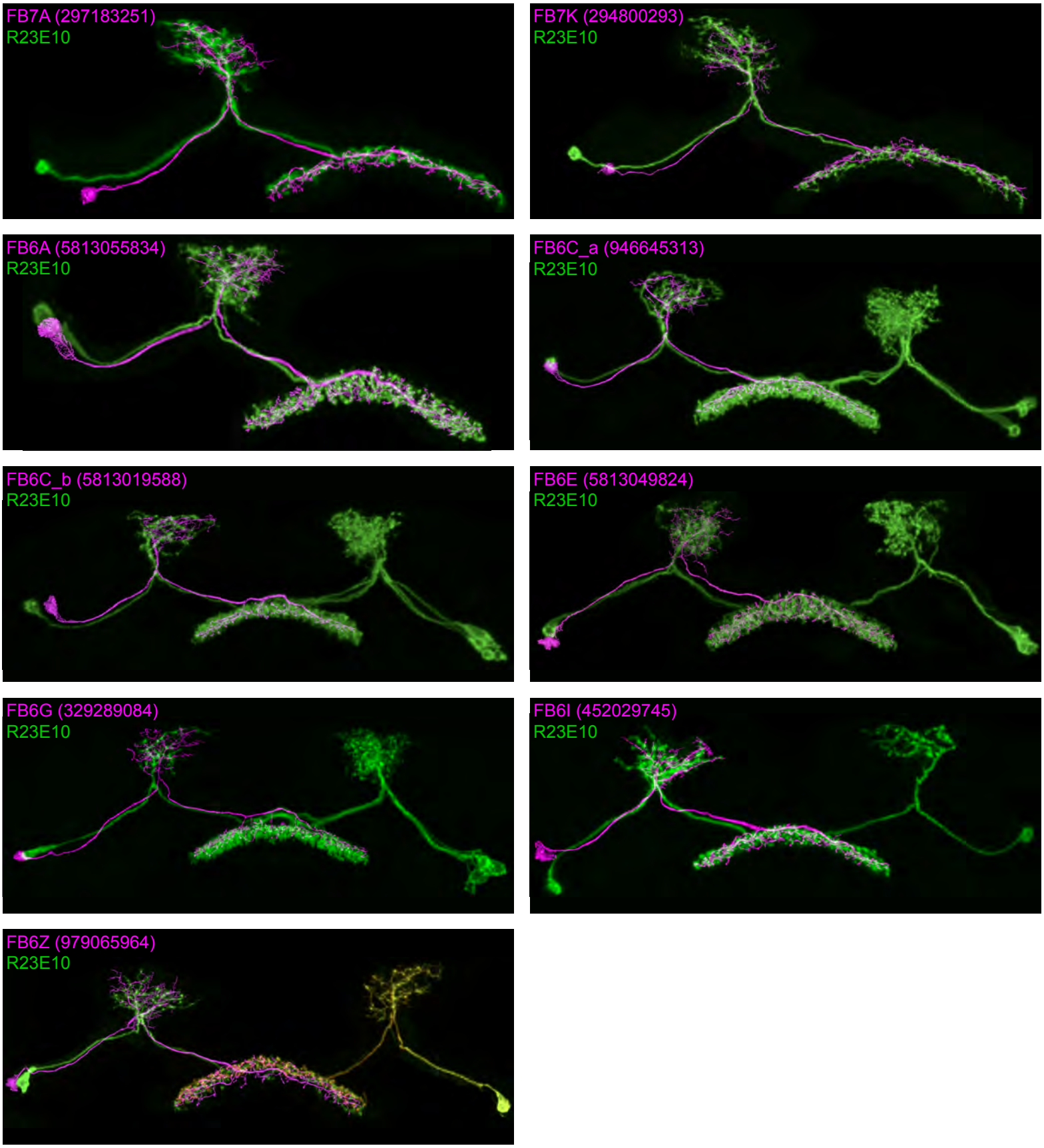


Figure 48—figure supplement 3: Overlap of individual R23E10 and dopamine neurons with corresponding EM neuron types.

Morphological renderings comparing individual R23E10 cells from the right hemisphere (green), generated using the MCFO stochastic labeling technique (Nern et al., 2015), to single EM neuronal morphologies (magenta). In every case but FB7K, one or more high-quality matches (i.e. those with a high degree of overlap between EM and LM processes) was obtained between single 23E10 neurons and their corresponding EM neuron type. See Videos (sleep) 1 and 2 for direct comparisons in 3D of layer 6 and layer 7 neurons, respectively.

Figure 48—figure supplement 4: Cell types not found in R23E10

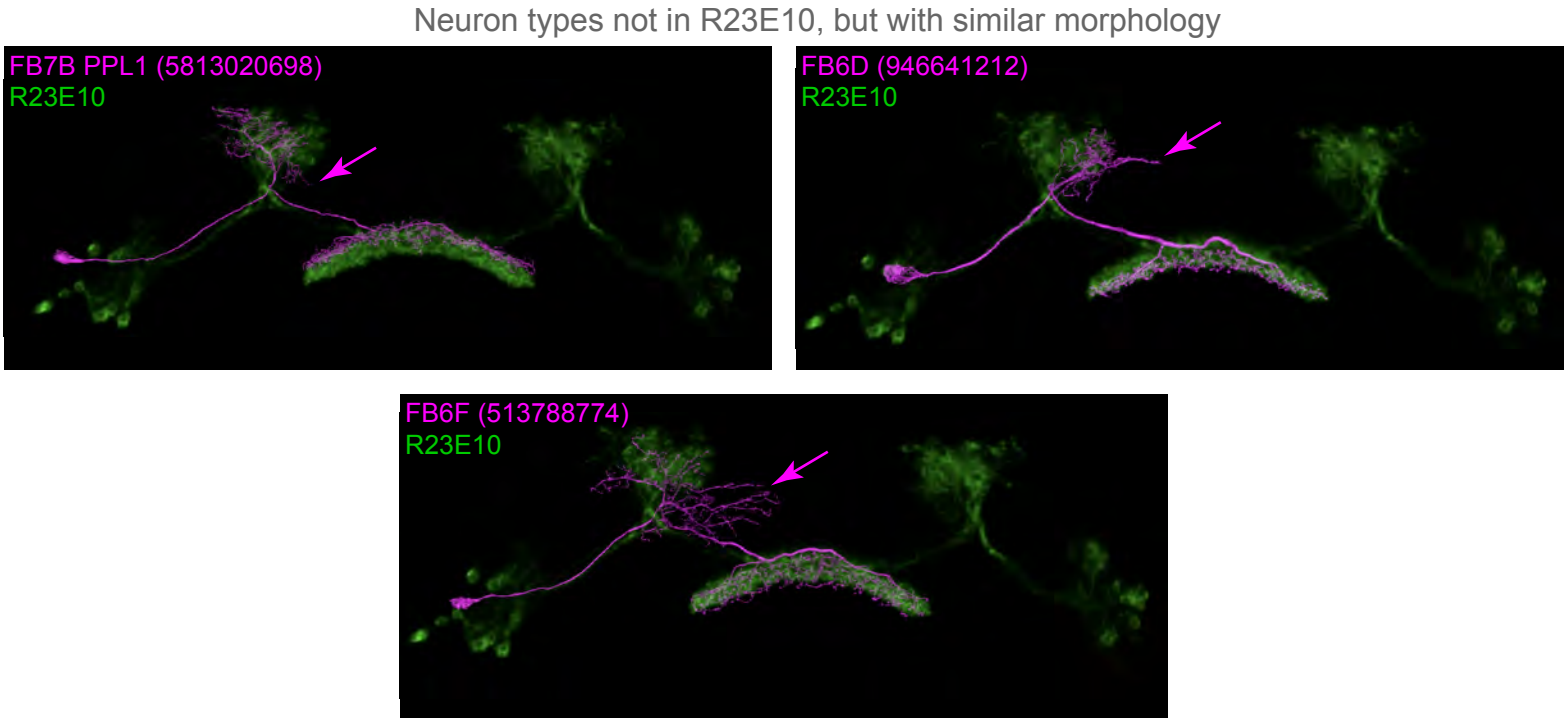


Figure 48—figure supplement 4: Cell types not found in R23E10, though they have similar morphology.

These three candidate EM neuron types contain processes, marked by magenta arrows, that lie well outside the R23E10 pattern.

Figure 48—figure supplement 5: Summary of the morphologies of all layer 8 and layer 7 tangential neurons

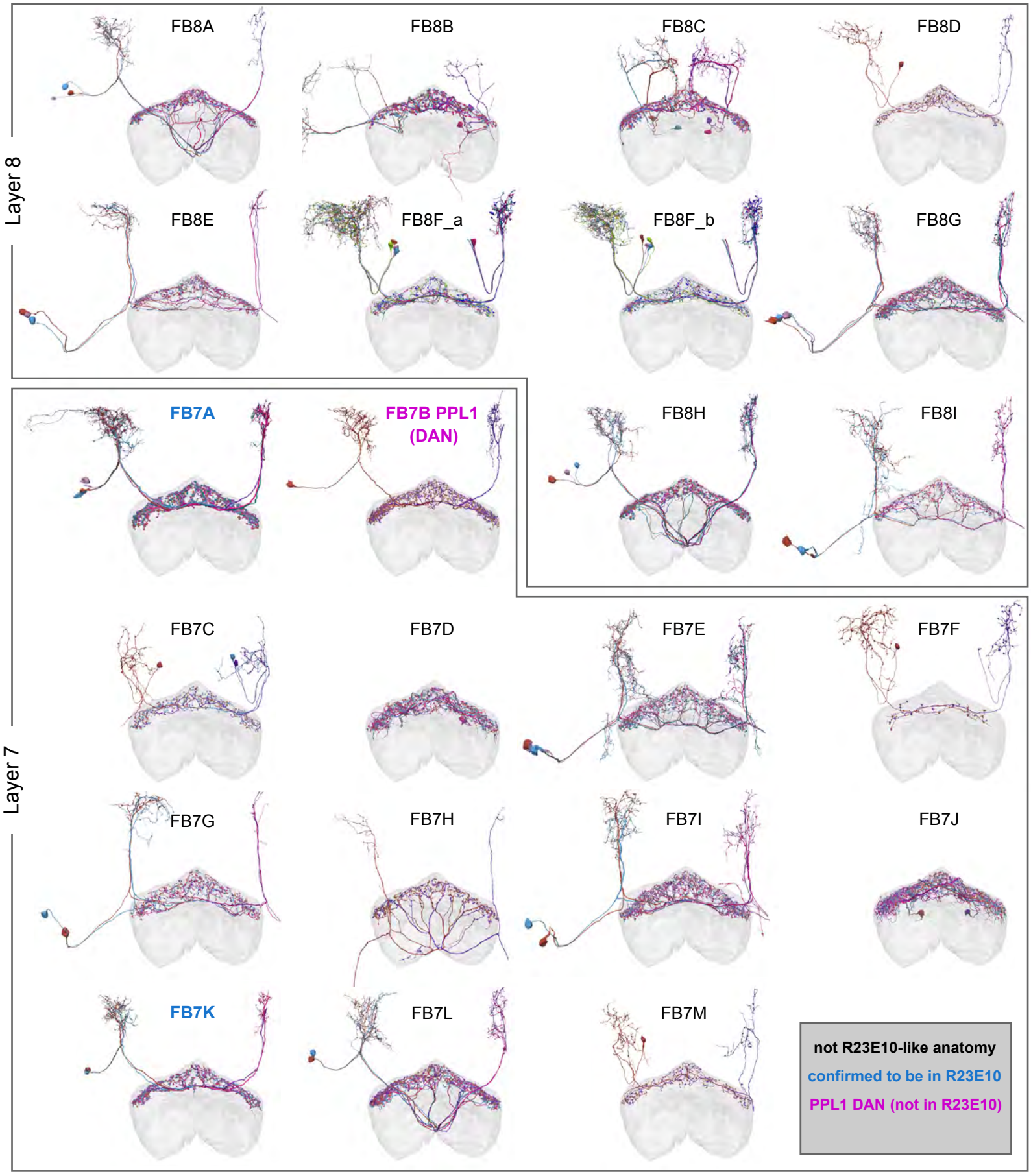


Figure 48—figure supplement 5: Summary of the morphologies of all layer 8 and layer 7 tangential neurons.

For each neuron type, the color of text indicates whether the type is confirmed to be in R23E10 (blue text), is confirmed to be a PPL1 dopamine type (magenta text), or does not have a general morphology consistent with R23E10 neurons (black text). In layer 7, we were able to identify high-quality matches (i.e. those with a high degree of overlap between EM and LM processes) with a subset of FB7A neurons and a moderate-quality match to FB7K. As presently defined, the FB7A neuron type contains three neurons per hemisphere. Two of these neurons send processes to the lateral portion of the superior neuropil—a feature not observed in R23E10—while the remaining neuron had a high-quality match in several R23E10 MCFO samples. Therefore, we include all FB7A neurons while recognizing that future work may further refine this neuron type and its relation to the R23E10 line.

Figure 48—figure supplement 6: Summary of the morphologies of all layer 6 tangential neurons

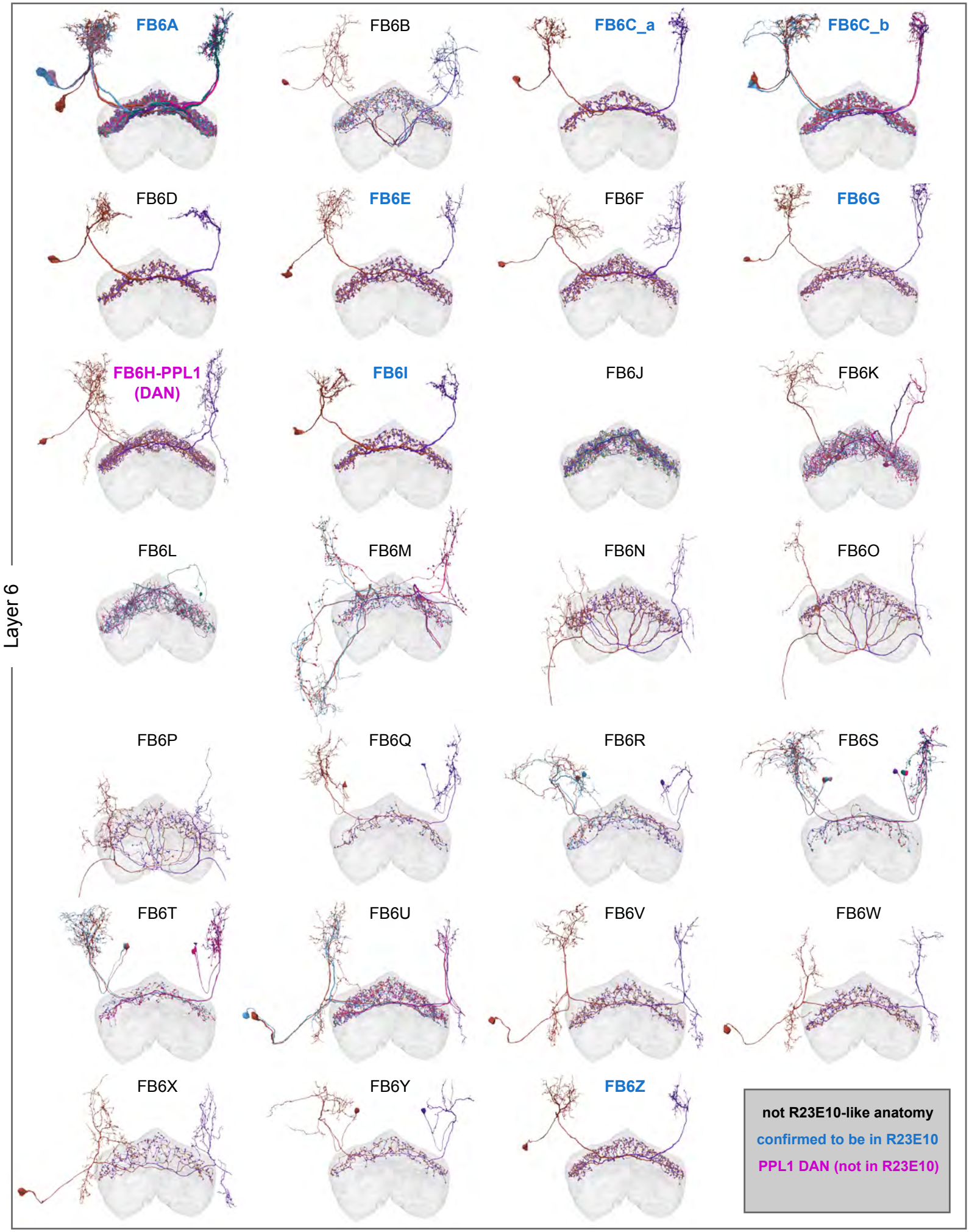


Figure 48—figure supplement 6: Summary of the morphologies of all layer 6 tangential neurons.

For each neuron type, the color of text indicates whether the type is confirmed to be in R23E10 (blue text), is confirmed to be a PPL1 dopamine type (magenta text), or does not have a general morphology consistent with R23E10 neurons (black text).

Figure 48—figure supplement 7: Summary of the morphologies of all layer 5 tangential neurons

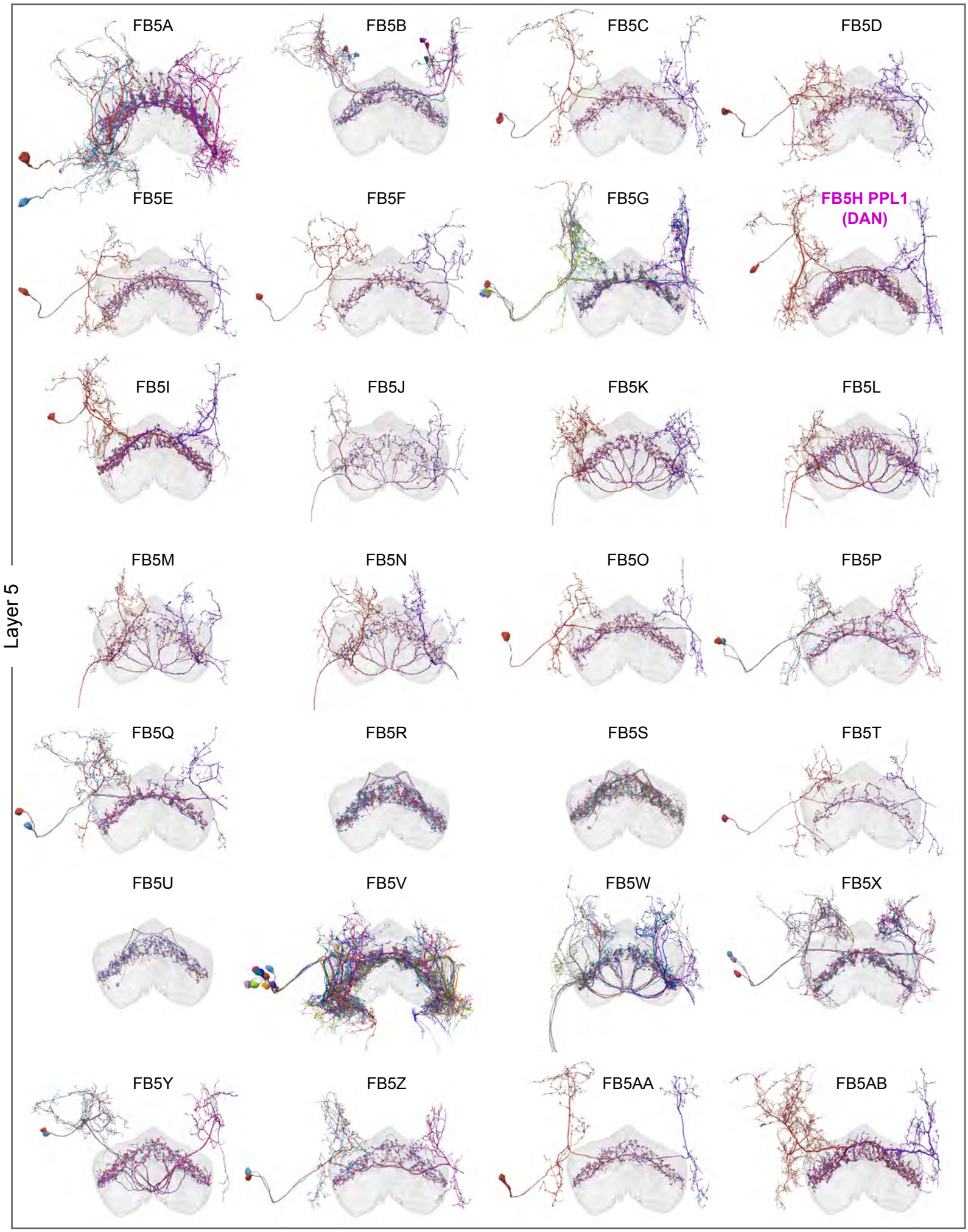


Figure 48—figure supplement 7: Summary of the morphologies of all layer 5 tangential neurons.

For each neuron type, the color of text indicates whether the type is confirmed to be in R23E10 (blue text), is confirmed to be a PPL1 dopamine type (magenta text), or does not have a general morphology consistent with R23E10 neurons (black text).

Figure 49: Identification of wake-promoting, PPL1 dopaminergic dFB tangential neuron types

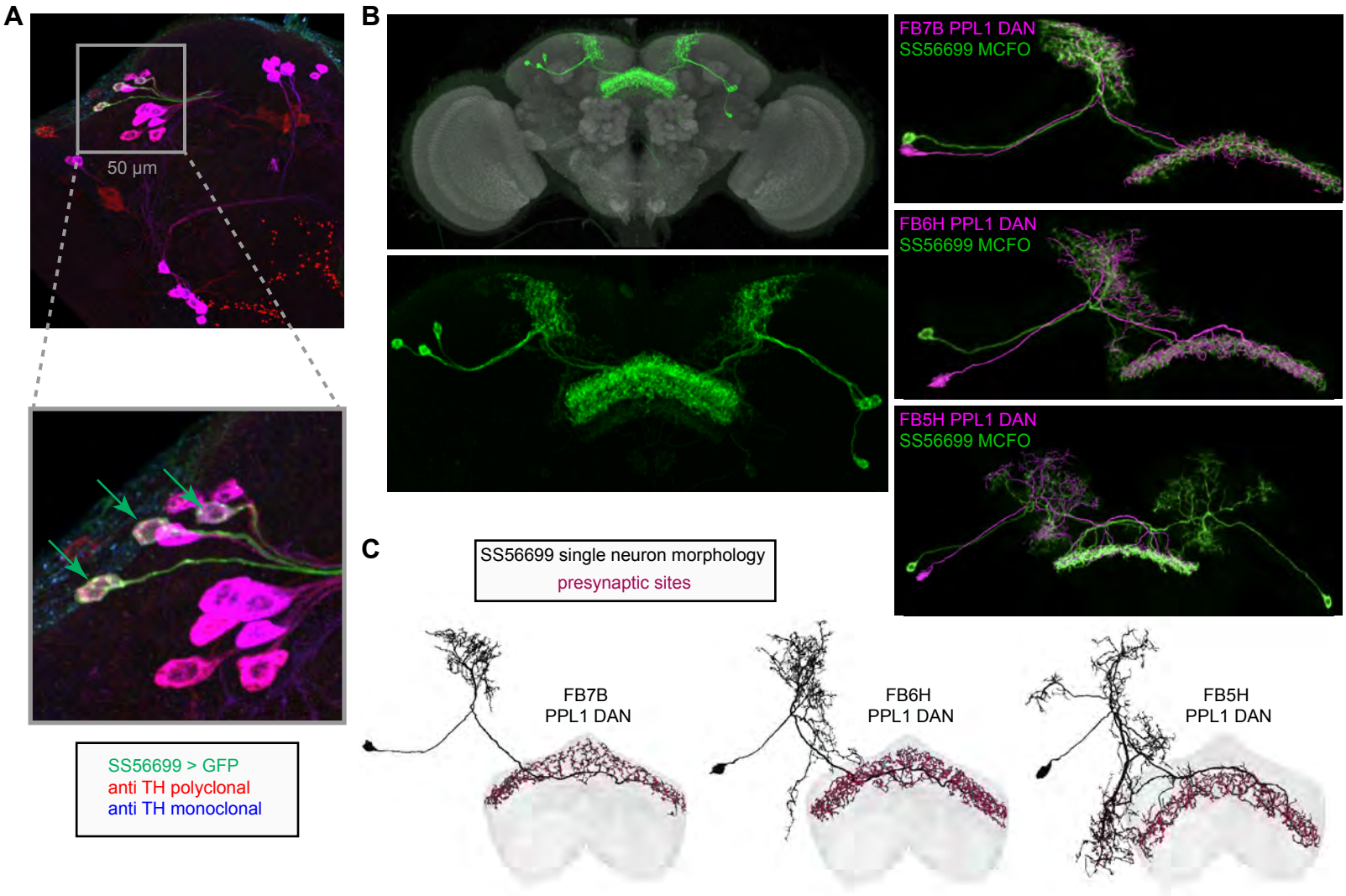


Figure 49: Identification of wake-promoting, PPL1 dopaminergic dFB tangential neuron types.

A) Confocal micrographs showing a portion of the expression pattern of a split-GAL4 line, SS56699 (green), focused on the cell bodies of the three neurons expressed in each brain hemisphere of this line along with immunohistochemical staining against TH using a polyclonal (red) and monoclonal (blue) antibody. Inset shows a zoomed-in view of the three SS56699 soma in the right hemisphere, marked by green arrows, which are all TH+. This result was consistent across 12 hemispheres from 6 brains.

B) Expression pattern of the SS56699 line with nc82 reference staining (top) and zoomed-in view of the expression pattern alone (bottom). One of hemidriver parents of this line uses an 11kb genomic segment of the TH tyrosine hydroxylase (TH) gene (see Aso et al., 2014a) to drive its expression. Morphological renderings comparing the three putative dFB dopaminergic neuron types (magenta)—FB7B (top panel), FB6H (middle panel), and FB5H (bottom panel)—to individual neurons from SS56699, generated by MCFO stochastic labeling (green; Nern et al., 2015).

C) Single neuron morphological renderings from each of the three identified PPL1 dopaminergic neuron types. Magenta circles mark presynaptic sites. See sleep Video 3 for 3D comparisons.

Figure 50: A potential sleep-wake flip-flop switch in the dFB

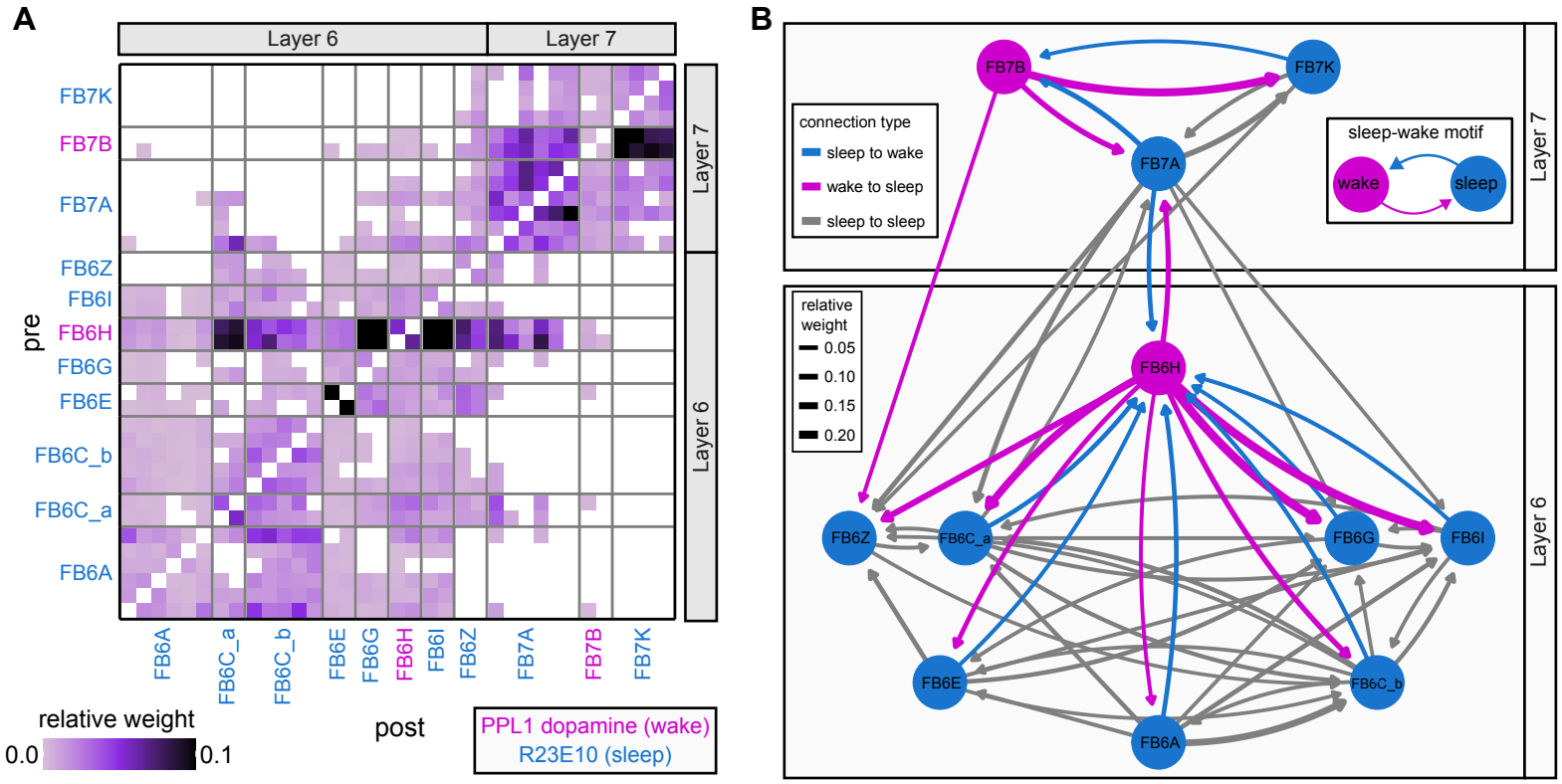


Figure 50: A potential sleep-wake flip-flop switch in the dFB.

A) Neuron-to-neuron connectivity matrix between R23E10 neurons (marked by blue text) and dopaminergic neurons (marked by magenta text). Note that most layer 6 neurons connect to other layer 6 neurons, and layer 7 neurons to other layer 7 neurons, but there are many fewer connections between layers.

B) Network graph showing the intra-FB connections between the R23E10 and dopaminergic types. Arrow width is proportional to connection strength, and arrow color indicates connection type. Node color indicates whether the neuron type belongs to a putative wake-promoting type (magenta) or a putative sleep-promoting type (blue). Connections within a type have been omitted for clarity, but can be observed in A.

Figure 50—figure supplement 1: Type-to-type connectivity matrix between R23E10 neuron types and dopaminergic neuron types

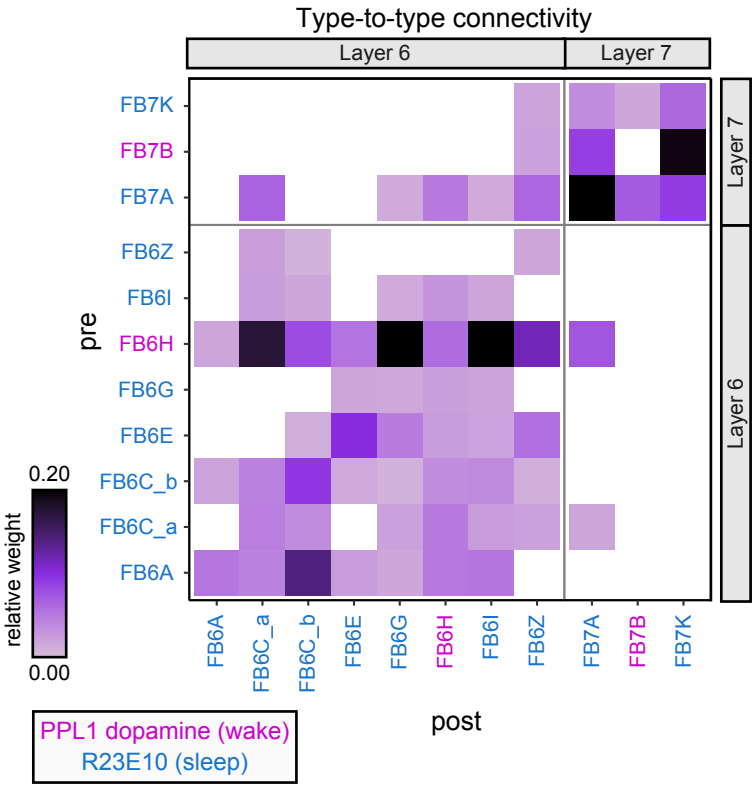


Figure 50—figure supplement 1: Type-to-type connectivity matrix between R23E10 neuron types (blue text) and dopaminergic neuron types (magenta text).

These data were used to construct the graph in **Figure 50B**. Compared to the neuron-to-neuron connectivity matrix in **Figure 50A**, this matrix has fewer weak connections due to the type-to-type significance criteria (see Materials and Methods).

Figure 51: Downstream targets of dFB sleep-wake neurons

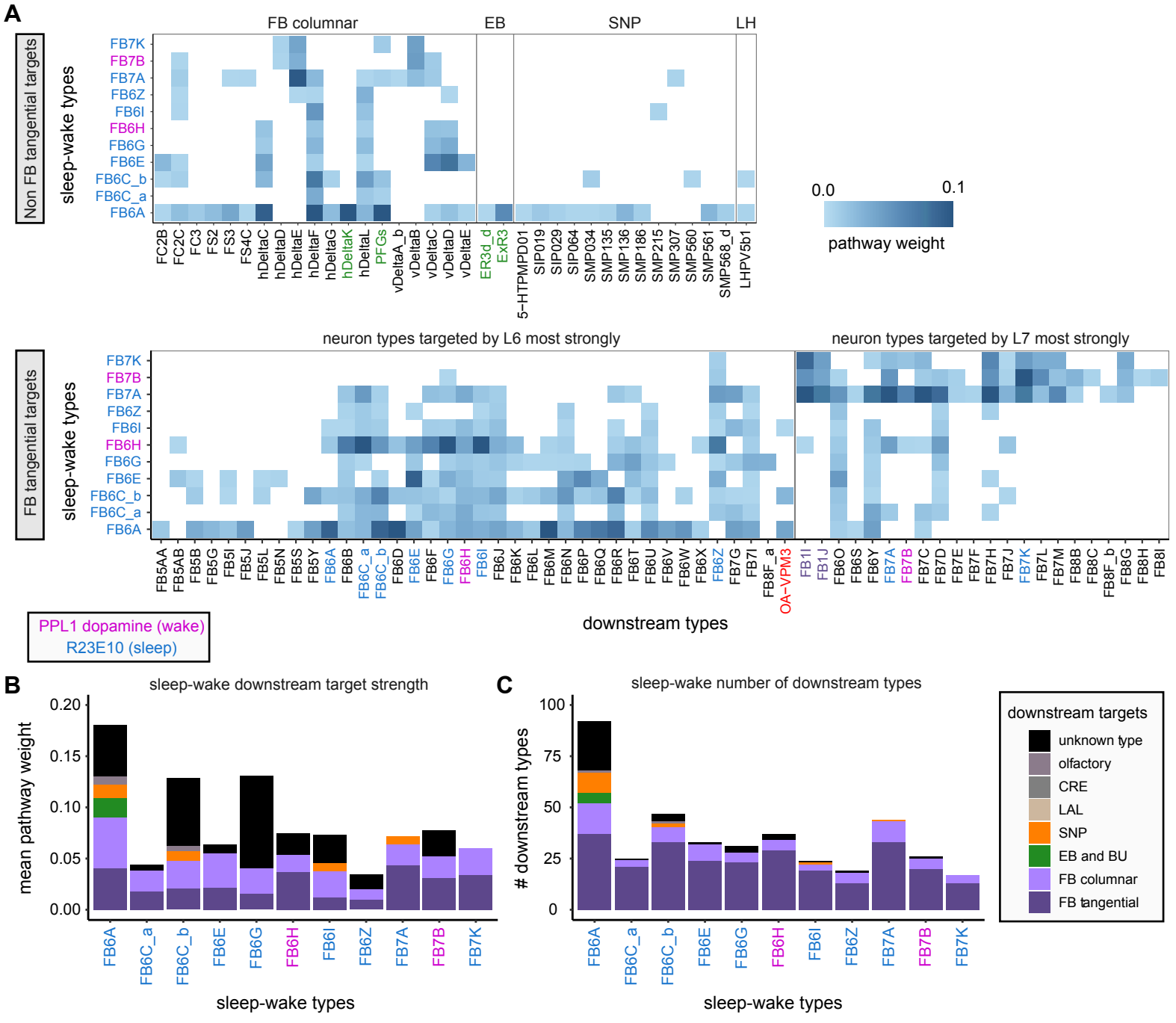


Figure 51: Downstream targets of dFB sleep-wake neurons

A) Pathway connectivity matrices (see Materials and Methods) showing the neuron types targeted by each of the sleep-wake neuron types. The downstream neurons are divided into groups, with non FB tangential targets shown in the top panel and FB tangential targets shown in the bottom panel. Pathways up to 2 steps downstream (that is, with one intermediate neuron) of the sleep-wake neuron types were considered. Green text marks neurons involved in EB-FB sleep-wake circuit (see **Figure 50**).

B) Mean pathway weight from each sleep-wake neuron type to other FB neuron types (tangential or columnar), neurons types with prominent arbors outside the FB (EB/BU, SNP, LAL, CRE, and olfactory), or unknown types (i.e. neurons that have not been assigned a type name).

C) Same as in (**B**) but plotting the number of downstream neuron types reached.

Figure 51—figure supplement 1: Direct downstream targets of dFB sleep-wake neurons

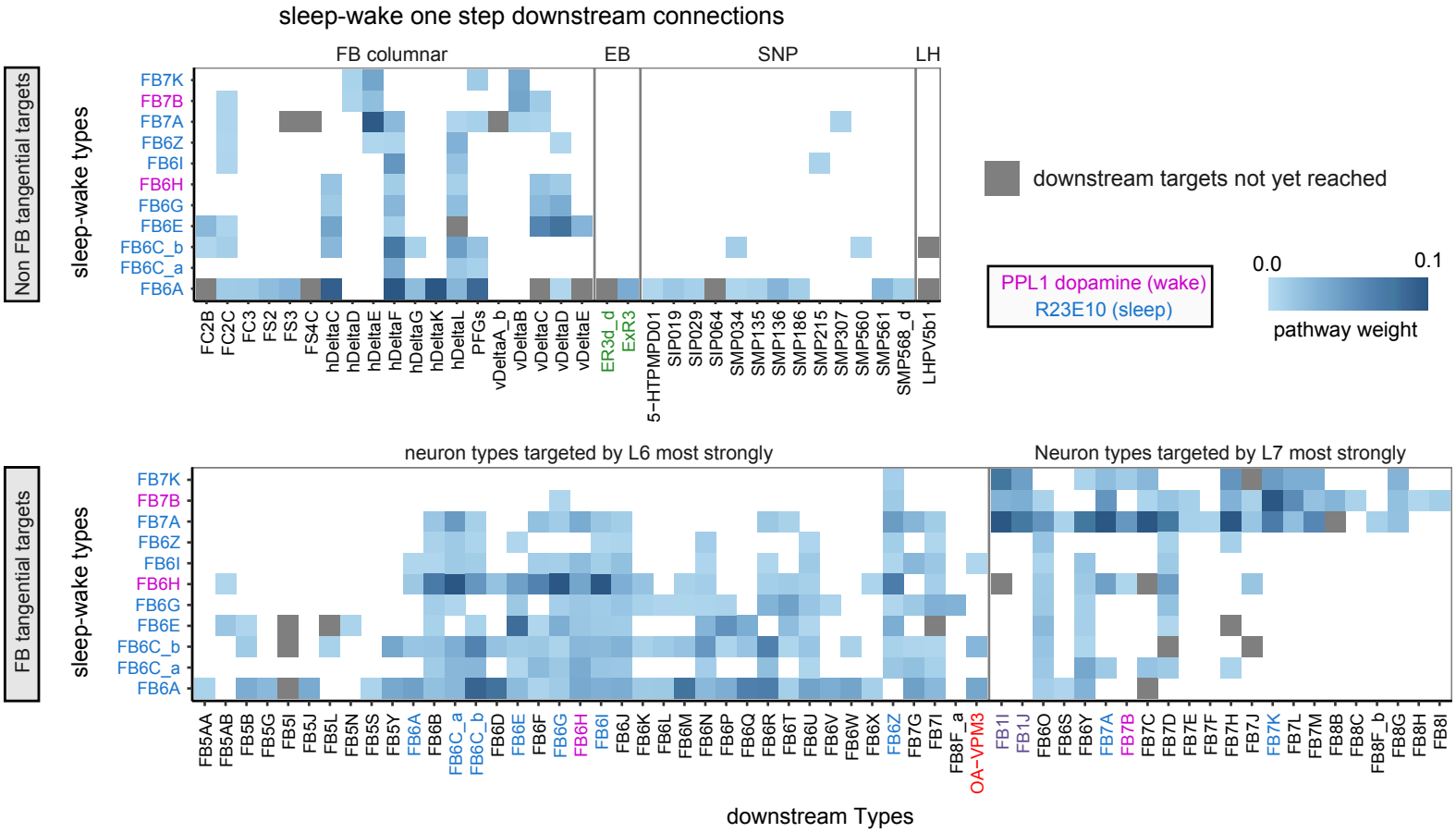


Figure 51—figure supplement 1: Direct downstream targets of dFB sleep-wake neurons.

Pathway connectivity matrices showing the non-FB tangential neuron types (top panel) and FB tangential neuron types (bottom panel) directly targeted by each of the sleep-wake neuron types. Gray boxes mark two-step downstream targets that are not contacted directly.

Figure 52: Upstream pathways leading to dFB sleep-wake neurons

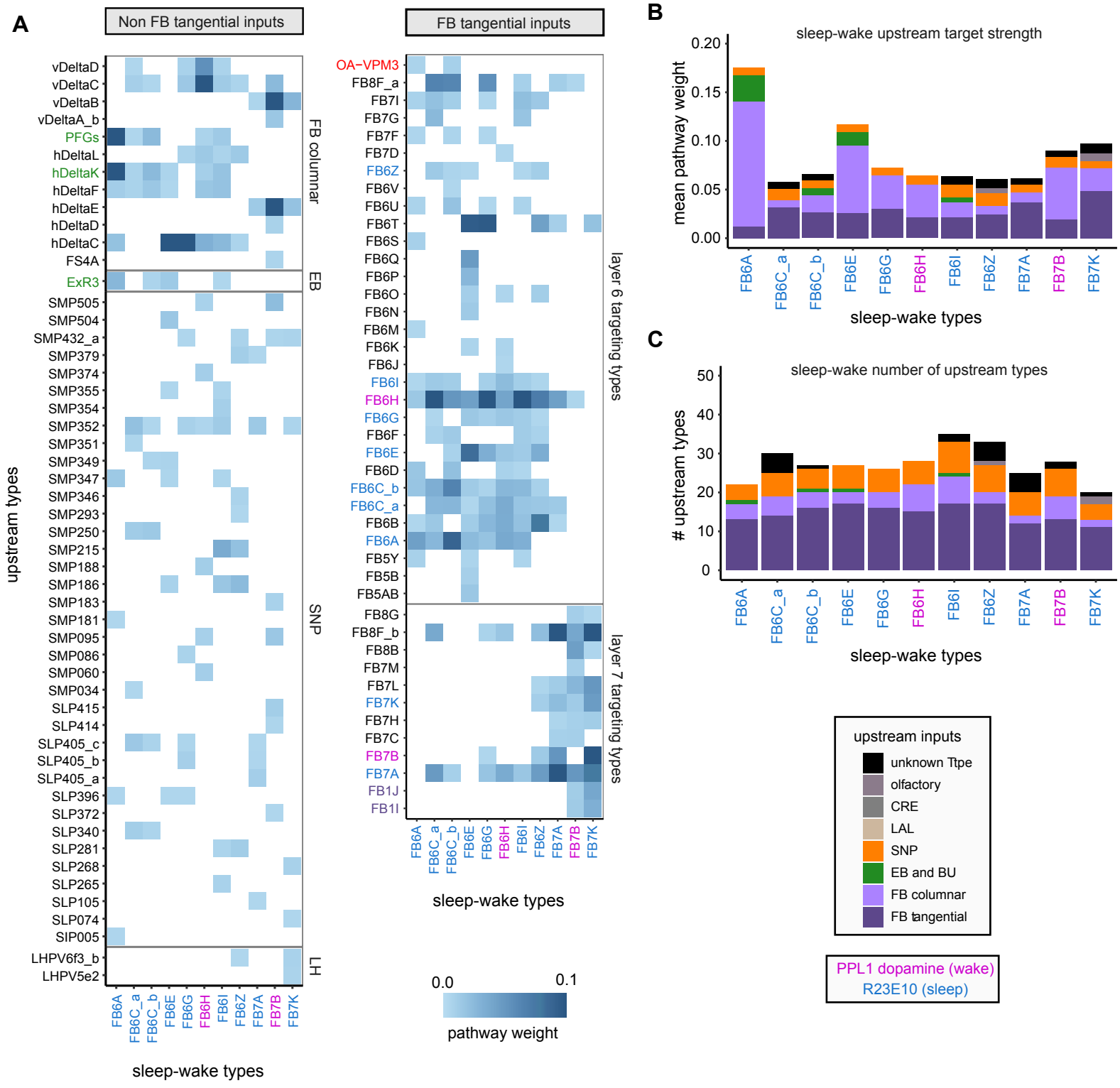


Figure 52: Upstream pathways leading to dFB sleep-wake neurons

A) Pathway connectivity matrices (see Materials and Methods) showing the neuron types that target each of the sleep-wake neuron types. The upstream neurons are divided into groups, with non-FB tangential targets shown in the left panel and FB tangential targets shown in the right panel. Pathways up to 2 steps upstream of the sleep-wake neuron types were considered.

B) Mean pathway weight to each sleep-wake neuron type from other FB neuron types (tangential or columnar), neurons types with prominent arbors outside the FB (EB/BU, SNP, LAL, CRE, and olfactory), or unknown types (see legend).

C) Same as in (**B**) but plotting the number of upstream neuron types.

Figure 52—figure supplement 1: Direct upstream pathways leading to dFB sleep-wake neurons

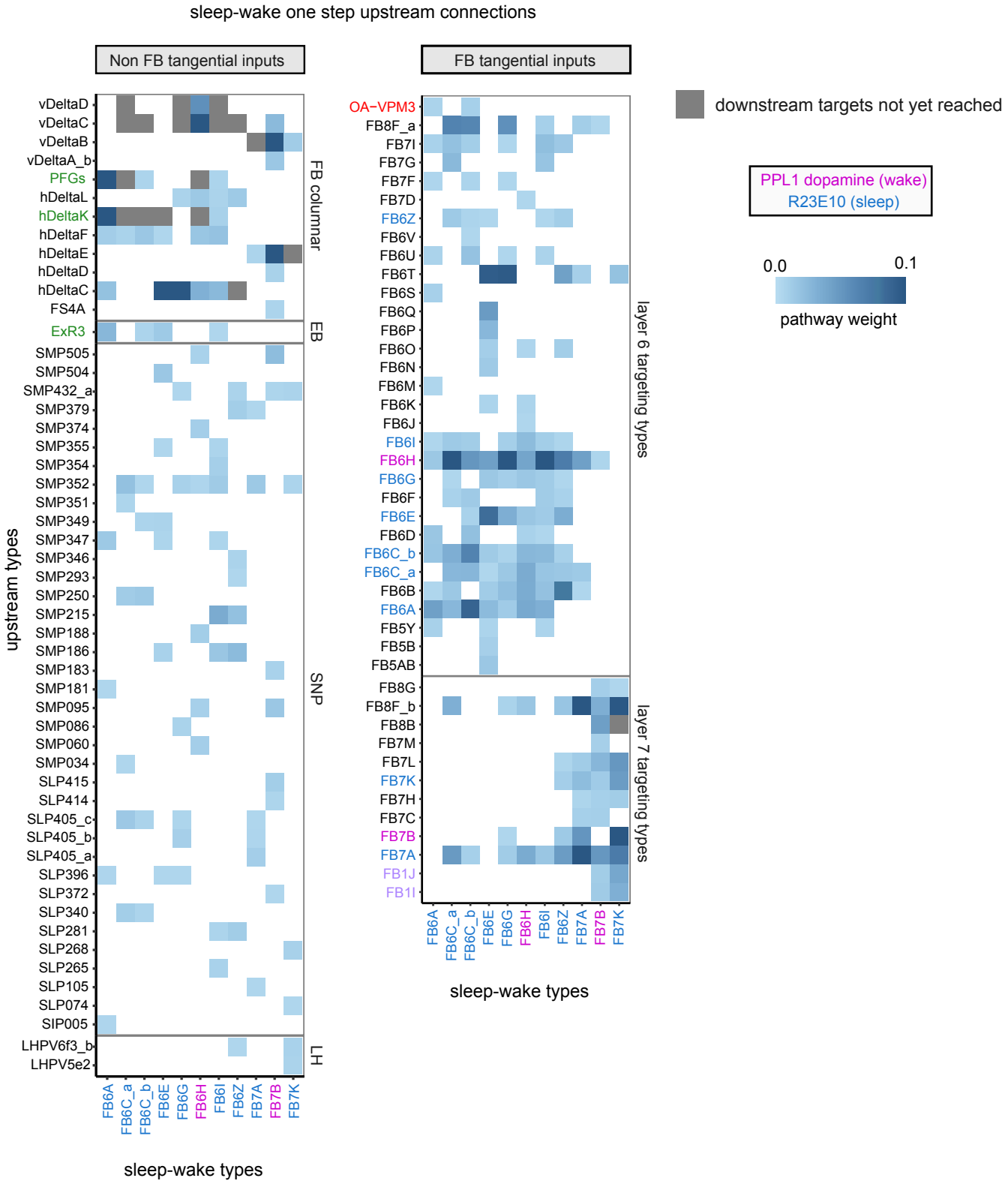


Figure 52—figure supplement 1: Direct upstream pathways leading to dFB sleep-wake neurons. Pathway connectivity matrices showing the non-FB tangential neuron types (left panel) and FB tangential neuron types (right panel) directly upstream of the sleep-wake neuron types. Gray boxes mark upstream targets that are not directly connected

Figure 53: A direct pathway linking sleep-wake neurons in the dFB and EB

FB-EB sleep-wake direct pathways

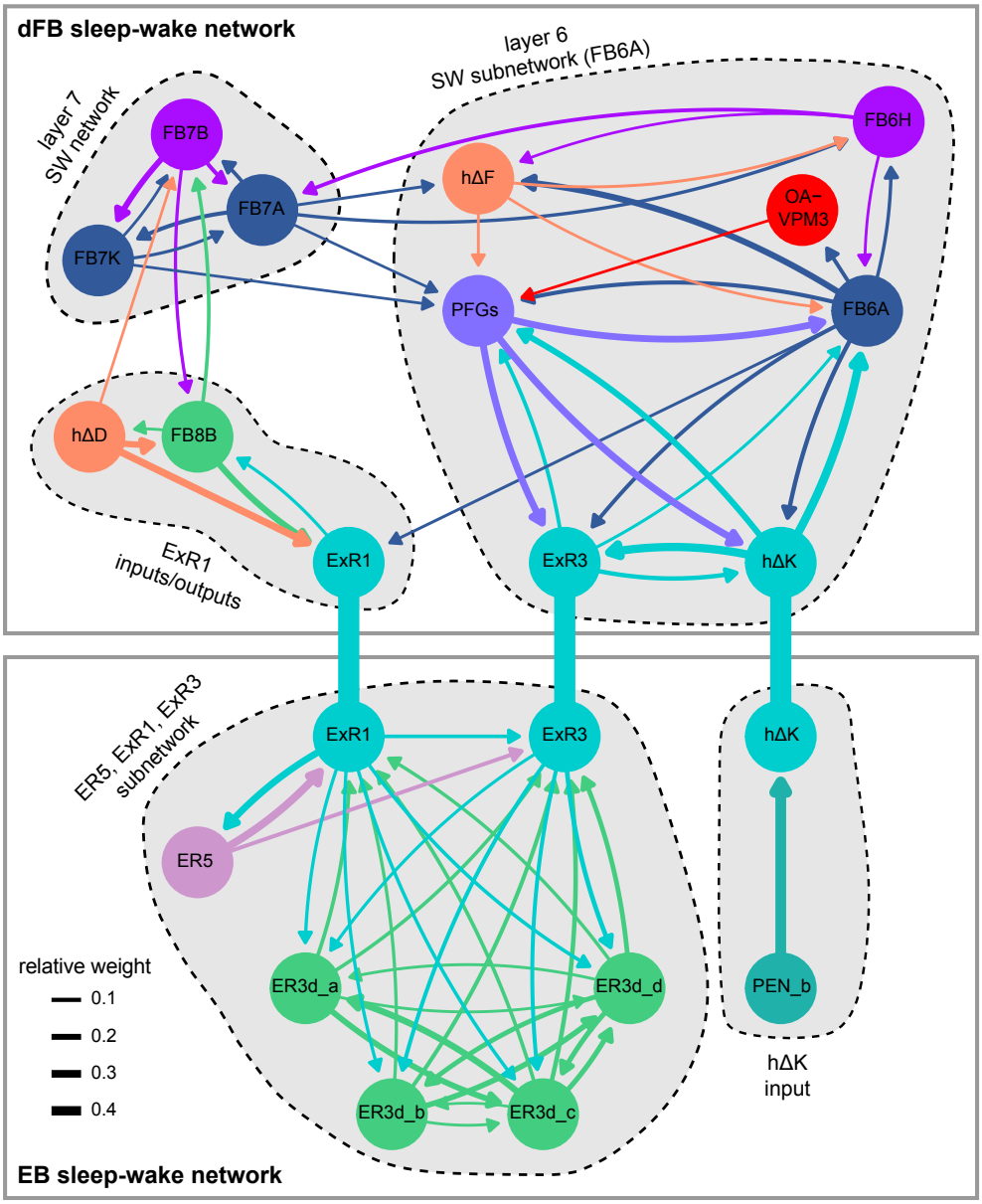
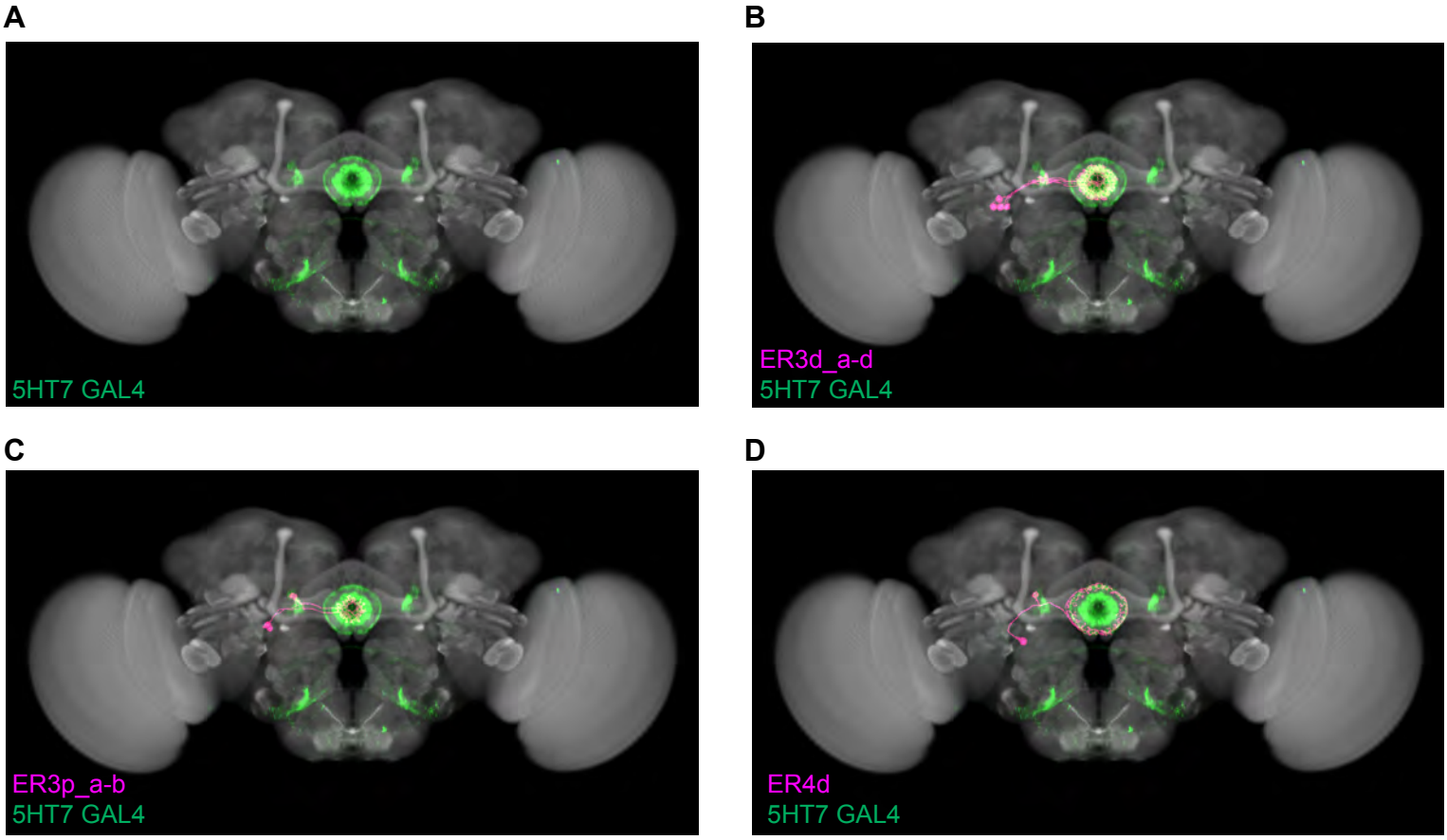


Figure 53: A direct pathway linking sleep-wake neurons in the dFB and EB.

Network graph showing the connections between ExR1, ExR3, and hΔK, along with some of their major upstream and downstream connections in the dFB (top panel) and EB (bottom panel). Note that these circuits are embedded in the highly recurrent dFB and EB networks, whose many neuron types and connections have been omitted for clarity.

Figure 53—figure supplement 1: EB neuron types in 5HT7-GAL4



Sleep Figure 53—figure supplement 1: EB neuron types in 5HT7-GAL4.

A) Front view of a 3D rendering of a confocal stack showing the expression pattern of 5HT7-GAL4, which contains EB neuron types that express the serotonin 5HT7 receptor and receive input from ExR3 (Liu et al., 2019). 5HT7-GAL4 labels neurons in the ER3d, ER3p, and ER4d populations.

B) Morphological rendering of ER3d (a-d) EM morphologies overlaid on 5HT7-GAL4 expression pattern. Individual neurons: ER3d_a (1261086734), ER3d_b (1261427885), ER3d_c (1261419142), ER3d_d (1261423534).

C) Morphological rendering of ER3p (a-b) EM morphologies overlaid on 5HT7-GAL4 expression pattern. Individual neurons: ER3p_a (1229288307) and ER3p_b (1260327246).

D) Morphological rendering of ER4d EM morphologies overlaid on 5HT7-GAL4 expression pattern. Individual neuron: ER4d (1198693217).

Figure 54: CX neurons downstream synapses outside the CX

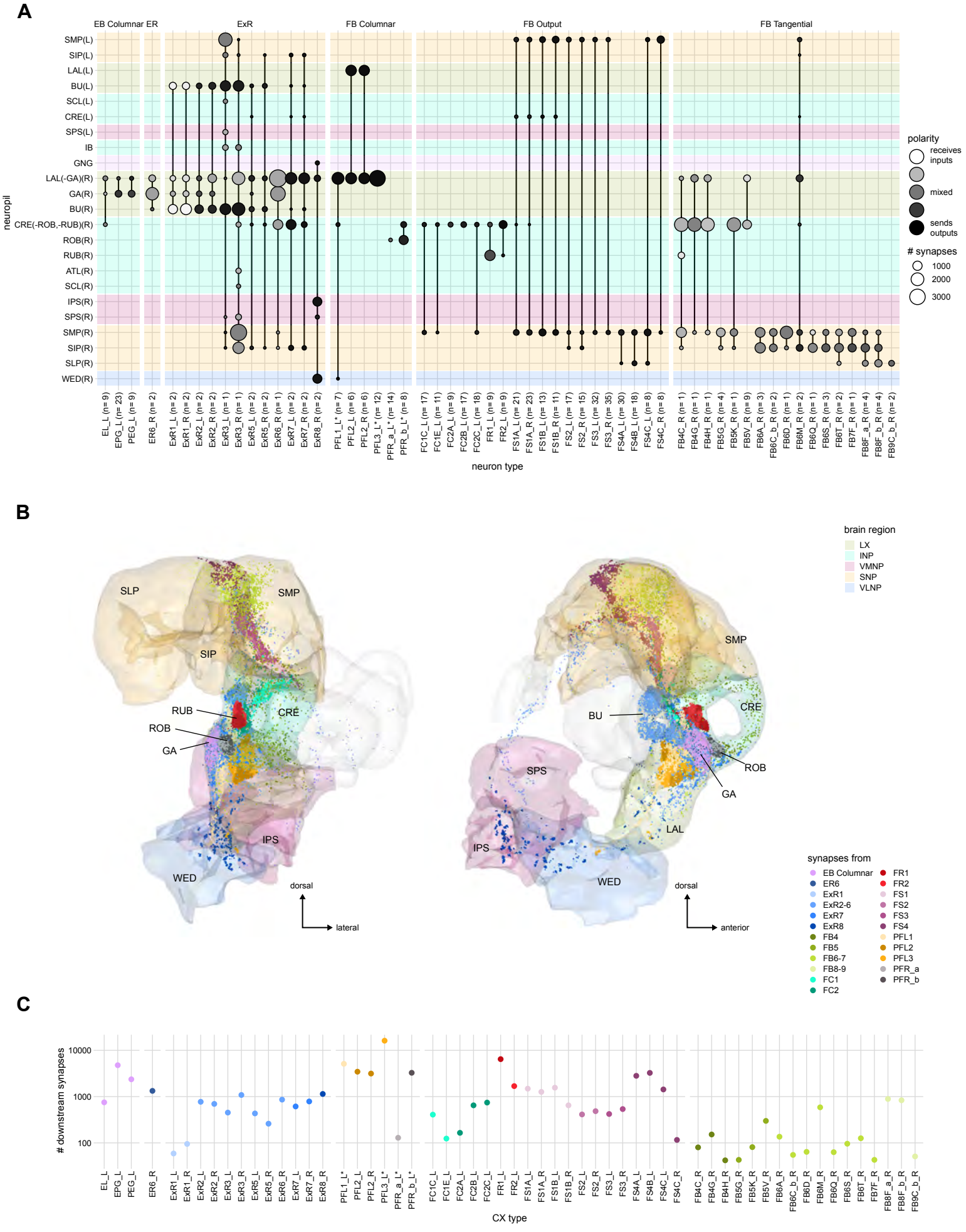


Figure 54: CX neurons with downstream synapses outside the CX

A) Neuropile innervation plot of all CX types having downstream connections outside the central complex. The CX is excluded to highlight the connections in outside neuropiles. The CX innervation of the same neurons can be found in **Figure 10** (EB columnar, ER neurons, ExR neurons), **Figure 28—figure supplement 1** (FB columnar), and **Figure 40** (FB tangentials).

B) Presynaptic site locations outside of the CX in the right hemibrain for the neuron types shown in A. Left: frontal view. Right: side view. The locations are overlaid on an anatomical rendering of the relevant neuropiles. Dot colors indicates the neuron type. The color code for neuropiles is identical in **A** and **B**.

C) The total number of synapses across all significant type-to-type connections outside of the CX in the right hemibrain for each of the types shown in **A** and B**.**

Figure 55: Divergence of output networks

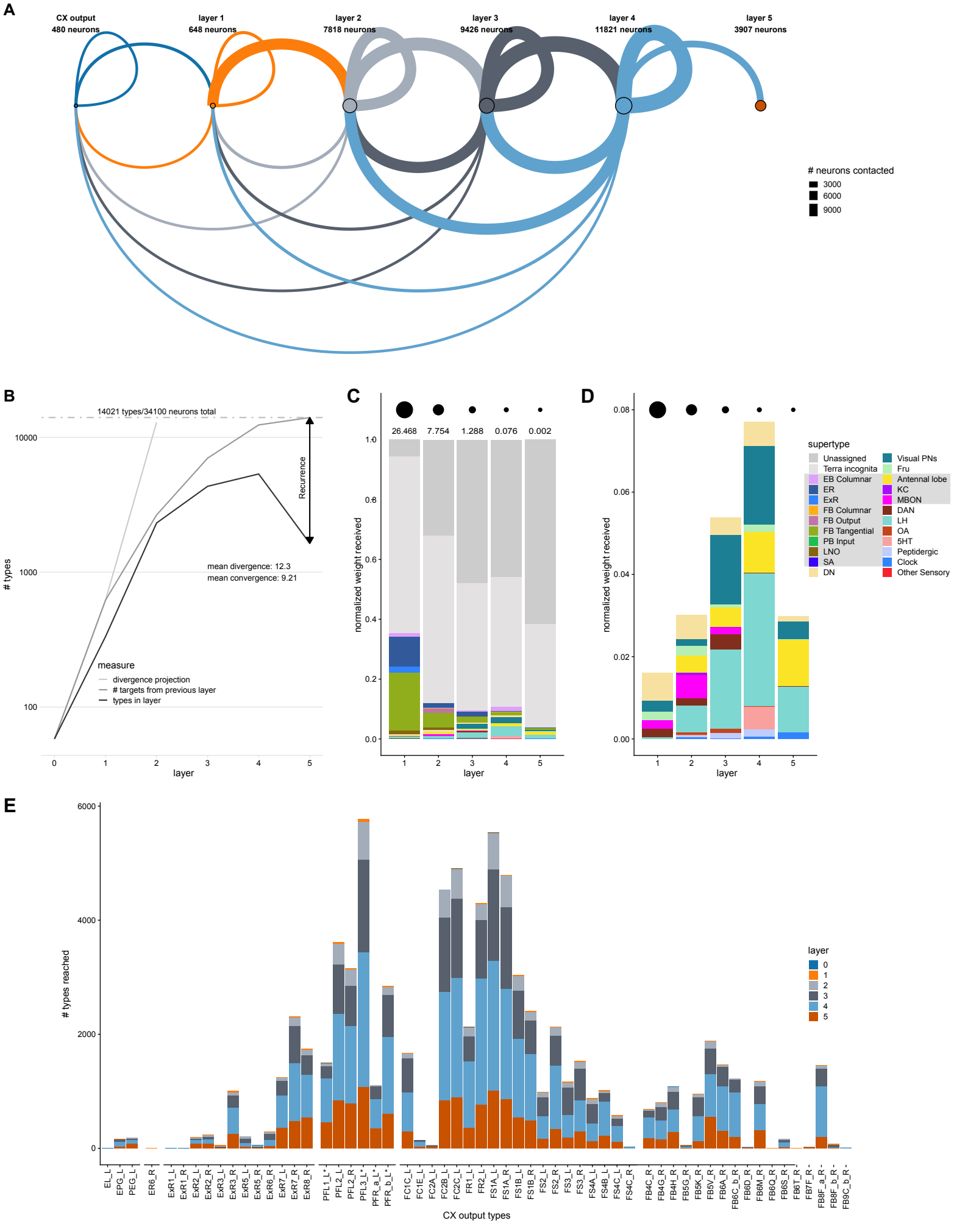


Figure 55: Divergence of output networks

A) Diagram of the number of neurons contacted while walking 5 steps downstream from central complex neurons that arborize outside of the central complex. Size of the circles represents the number of new neurons in each layer. The thickness of the connecting lines indicates the number of neurons reached in the same layer (upper loops), in the next layer (top arc) or in previous layers (bottom arcs). Color of the connector indicates the layer of origin.

B) The number of types per layer (black), the total number of targets of the previous layer (to any layer, dark gray), and the projected number of types from the mean divergence of the previous layer (light gray). The difference between the total number of targets (dark gray) and the number extrapolated from the divergence (light gray) reveals the convergence of the output pathways. The difference between the number of types per layer (black) and the total number of targets (dark gray) reveals the recurrence of the output pathways. On average, each type connects 12.3 other types (divergence of 12.3), and is contacted by 9.21 other types (convergence of 9.21). Note that the totals here exceed the number of neurons in the database as they include simulated pathways on the side of the brain not present in the volume (see Materials and Methods). Of the 34100 neurons reached, 21363 (out of 26190 for the entire dataset) are in the hemibrain dataset, and 12737 are mirror symmetric neurons from existing neurons inferred from symmetric connections.

C) Relative type composition of the different layers weighted by the pathway weight (see Methods and Methods Figure 1) they receive from the CX. Circles on the top row represent the total pathway weight received in every layer. Reflecting the composition of the database, the majority of neurons present either belong to terra incognita or are unidentified. Note that in the first layer, most identified targets are CX types. The total pathway weight decreases as the layer gets farther away from the CX, as is expected with the metric used, which multiplies relative weights across a pathway then sums pathways ending on the same neuron.

D) Same as **C**, but zoomed in onto known types excluding CX types. Types with a gray background in the legend are those for which most existing neurons of that type are present in the database. The fraction of known targets increases to reach a maximum in the fourth layer.

E) Number of types reached outside of the CX for every output CX neuron type (that is, with downstream synapses outside of the CX) in different downstream layers. Note that very small numbers are not visible on this scale.

Figure 56: CX to CX interactions

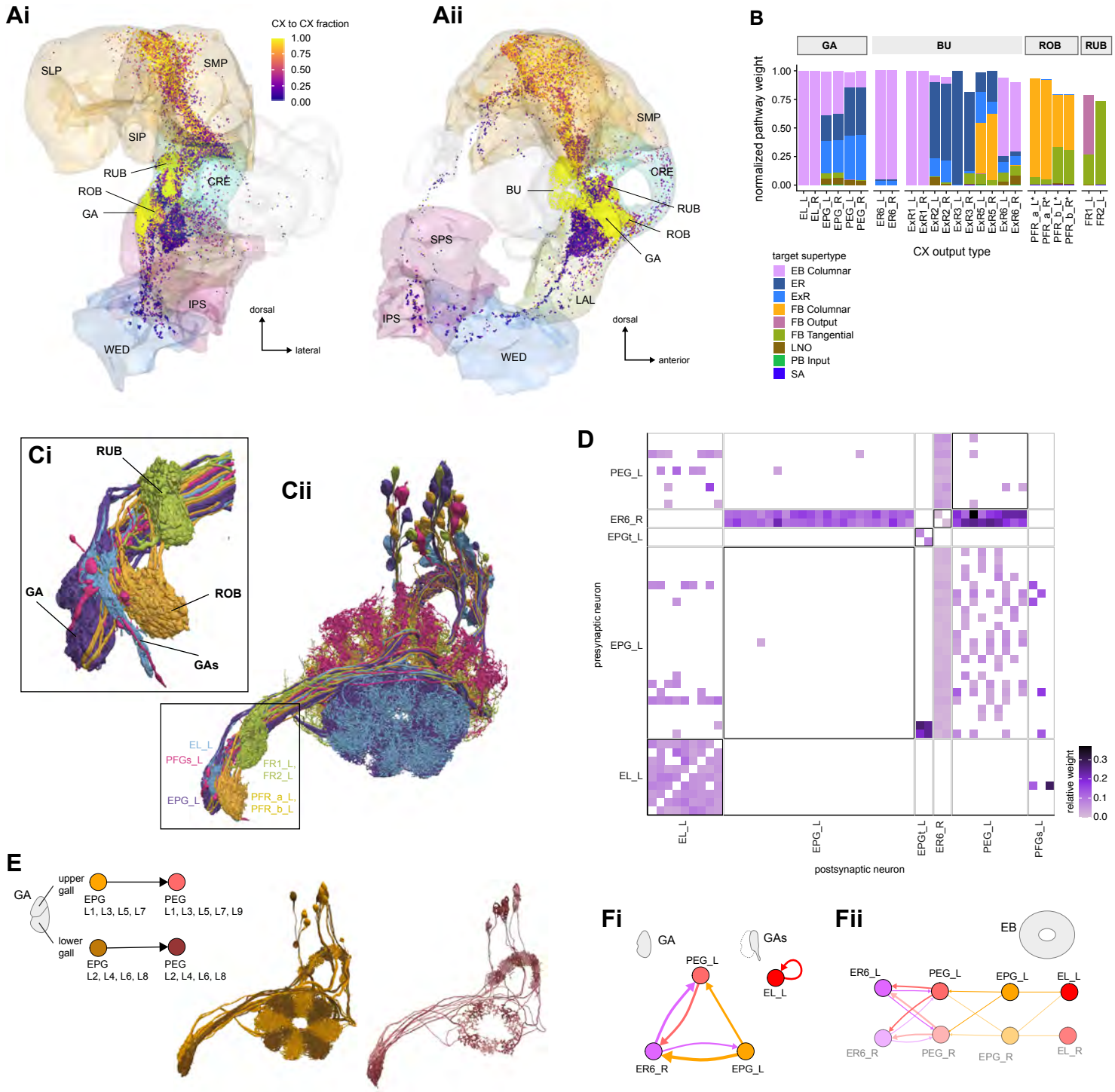


Figure 56: CX to CX connections

A) Downstream synapses of potential CX output neurons, colored by the fraction of their target pathways that contribute to pathways coming back to the CX (see Materials and Methods). The GA, BU, ROB and RUB contribute most of their outputs to the CX and the LAL almost none, while the upper neuropiles are mixed.

B) Pathway weights of all pathways that start in the GA, BU, ROB and RUB and end on another CX neuron. The weights are normalized for each type of origin. If the normalized pathway weight is 1, it corresponds to a neuron for which all output pathways come back to the CX. Connections are separated by the supertypes that these recurrent pathways reach. EB neurons mostly reach other EB neurons whereas FB neurons mostly reach FB tangential neurons (in green).

C) Morphological renderings of 7 selected neuron types that innervate the gall (GA), gall surround (GAs), round body (ROB) and rubus (RUB). **Ci**: Closeup of the four structures. **Cii**: Illustration of the full morphology of the 7 neuron types, showing the left population only.

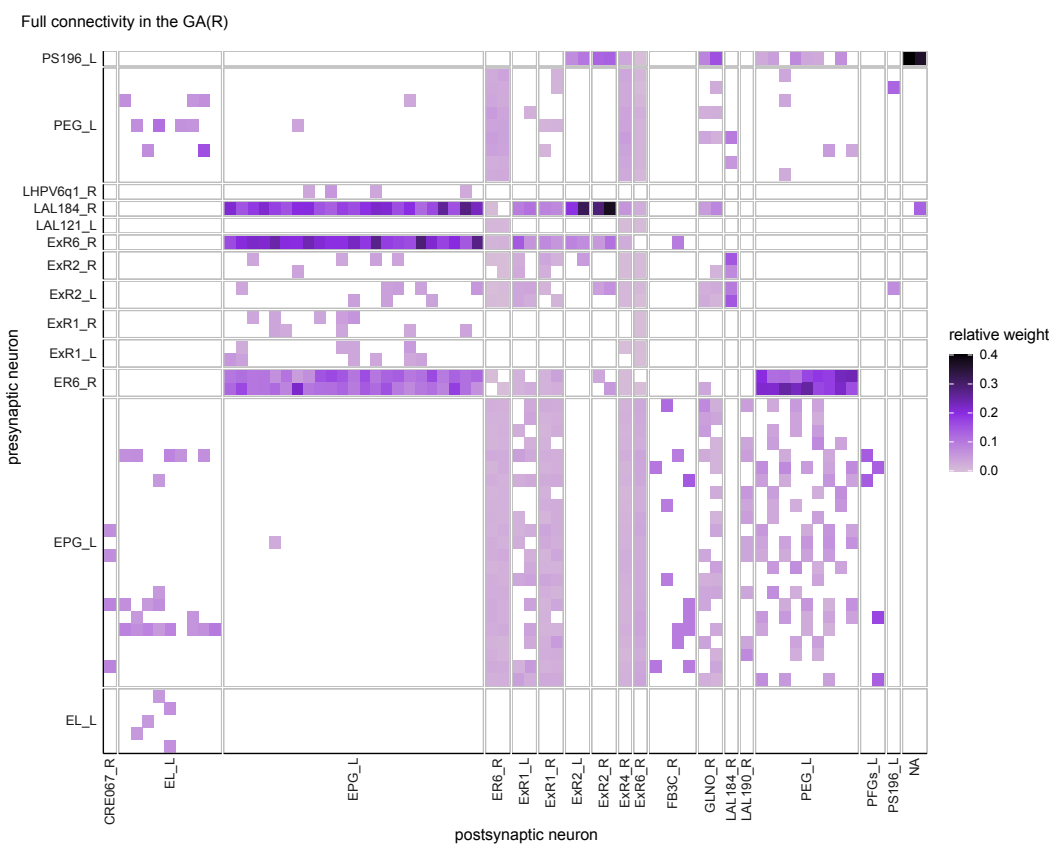
D) Connectivity matrix of neurons that arborize in the right gall and gall-surround region. All connections outside of the CX regions (EB, PB, FB, NO) were considered, because the GA region of interest does not capture the gall surround. PFG neurons were included in the analysis, but did not make any significant connections.

E) Illustration of selective connectivity between EPGs to PEGs from odd and even wedges in the upper and lower gall, respectively. Left: Schematic. Middle: Rendering of EPGs targeting the right gall with those from odd edges colored in orange and those from even edges colored in blue. Right: Rendering of PEGs shown analogously as EPGs.

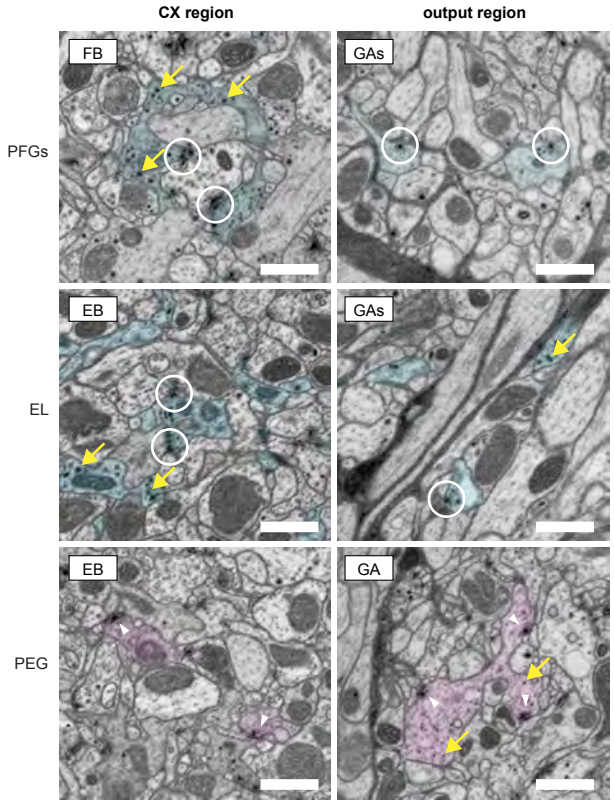
F) Connectivity graphs on the level of neuron types showing any connections with at least 0.05 relative weight. **Fi**: Connectivity in the gall and gall-surround. **Fii**: Connectivity in the EB between the same neuron types as in **Bi**, including the neurons from the other hemisphere.

Figure 56—figure supplement 1: Gall and Gall surround

A



B



C

	FB /EB		OUTPUT REGIONS	
	Synapse	*DCV*	*Synapse*	*DCV*
PFG	non-Tbar	Dark	non-Tbar	Dark
EL	non-Tbar	Dark	non-Tbar	Dark
PEG	Tbar	Dark&Small	Tbar	Dark&Small
FC1A	Tbar	Light	Tbar	Light
FC1B	Tbar	Light	Tbar	Light
FC1C	Tbar	Light	Tbar	Light
FC1D	Tbar	Light	Tbar	Light
FC1E	Tbar	Light	Tbar	Light
FC1F	Tbar	Light	non-Tbar	Light
FC2A	Tbar	Dark	Tbar	Dark
FC2B	Tbar	Dark	Tbar	Dark
FC2C	Tbar	Dark	Tbar	Dark
FC3	Tbar	Dark&Small	Tbar	Dark&Small

Figure 56—figure supplement 1: Gall and Gall surround

A) Neuron-to-neuron connectivity matrix of all neurons that make connections in the GA(R) region.

B) Neuronal profiles of PFGs, EL and PEG neurons in the EM micrographs taken from the FB/EB regions (left panels) and the GA/GAs output terminal regions (right panels). The presynaptic densities that are observed for PFG and EL neurons (circled in white) are not traditional T-bar style synapses, while those in PEG neurons have clear T-bars (white arrowheads). Dense-core vesicles (DCVs) in PFG and EL neurons are larger than those in PEG (yellow arrows). Scale bars: 500 nm.

C) Table summarizing the finding of DCVs and synapse types for various output neurons.

Figure 56—figure supplement 2: Round body

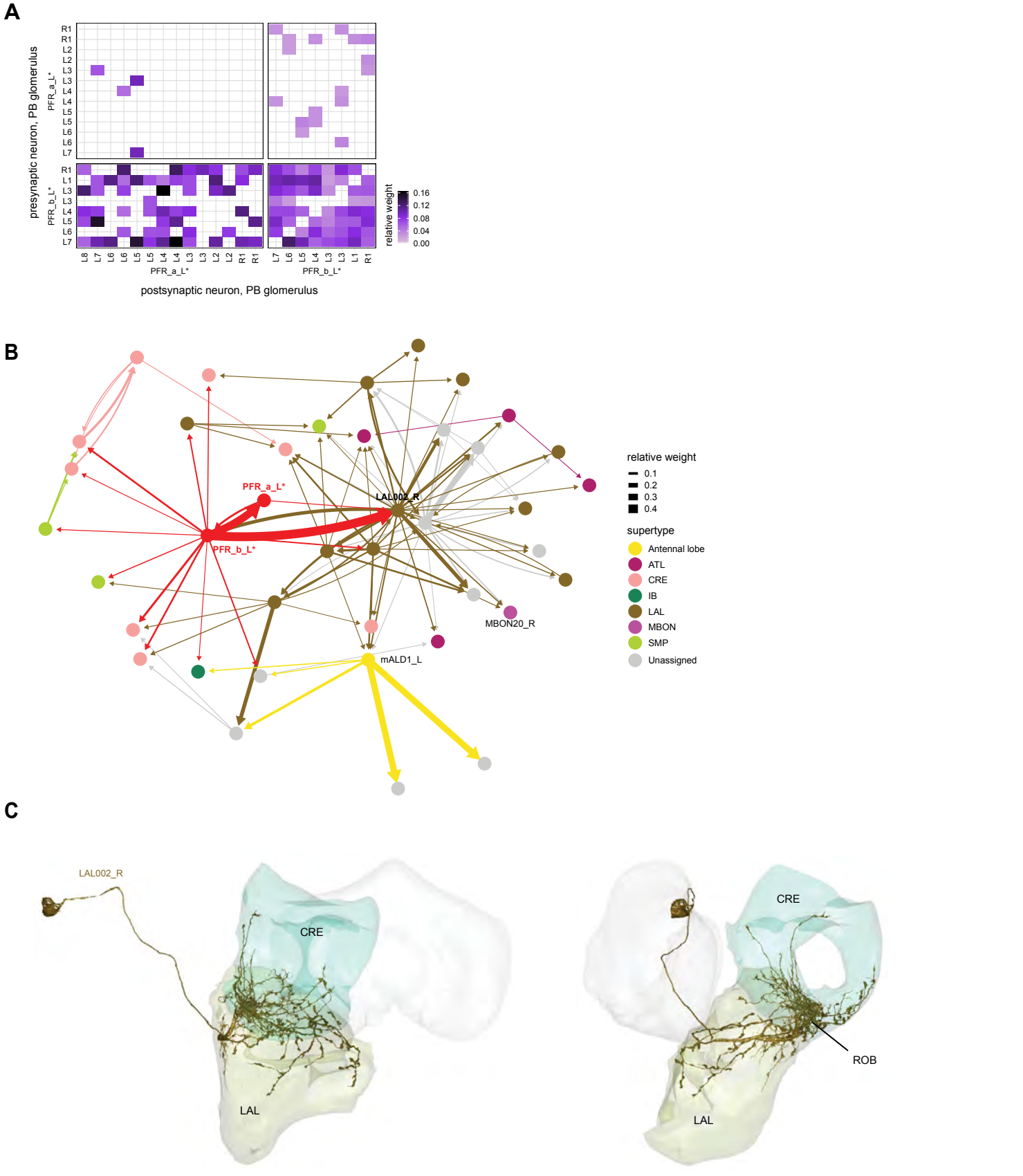


Figure 56—figure supplement 2: Round body

A) Neuron-to-neuron connectivity matrix of PFR to PFR connections in the round body. PFR_b neurons contact both PFR_a neurons and themselves in the ROB in a homogeneous manner.
B) Connectivity graph of output pathways from PFR_a and PFR_b
C) Morphological rendering of LAL002_R

Figure 56—figure supplement 3: FR connectivity in the rubus

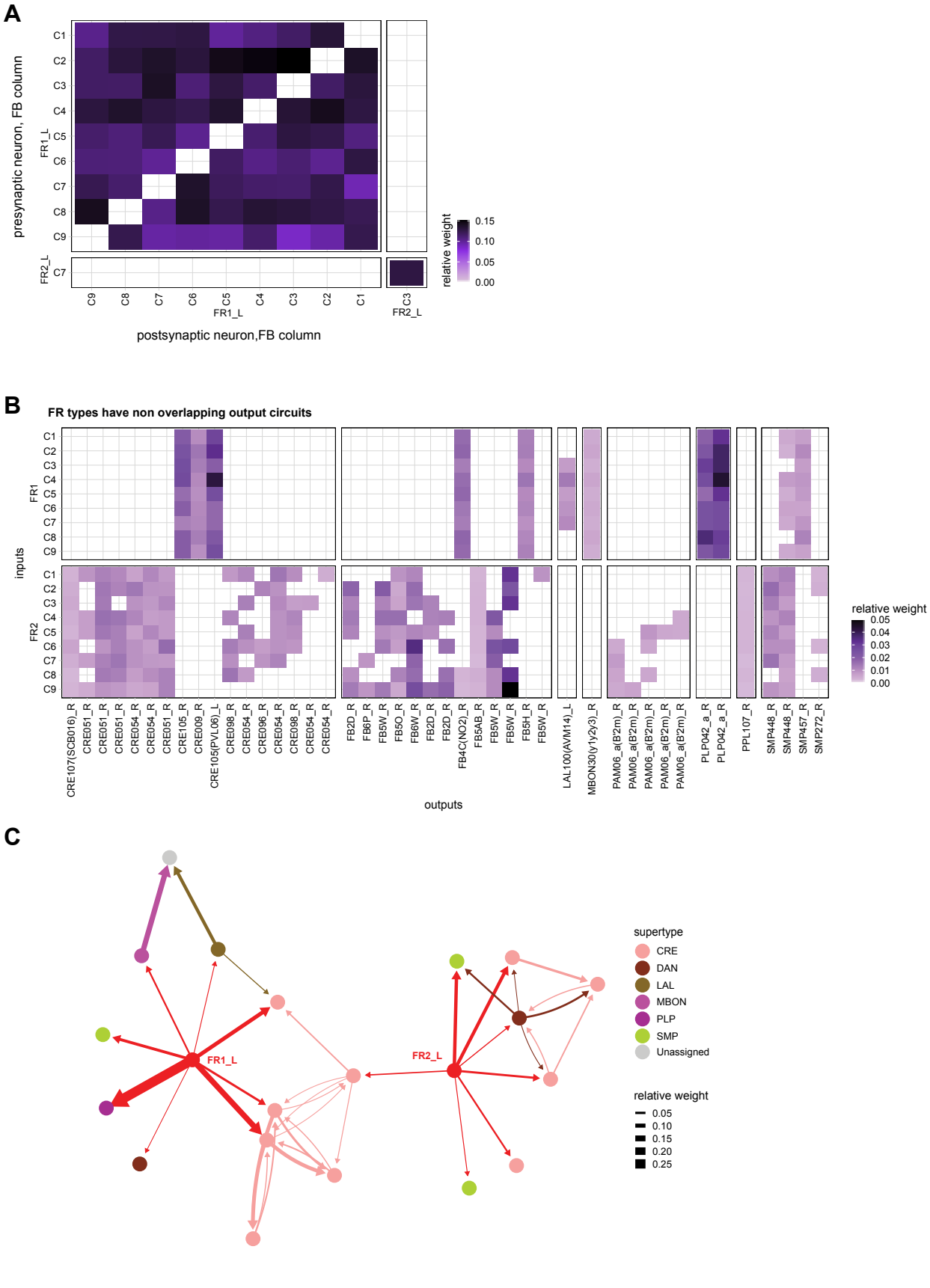
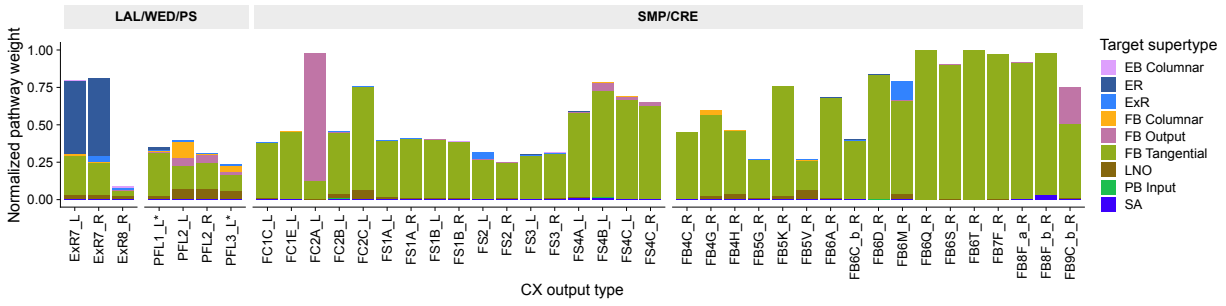


Figure 56—figure supplement 3: FR connectivity in the rubus

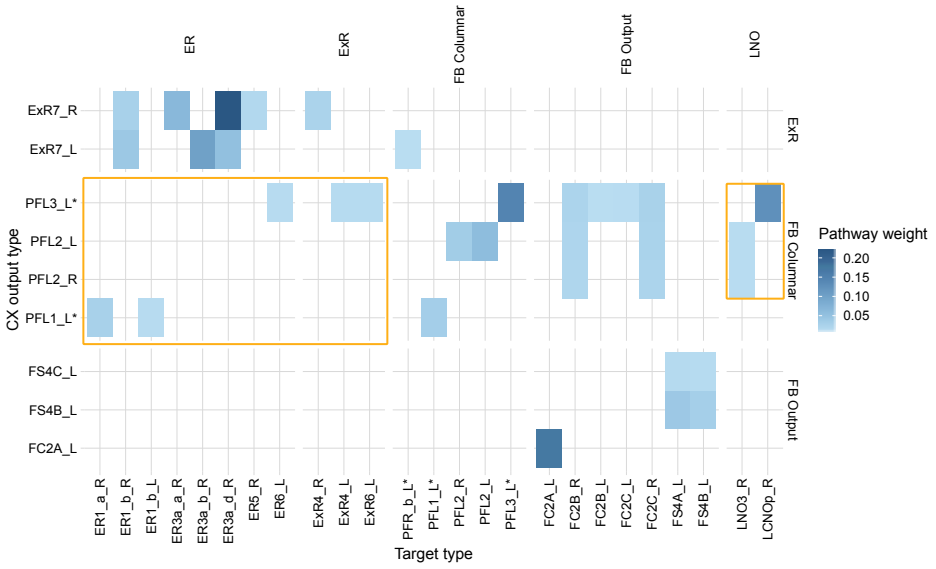
A) Neuron-to-neuron connectivity matrix of FR-to-FR connections in the RUB. FR1 neurons form all to all connections between themselves, FR2 is not involved in direct CX to CX connections in the RUB.
B) Neuron-to-neuron connectivity matrix of non-CX targets of FR neurons in the RUB.
C) Connectivity graph of output pathways from FR1 and FR2.

Figure 57: Other CX to CX connections

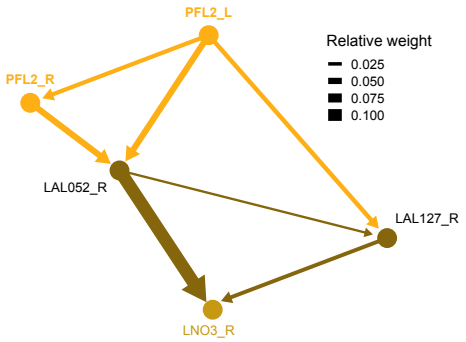
A



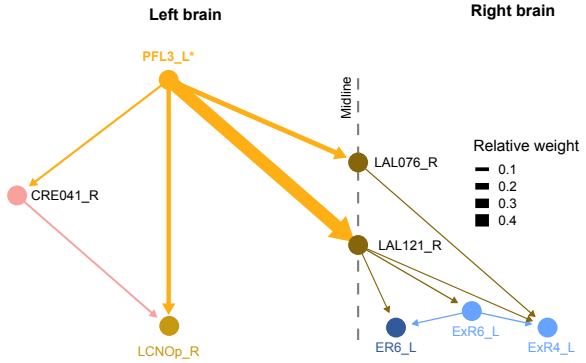
B



Ci



Cii



Ciii

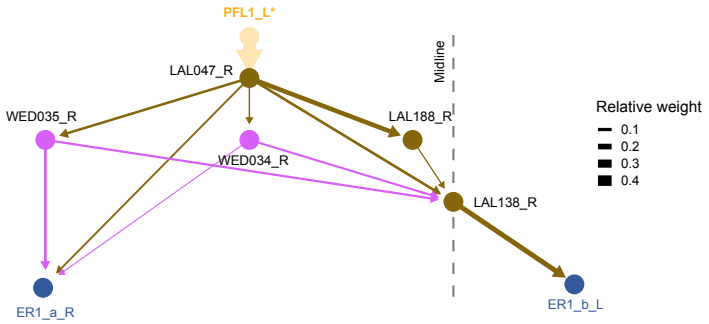


Figure 57: Other CX to CX connections

A) Pathway weights (see **Methods Figure 1**) of pathways that end on another CX neuron for neurons innervating the neuropiles contributing the less CX to CX pathways, LAL, WED, PS, CRE and SMP . The weights are normalized for each type of origin (see Materials and Methods). The connections are separated by the supertypes that these recurrent pathways reach.

B) Pathway connectivity matrix of those same neuron, excluding the FB tangential neurons. Connections from the FB to EB and NO neurons are highlighted.

C) Pathways from PFL neurons to EB and NO neurons.
Ci PFL2 reaches LNO3 in 2 steps.
Cii PFL3 connects ipsilaterally to LCNOp and contralaterally, through midline crossing LAL interneurons, to ER6, ExR6 and ExR4.
Ciii PFL1, reaches through a multilayered network, ER1_a ipsilaterally and ER1_b contralaterally.

Figure 57—figure supplement 1

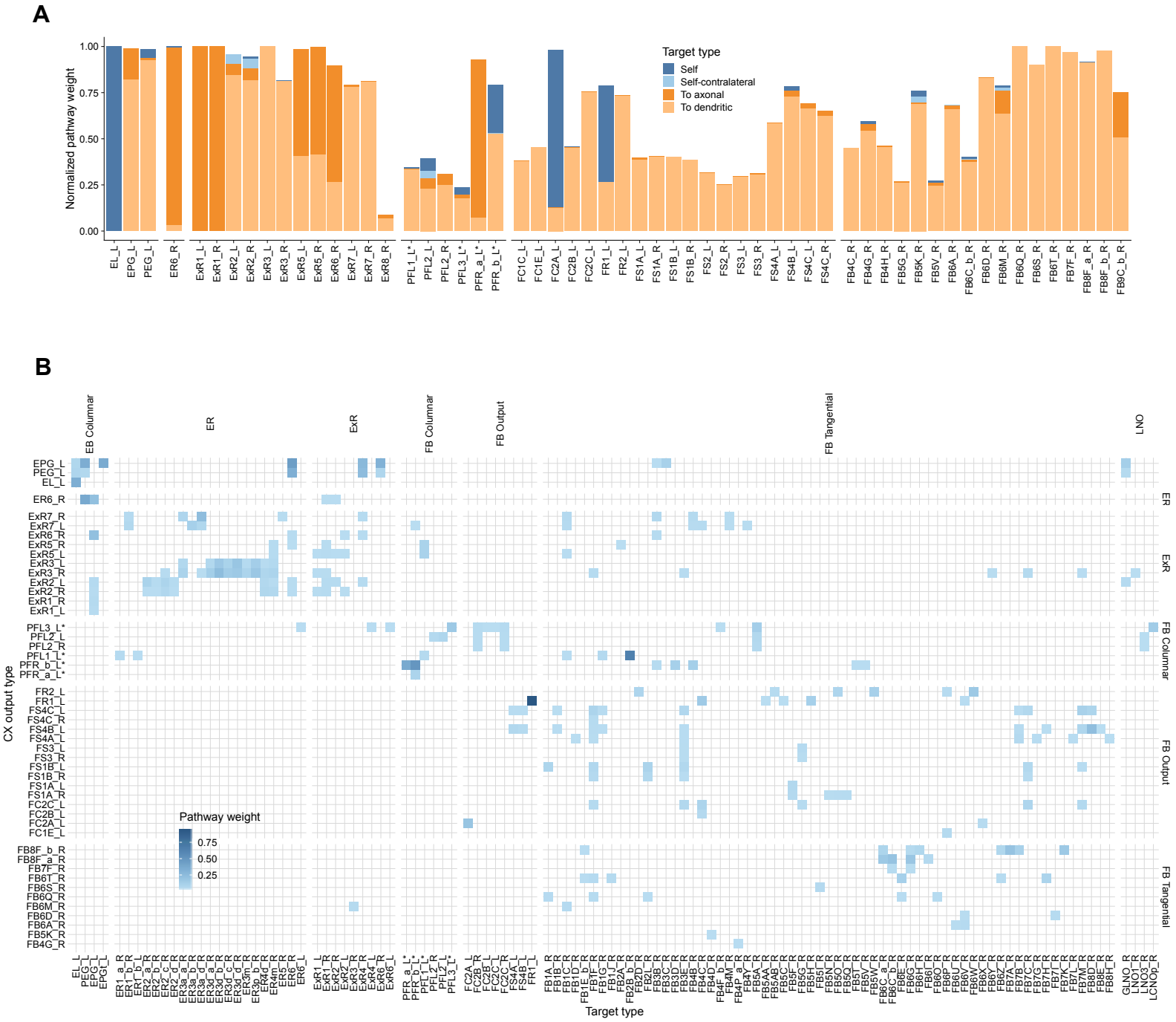


Figure 57—figure supplement 1: All CX-to-CX connections

A) Pathway weights (see **Methods Figure 1**) of all pathways that end on another CX neuron. The weights are normalized for each type of origin (see Materials and Methods). If the normalized pathway weight is 1, it corresponds to a neuron for which all output pathways come back to the CX. TConnections are separated between axonal, dendritic, self, and self-contralateral. “Self” pathways end on the same neuron they started with, whereas “self-contralateral” pathways end on the mirror symmetric starting neuron. “Axonal” pathways end on a neuron for which more than 75% of the synapses located outside of the CX are output synapses.

B) Connectivity matrix of all CX-to-CX pathways outside of the CX, filtered for pathway weights larger than 0.5%.

Figure 58: CX to CX motifs

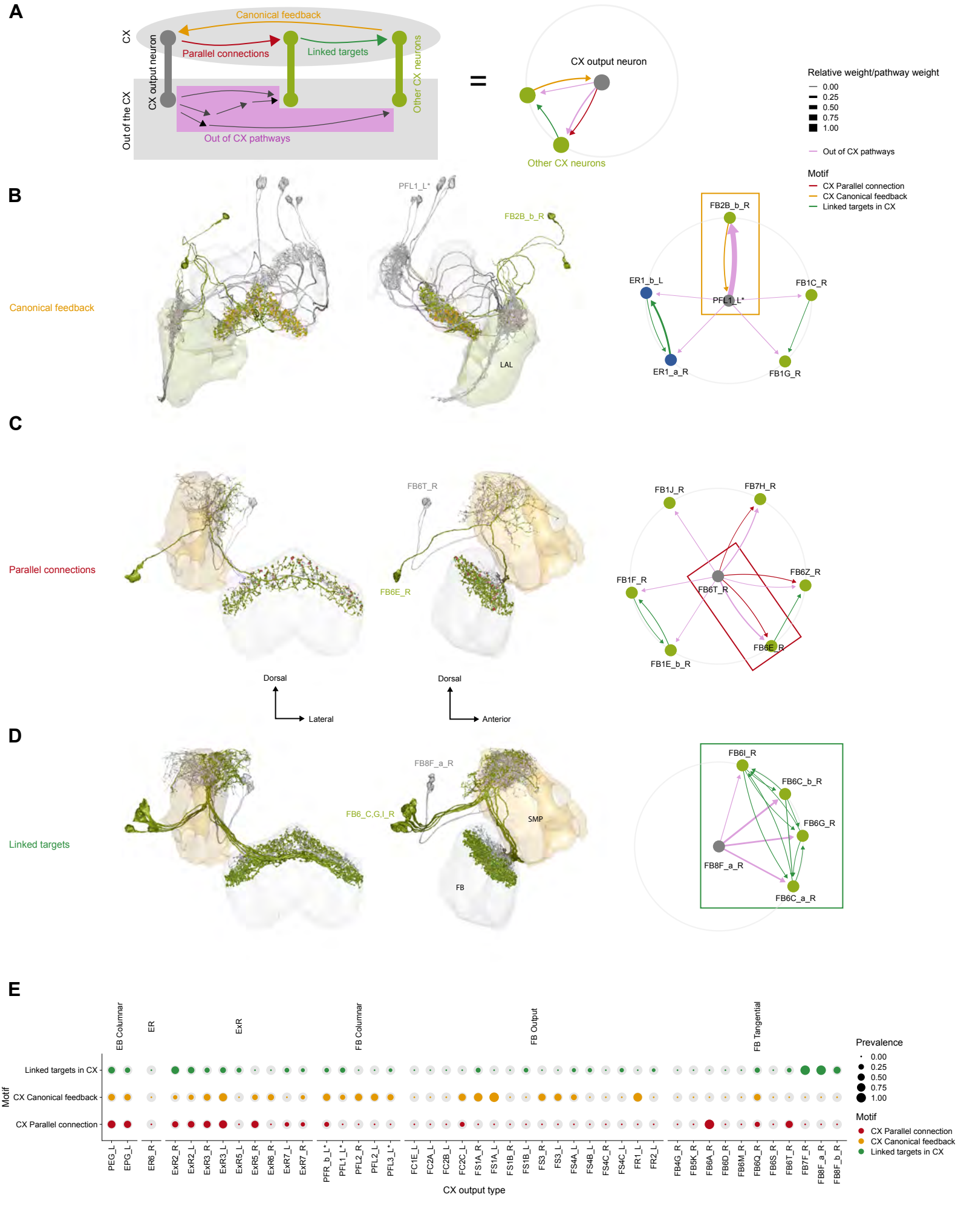


Figure 58: CX to CX motifs

A) Schematic of the 3 motifs considered (left) and their equivalent representation in a compact circular network plot (right). The CX output type of interest is in gray on the left and at the center of the circular diagram. It reaches other CX neurons (in green) through pathways that can be constituted of multiple steps (in pink). Motifs are formed by the relation between those pathways outside of the CX and the connections formed inside of the CX by the same CX neurons. "Canonical feedback" corresponds to the target of the pathway contacting the source type in the CX (in yellow). "Parallel connections" occur when the source neurons also contact the pathway target neuron inside the CX (in red). "Linked targets" are neurons connected in the CX that are targets of the same neuron outside of the CX (in green).

B) **B-D** Example motifs. Left: Frontal and lateral anatomical renderings. Right: circular motif graph showing the motif in the context of all the CX to CX motifs the type of origin is implicated in. **B** FB2B_b forms a canonical feedback loop with PFL1 neurons: PFL1 contacts FB2B_b in the LAL while FB2B_b contacts PFL1 in the FB.

C) Parallel connections: FB6T contacts FB6E both in the SIP/SMP and FB.

D) Linked targets: FB8F_a contacts four FB6 neurons who are themselves interconnected in the FB.

E) Prevalence of the 3 motifs for all the potential CX output types. The colored circles represent the prevalence of each specific motif. The gray circles represent the total number of all the motifs of the same type that could form given that type's partners outside of the CX. EB columnars, ExR2s, and ExR3s form a large proportion of all the possible motifs, reflecting the high level of recurrence in EB circuits.

Figure 59: Feedforward output networks

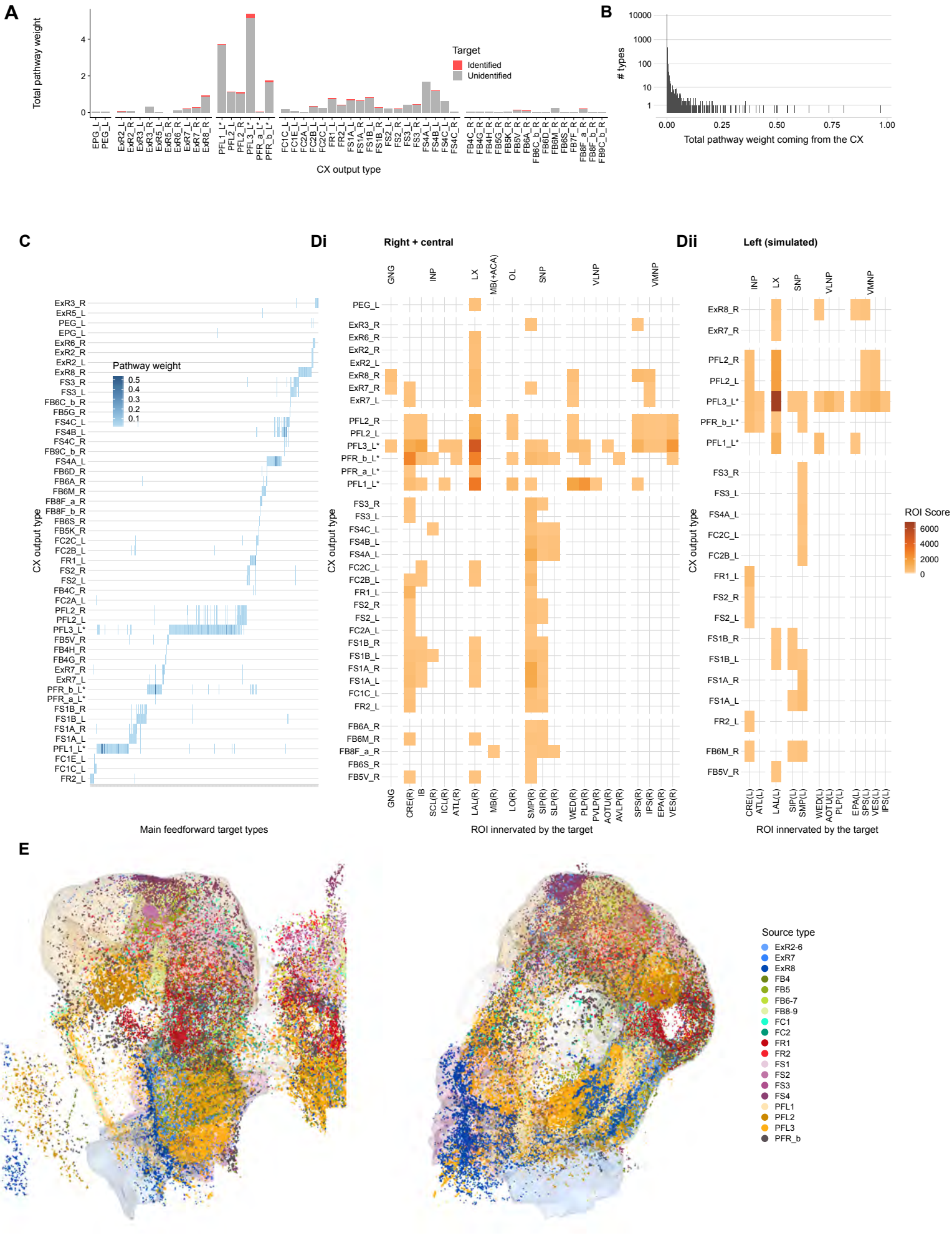


Figure 59: Feedforward output networks

A) Total pathway weights contributed by the different CX output neurons (summed over all neurons in the graph, see Materials and Methods and **Methods Figure 1**). Color indicates if the receiving types are identified or not (unidentified means that they are part of the *terra incognita* neurons or utterly unknown). Compare to **Figure 54C**: some types (for example, FRs,FSs) reach a large number of neurons but have much lower pathway weights than other prominent types (for example, PFLs). This arises because these types only make modest contributions to their targets.

B) Histogram of total pathway weight received from the CX for every downstream type. Most neurons receive very weak inputs from the CX.

C) Connectivity matrix from the CX to every type in the graph receiving more than 0.5% total pathway weight (filtered for individual weights>0.5%). The CX output types are ordered according to the similarity of their output vectors (see Materials and Methods) Most important targets are influenced mainly by a single CX type. PFL3 neurons contact more types than any other CX type.

D) Downstream neuropile innervation of the targets of CX output neurons, starting on the right side of the brain. The innervations are weighted by the pathway weight they receive. The ROI score is the sum of pathway weights for all the targets in the ROI times the number of downstream synapses in the same ROI.

Di Innervation measured (right and central neuropiles).

Dii Innervation simulated (left neuropiles) from the known symmetric types. This is necessarily an underestimate of the extent to which the CX pathways reach the contralateral side of the brain.

E) Downstream synapses of targets receiving more than 0.5% of pathway weight from the CX, colored by their main contributor.

Figure 59—figure supplement 1: Clustering at different dephts

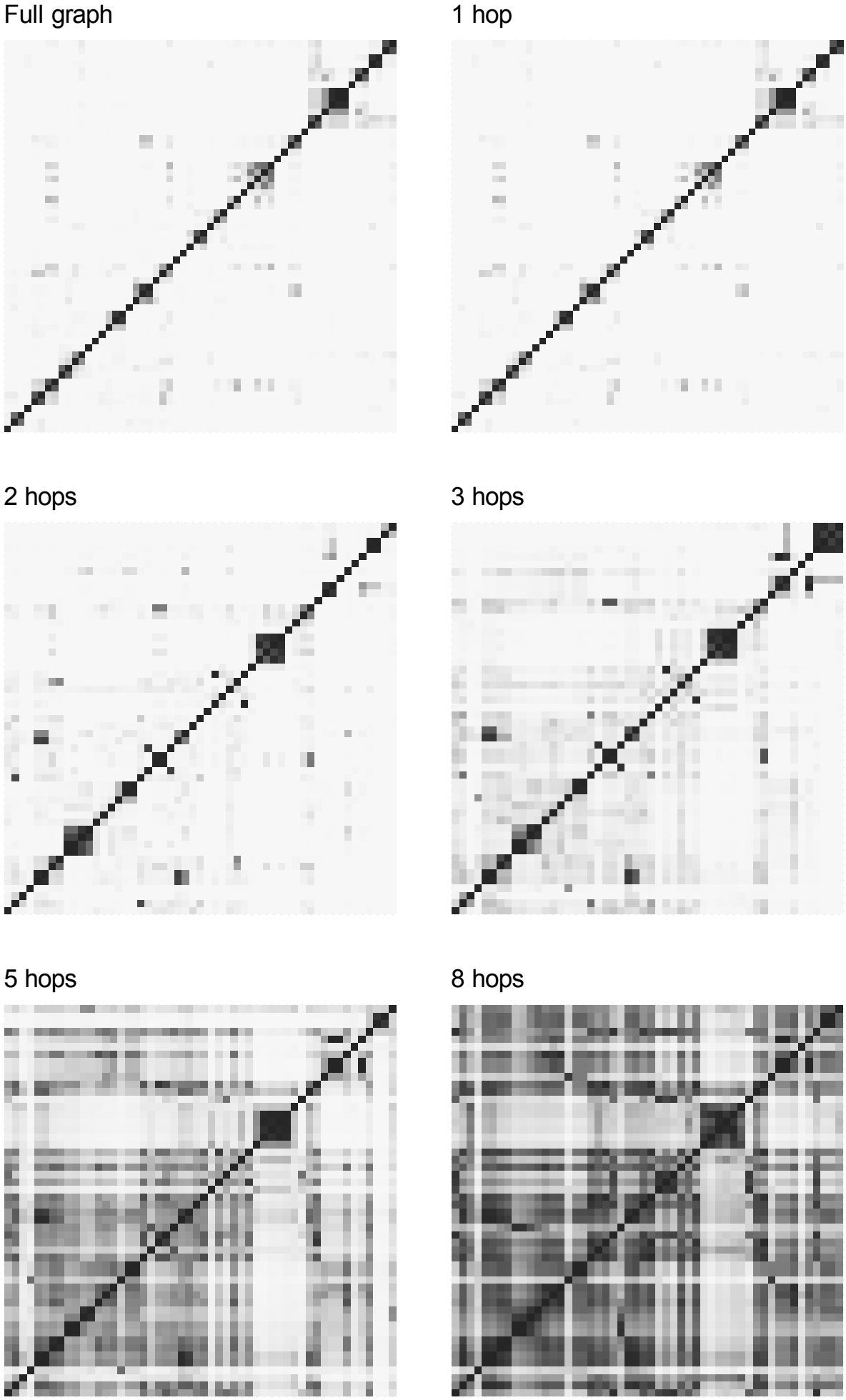


Figure 59—figure supplement 1: Clustering at different dephts

The cosine distances were computed for all connections at a given pathway length (labeled at top). Modularity is strong at the onset (and for the full graph as the short paths dominate) and remains strong relatively deep in the network. CX outputs were clustered on their full connectivity profile (the pathway weights they contribute to every neuron in the network). For all panels the ordering is the clustering order obtained on the full pathway weight distances.

Figure 59—figure supplement 2: modularity of output networks

Figure 59—figure supplement 2: Modularity of output networks

A) The full graph of all strong feedforward targets of the CX, in a stress minimizing layout (see Methods). **A** Nodes are colored by their main CX contributor.

B) Nodes are colored by the results of the label propagation community detection algorithm (see Methods). Neurons that receive their main input from the same CX neuron tend to form communities (sets of nodes that are more connected between themselves than they are with the rest of the network).

C) Average connection strength between two neurons as a function of their main CX contributor (filtered for average connections larger than 0.1%). Connections between neurons that share their main CX input (on the diagonal) tend to be stronger.

Figure 59—figure supplement 3: Same as Figure 54B and Figure 59E, for PFL or FS neurons alone

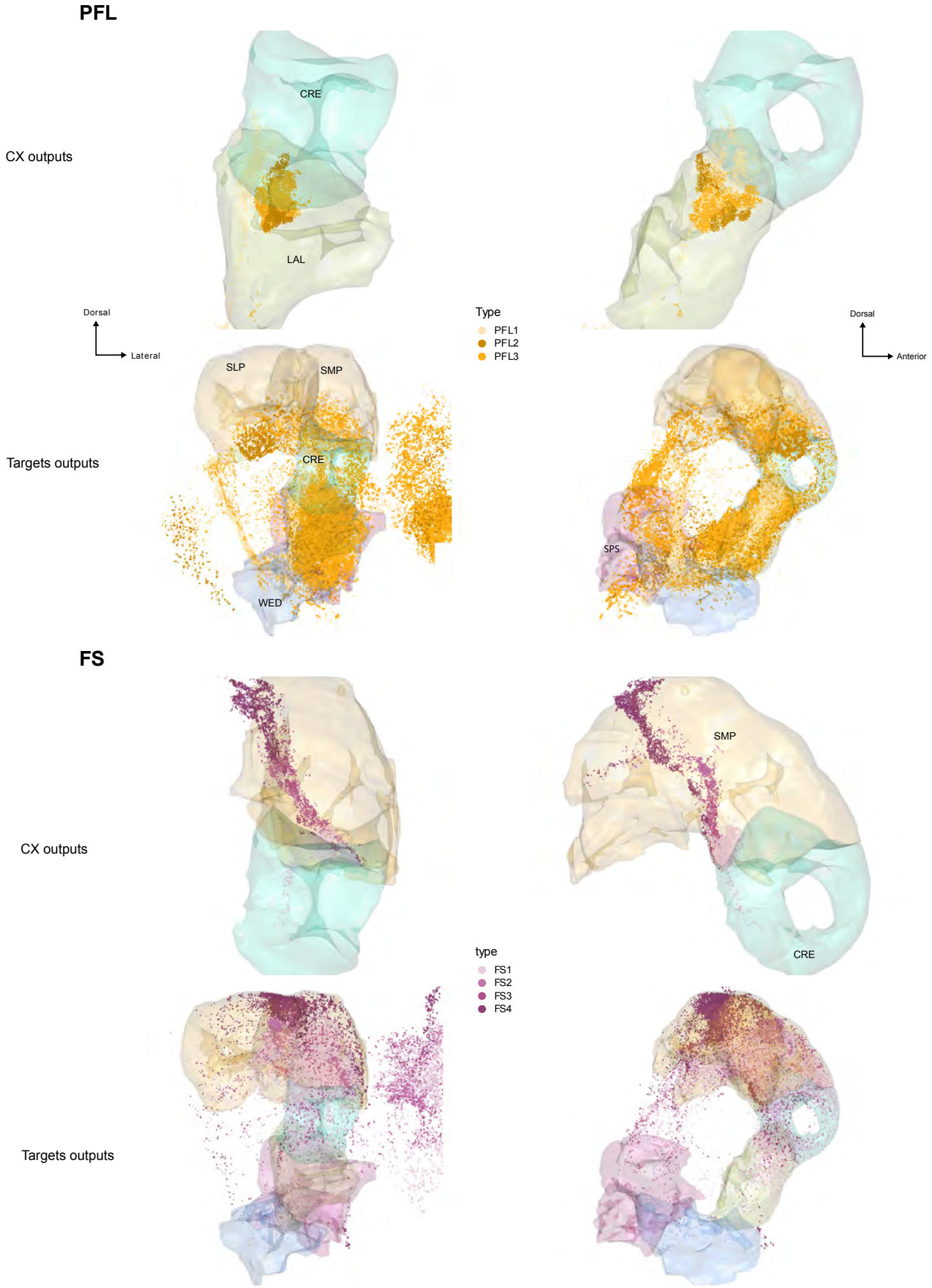


Figure 59—figure supplement 3: Same as Figure 54B and Figure 59E, for PFL or FS neurons alone.

Figure 59—figure supplement 4: Same as Figure 54B and Figure 59E, for FC or FR neurons alone

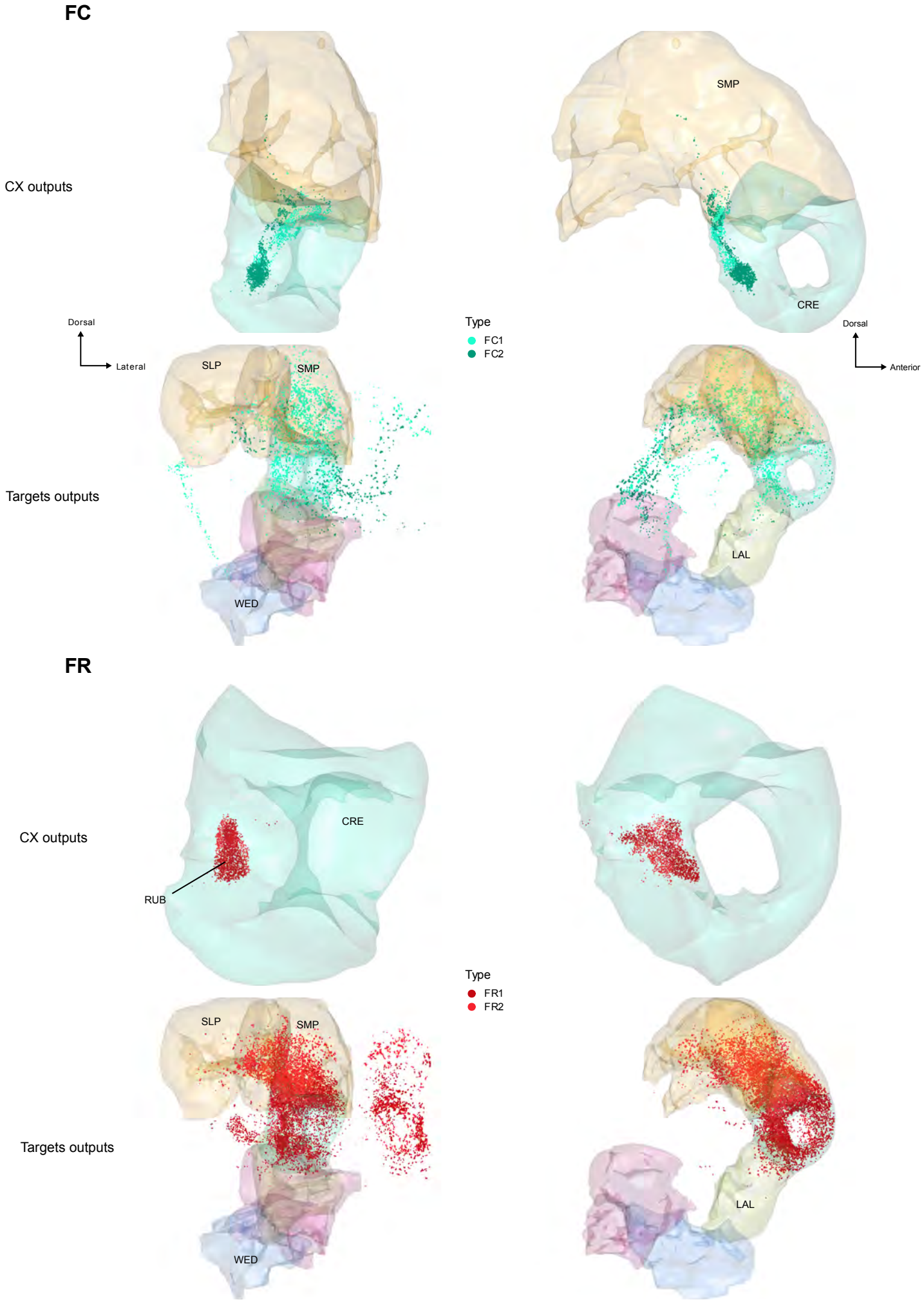


Figure 59—figure supplement 4: Same as Figure 54B and Figure 59E, for FC or FR neurons alone.

Figure 59—figure supplement 5: Same as Figure 54B and Figure 59E, for PFR or ExR neurons alone

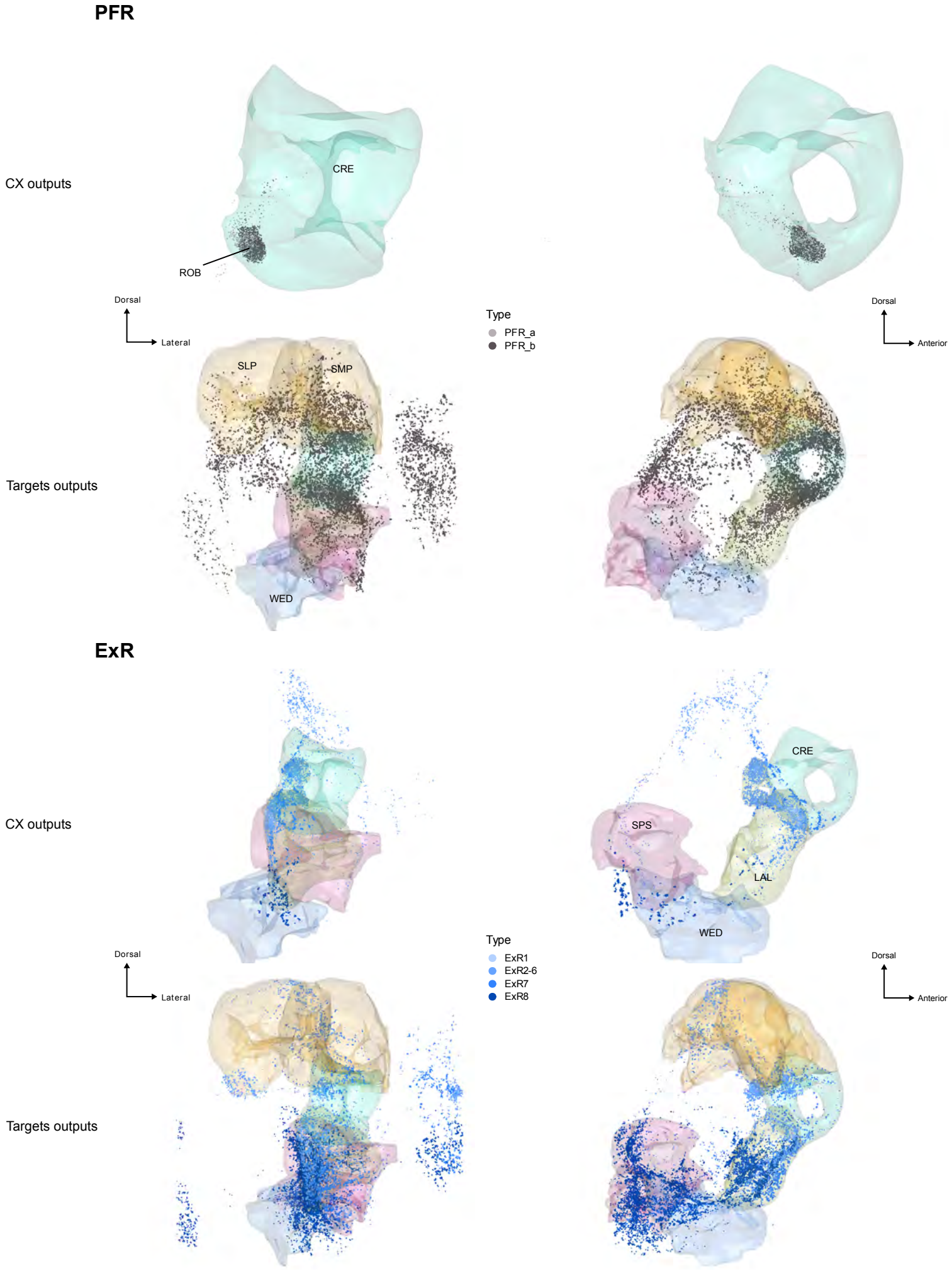


Figure 59—figure supplement 5: Same as Figure 54B and Figure 59E, for PFR or ExR neurons alone.

Figure 60: Connections to identified types

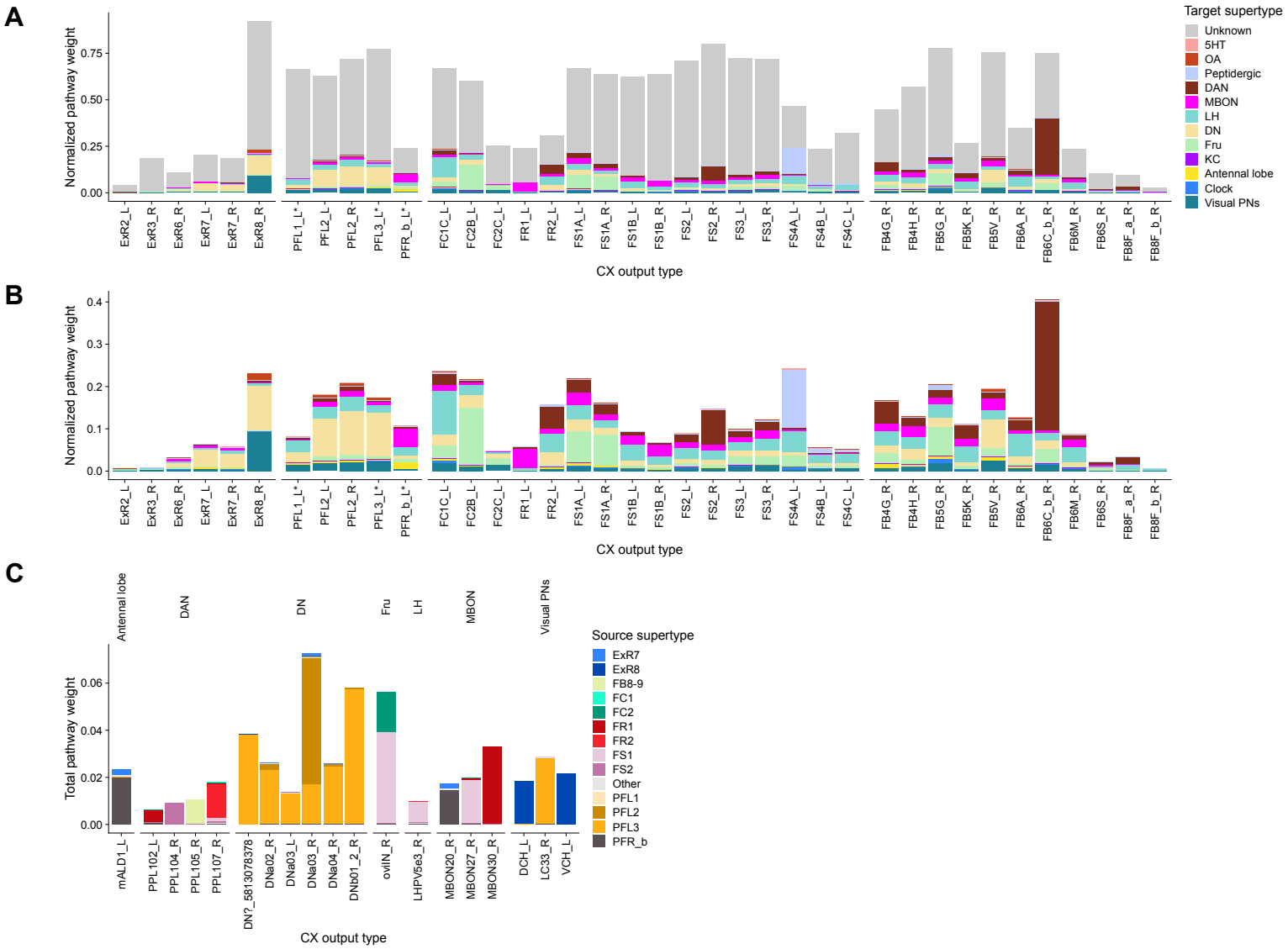


Figure 60: Connections to identified types

A) Normalized pathway weights (see **Methods Figure 1** and Materials and Methods) of pathways that end on various known types. Only CX neurons that contribute at least 0.5% of their outputs to feedforward networks are included. “Unknowns” correspond to pathways that never reach a known type.

B) Same as **A**, but zoomed in on the known types.

C) Pathway weights from the CX received by the most prominent known targets (getting at least 0.5% of their inputs from CX pathways), colored by the CX types of origin.

Figure 61: Connections to MBONs, dopaminergic and antennal lobe neurons

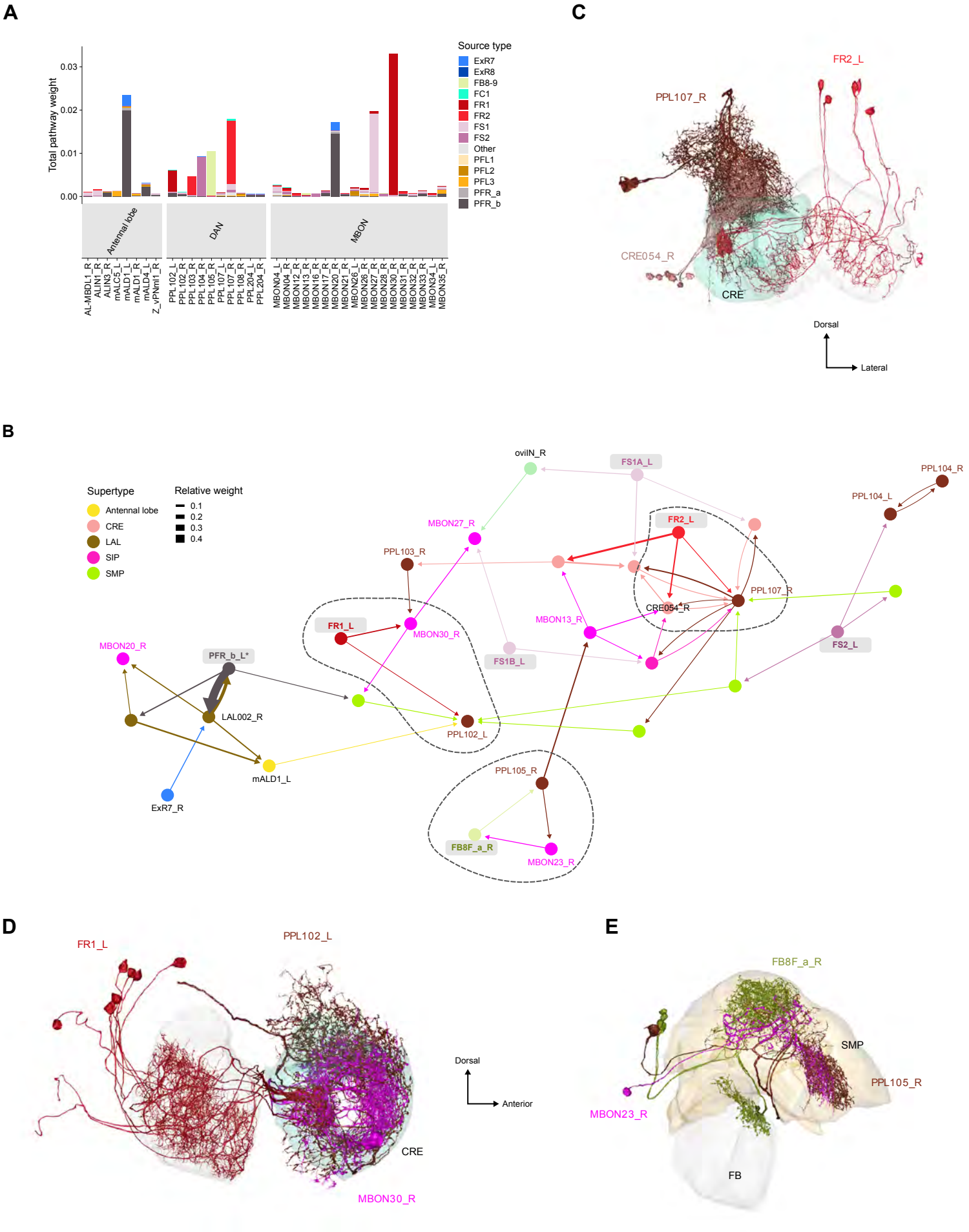


Figure 61: Connections to MBONs, dopaminergic and antennal lobe neurons

A) Pathway weights (see **Methods Figure 1**) from the CX to MBONs, dopaminergic (PPL DANs) and antennal lobe neurons for which the total pathway weight is superior to 0.05%, colored by the CX type of origin.

B) Network diagram showing the interconnections between those pathways. Circled areas correspond to the morphological renderings in **C**, **D**, and **E**. CX neuron have a grey background label. The LAL 002 neuron is a small neuron that receives a large fraction of its input from PFR_b via very targeted innervation of the ROB. Note that the LAL002 neuron also receives some input from ExR7 neurons. The PFR_b/LAL002 pathway also feeds the mALD1 neuron, which sends projections to a large number of regions across the brain. This giant neuron collects much of its input in the LAL and CRE, among other regions, and makes presynaptic contacts in many regions across the protocerebrum, including to the main MBON/PPL CX output network via PPL102. Its function is unknown.

C) FR1 to PPL102 and MBON30, anatomical rendering.

D) FR2 to PPL107 and CRE054, anatomical rendering.

E) FB8F_a/MBON23/PPL105 loop, anatomical rendering

Figure 61—figure supplement 1: Main circuits converging onto oviIN and MBON27

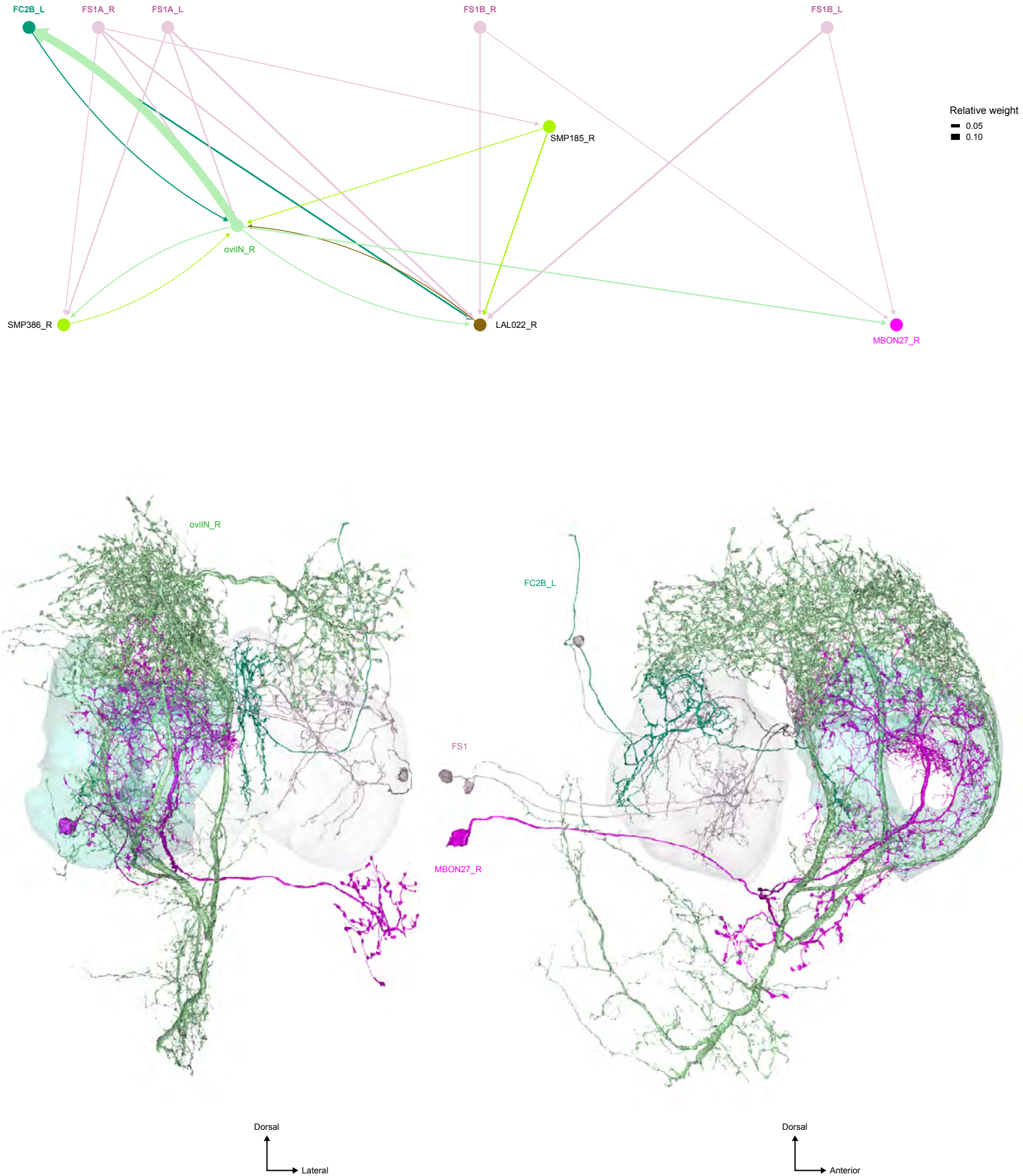


Figure 61—figure supplement 1: Main circuits converging onto oviIN and MBON27.

A) Network diagram showing the FS and FC connections to oviIN and MBON27.
B) Anatomical rendering of example FS and FC neurons, oviIN and MBON27.

Figure 62: connections to visual projection neurons

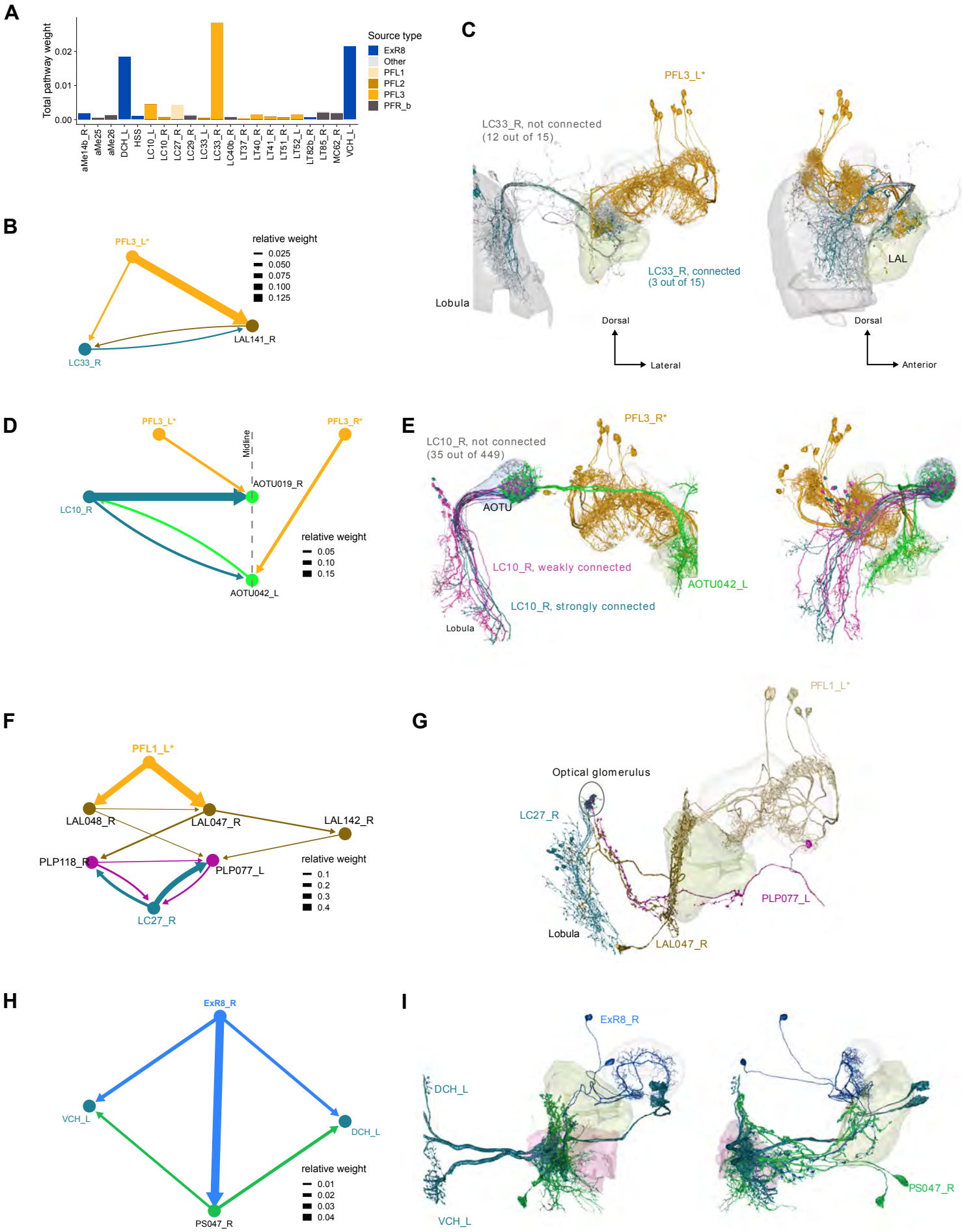


Figure 62: Connections to visual projection neurons.

A) Total pathway weights (see Outputs Figure 2 – SI 1) from the CX to vPNs for which the total pathway weight is greater than 0.05%, colored by the CX type of origin.

B) **B,C** PFL3 interacts with a subpopulation of LC33 neurons. **B** Network diagram. PFL3 synapses onto LC33 in the LAL both directly and through one of its strong targets (LAL141).

C) Morphological rendering showing which subset of LC33 neurons is associated with PFL3 neurons.

D) **D,E** PFL3 interacts with LC10 neurons through AOTU-LAL neurons. **D** Network diagram showing how PFL3 neurons interact with LC10 neurons both ipsilaterally and contralaterally, through AOTU-LAL neurons. The midline is denoted by the vertical dotted line.

E) Morphological rendering showing that PFL3 interacts with LC10 along a dorso ventral axis in the AOTU, corresponding to the antero-posterior axis in the lobula. Connections are stronger on the ventral side of the AOTU, corresponding to LC10 neurons innervating the posterior part of the lobula.

F) **F,G** PFL1 interacts with LC27 neurons. **F** Network diagram showing that the connection is through two layers constituted by LAL and PLP neurons, respectively.

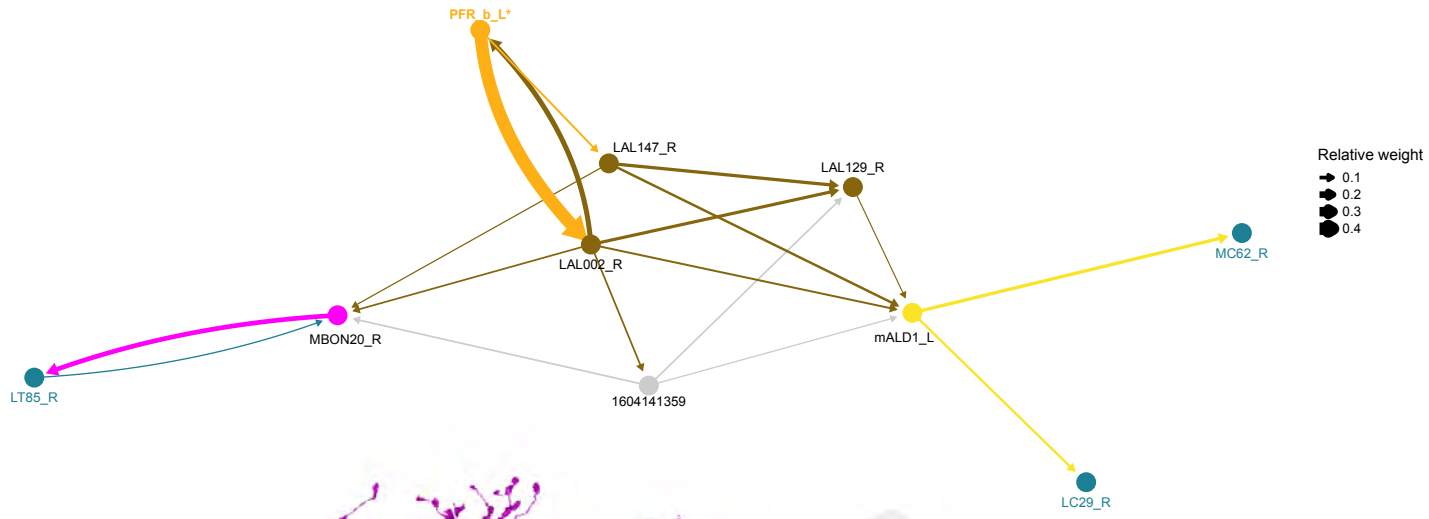
G) Morphological rendering showing that the PLP neurons specifically target the LC27 glomerulus.

H) **H,I** ExR8 contacts VCH and DCH, centrifugal neurons of the horizontal fiber system. **H** Network diagram showing that ExR8 reaches CH neurons both directly and indirectly through a PS neuron.

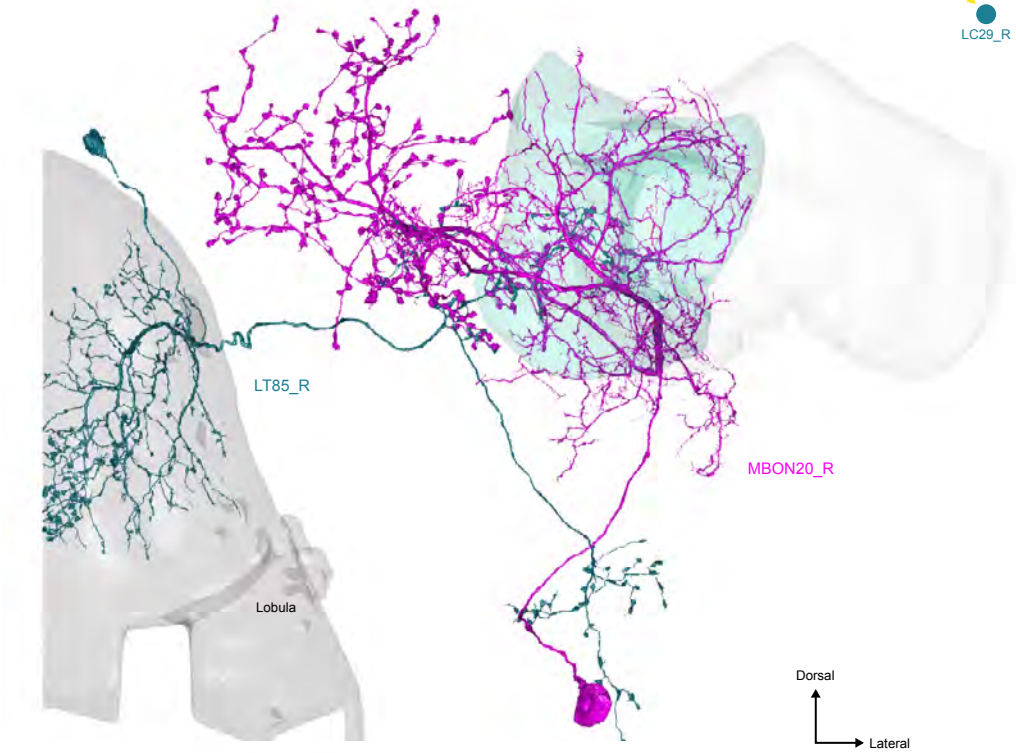
I) **I** Morphological rendering. PS047 innervation closely follows ExR8 innervation.

Figure 62—figure supplement 1: PFRb to Visual PNs

A



B



C

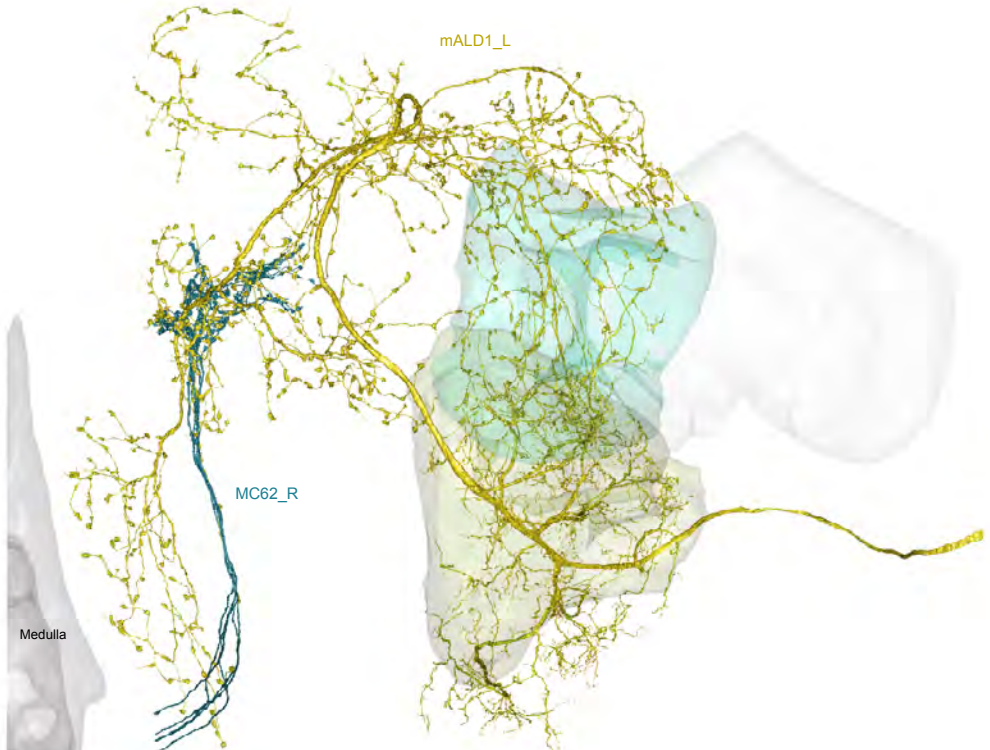


Figure 62—figure supplement 1: PFR_b to visual PNs

A) Network diagram. PFR_b reaches visual PNs through the MBON20 and mALD1 neurons.
B) Morphological rendering of MBON20 with its visual target, LT85.
C) Morphological rendering of mALD1 with one of its visual targets, MC62.

Figure 62—figure supplement 2: : PFL3 and LC33 neuron to neuron connectivity

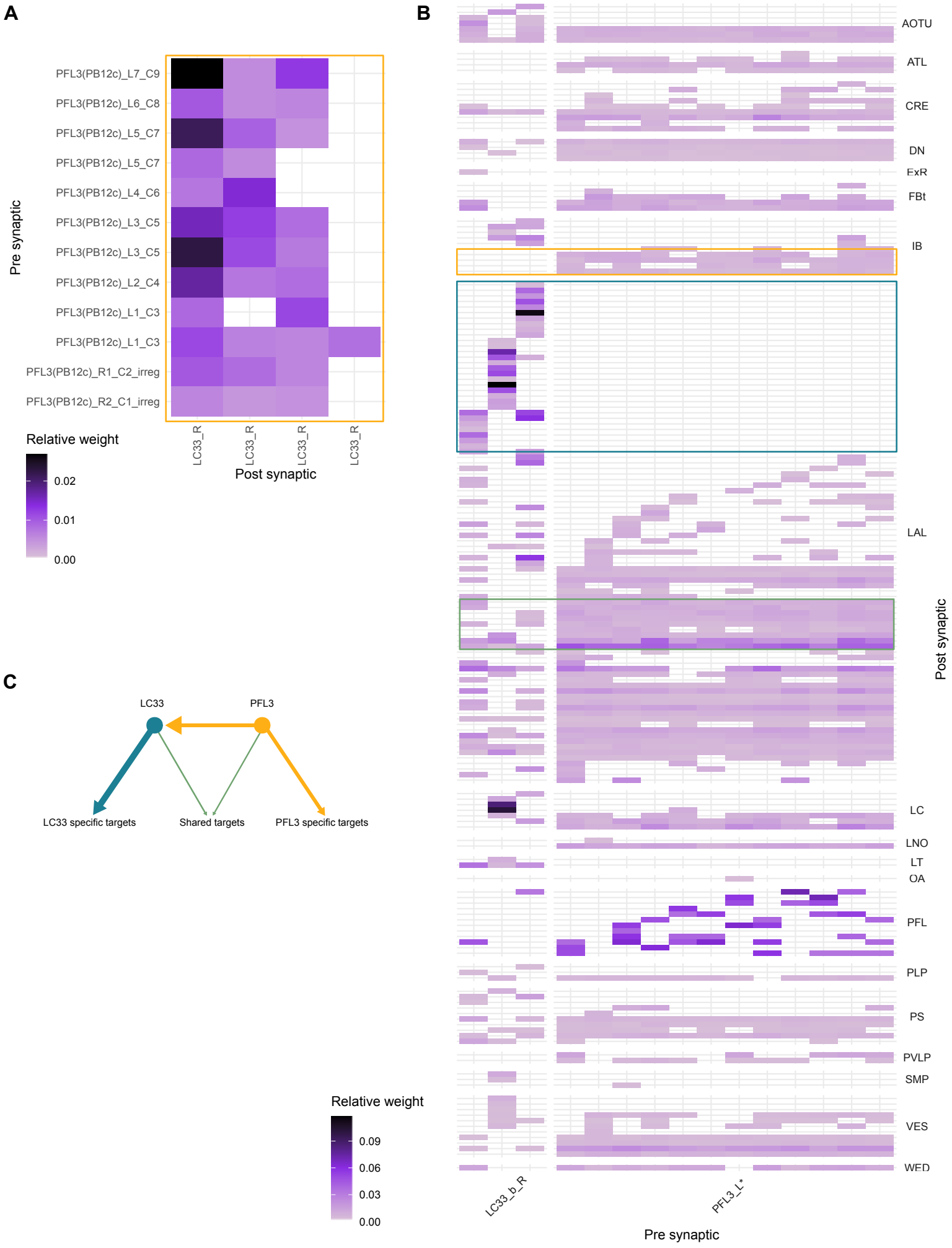


Figure 62—figure supplement 2: PFL3 and LC33 neuron to neuron connectivity

A) Neuron-to-neuron connectivity of PFL3 to LC33 connections. Only 4 LC33s receive any PFL3 synapses (and only 3 consistently so).

B) Post-synaptic connectivity of PFL3 and LC33 neurons. For ease of display, this matrix has its axis flipped compared to the convention used in the paper. Note that the downstream connectivity of LC33 neurons is not consistent, and only partially overlaps with that of PFL3 neurons. Targets of LC33 neurons that are not contacted by PFL3 is circled in teal. In yellow, targets of PFL3 not contacted by LC33. In green an example of shared connectivity. Even in that case, usually only one of the LC33 neurons contacts the same neuron as the PFL3s.

C) Schematic representation of the degree of overlap between LC33 and PFL3 targets.

Figure 63: connections to descending neurons

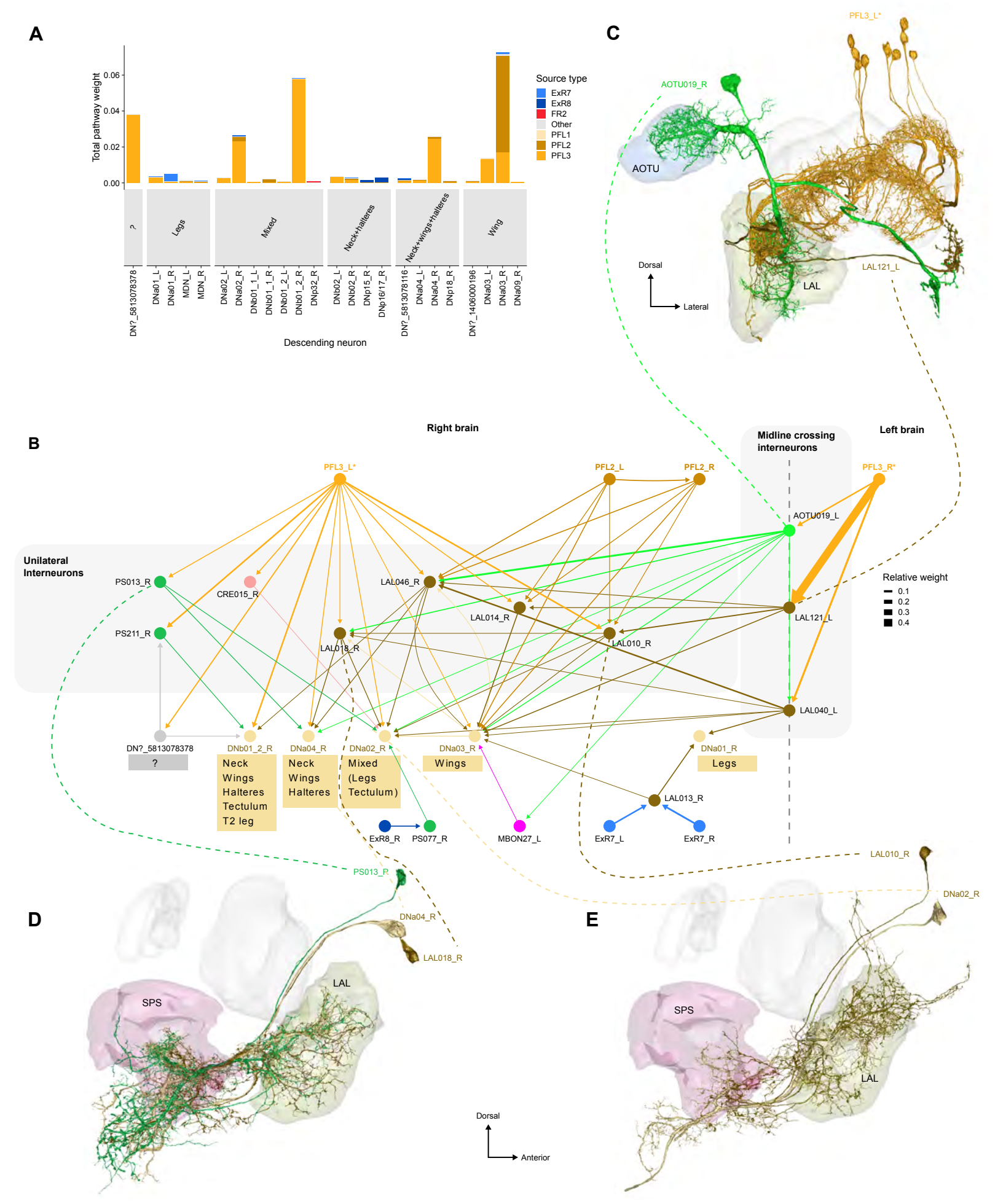


Figure 63: Connections to descending neurons.

A) Pathway weight (see **Methods Figure 1** and Materials and Methods) from the CX to DNs for which the total pathway weight is greater than 0.05%, colored by CX type of origin and separated by their VNC innervation.

B) Network diagram of the main CX to DN connections, restricted to DNs on the right side of the brain. DN connections primarily come from PFL2 and PFL3, with smaller contributions made by ExR7 and ExR8. PFL2 reaches DNs through ipsilateral networks while PFL3 also reaches them via LAL interneurons crossing the midline. Much of the circuit is shared between PFL2 and PFL3 pathways. VNC innervations for each DN type are indicated below, highlighting their diversity.

C) Morphological rendering of PFL3 with two of its midline crossing targets, AOTU019 and LAL121.

D) Morphological rendering of PS013, DNa04 and LAL018. Note how LAL018 innervations follow those from DNa04

E) Morphological rendering of DNa02 and LAL010.

Figure 63—figure supplement 1: Other connections to DNs

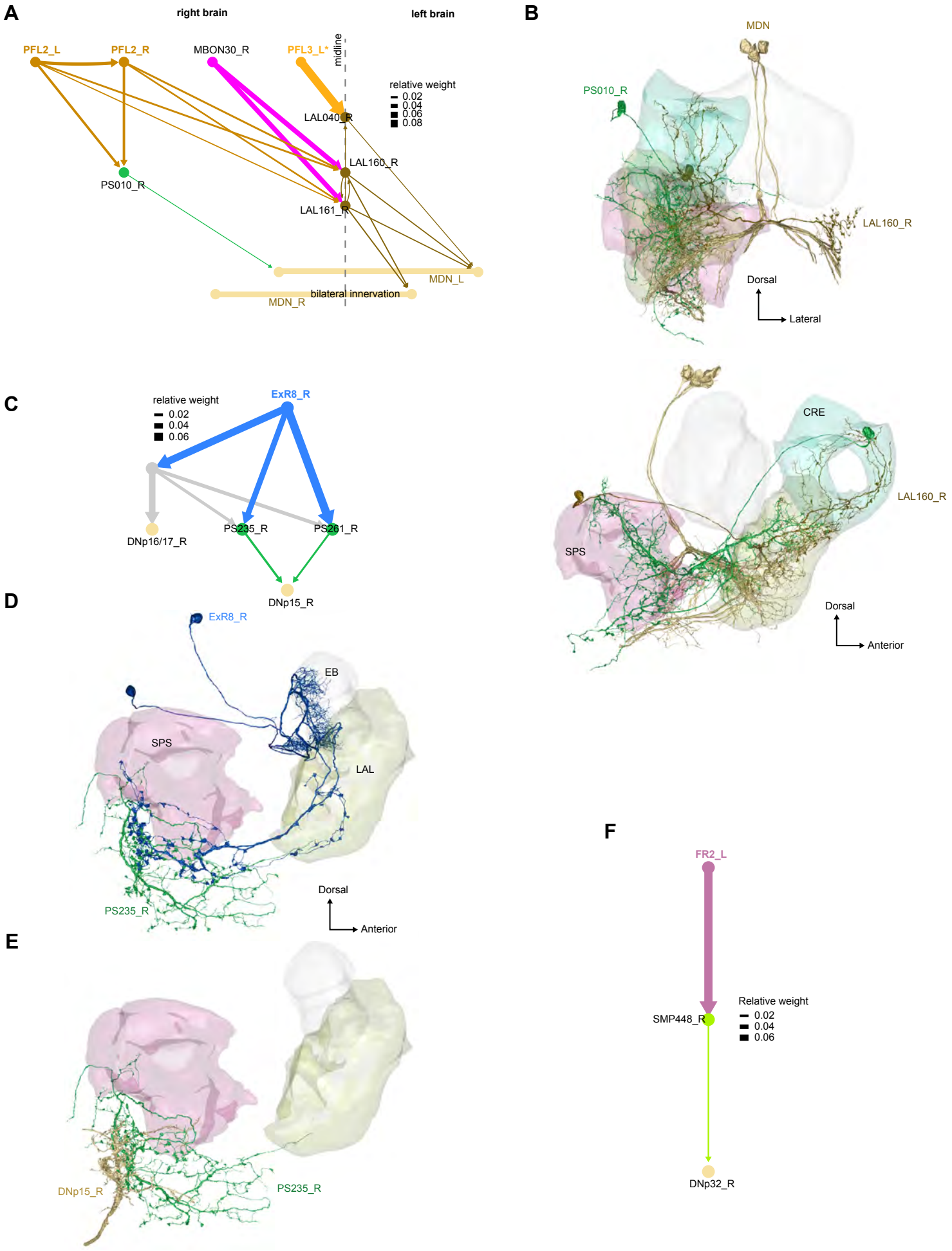


Figure 63—figure supplement 1: Other connections to DNs

A) Network diagram showing the connection from PFL2,3 in the right LAL to the bilateral MDNs. LAL160, a midline crossing LAL neuron linking PFL2 to the MDN, is also targeted by MBON30.
B) Anatomical rendering of PFL2, LAL160, PS010 and MDN_R.
C) Network diagram of the indirect connections between ExR8 and DNp15 and DNp16/17.
D) Morphological renderings ofExR8 and PS235
E) Morphological renderings of PS235 and DNp15
F) Network diagram of the indirect connection between FR2 and DNp32.

Figure 64: PFL3 outputs distribution and possible implications for the downstream LAL network

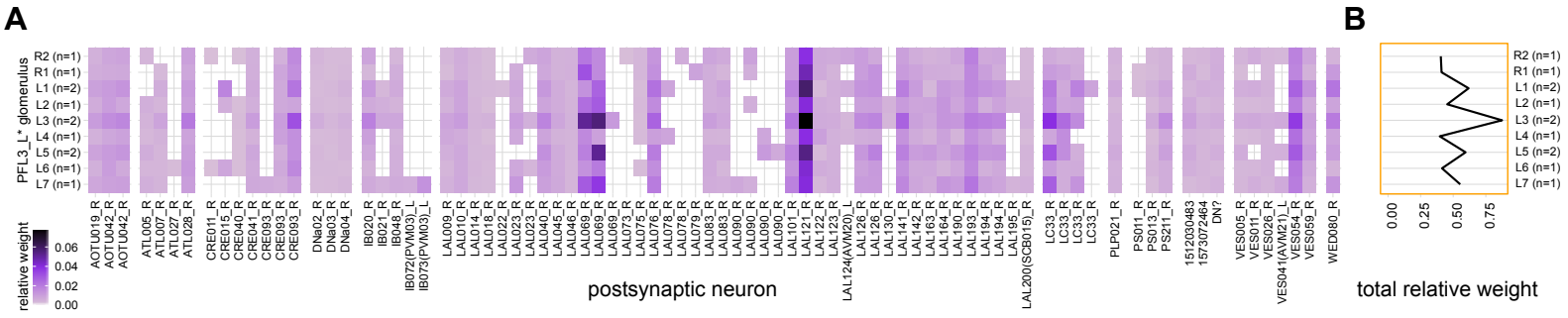


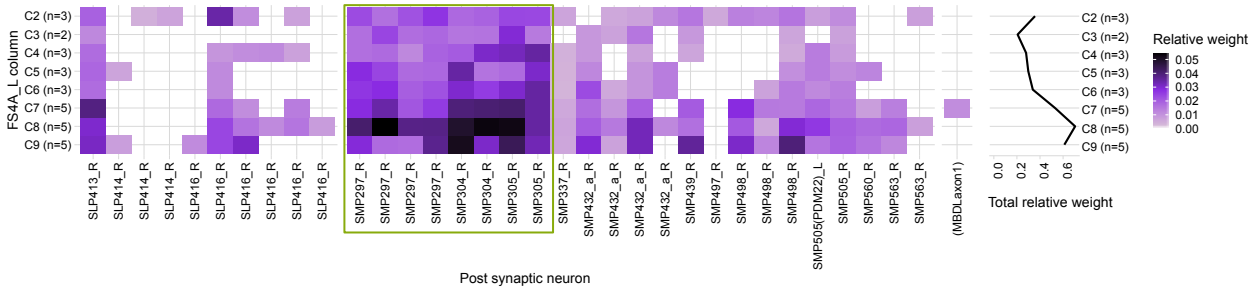
Figure 64: PFL3 outputs distribution and possible implications for the downstream LAL network

A) Connectivity matrix between PFL3_L and its direct downstream partners outside of the CX, on the right side of the brain. PFL3 neurons are binned by PB glomerulus (numbers of neurons per glomerulus are indicated in parenthesis on the y axis).

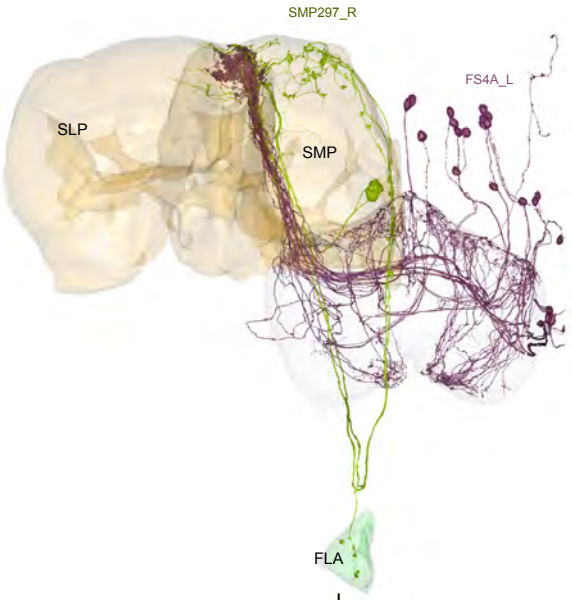
B) Sum of the relative weights across glomeruli. Connections are strongest for glomerulus L3.

Figure 65: FS4A asymmetric connection to the flange

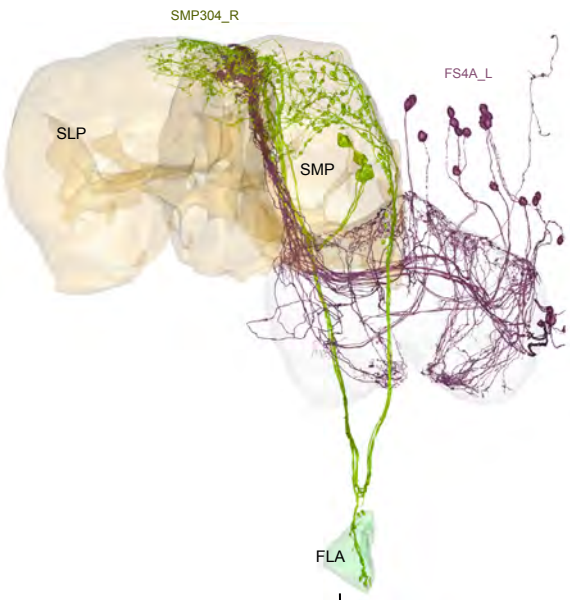
A



Bi



Bii



Asymmetric feeding behaviors?

Figure 65: FS4A asymmetric connection to the flange

A) Connectivity matrix between FS4A_L and its direct downstream partners outside of the CX, on the right side of the brain. FS4A neurons are binned by FB column (numbers of neurons per column are indicated in parenthesis on the y axis). Right: sum of the relative weights across columns. Connections are biased towards columns C7-C9.

B) Morphological rendering of two of the strongest direct targets of FS4A, SMP297 and SMP304 (also circled in green in the connectivity matrix in A). All strong direct targets of FS4A project to the FLA, potentially participating in the control of feeding behaviors. Given that FS4A neurons are columnar and project unilaterally in the SMP, this raises the possibility that it controls feeding behaviors in a directed fashion. Besides, the asymmetry in FB innervation also suggests that this behavior could have a default directionality corresponding to the border columns of the FB.

Figure 66: Mapping multi-sensory cues to a flexible head direction representation

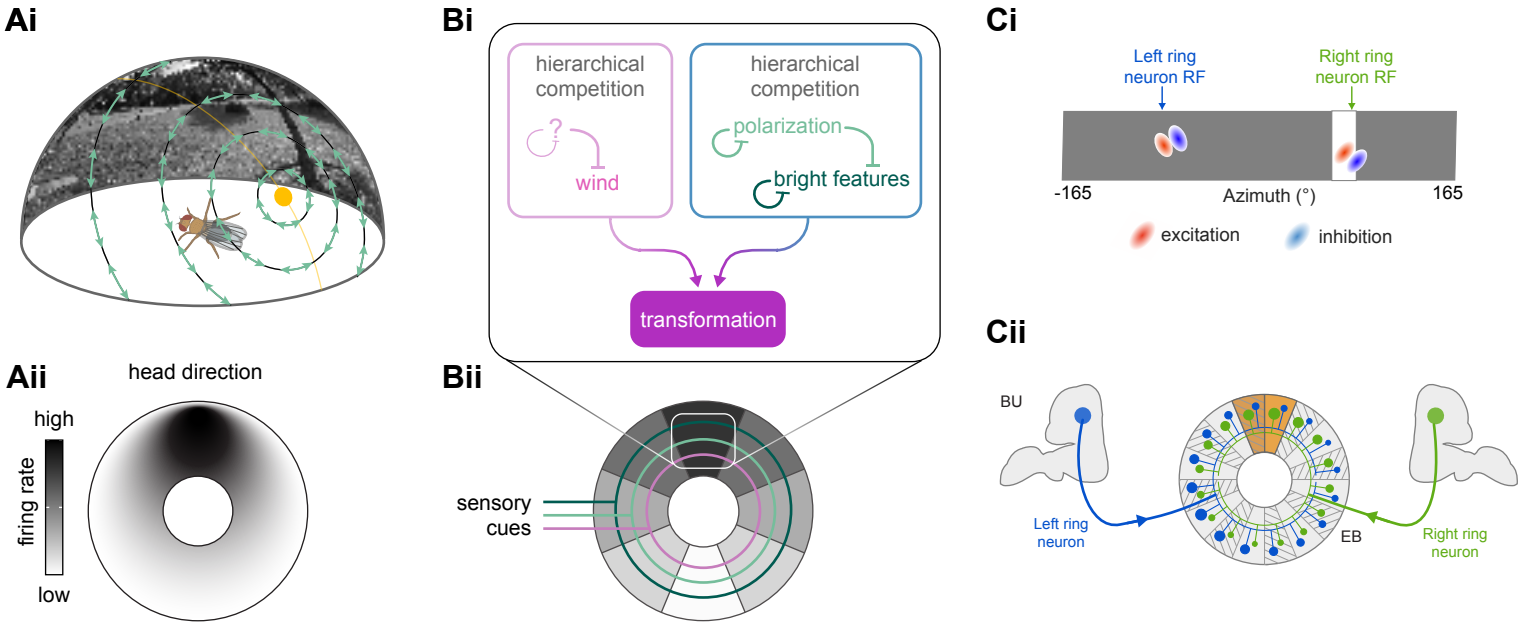


Figure 66: Mapping multisensory cues to a flexible head direction representation

A) Flies have access to a number of directional sensory cues, such as mechanosensory information about wind direction (Okubo et al., 2020), celestial visual cues related to the polarization pattern of the sky (Hardcastle et al., 2020) or the azimuthal direction of the sun (Giraldo et al., 2018), and terrestrial visual cues such as prominent visual landmarks (Seelig and Jayaraman, 2013). Importantly, these directional cues are all in a head-centered reference frame (**Ai**). Various ring neuron types convey this sensory information to the EB, which contains an activity bump that encodes the fly's allocentric head direction (Seelig and Jayaraman, 2015) (**Aii**). Plasticity between ring neurons and the compass allows flies to learn a flexible mapping between these sensory cues and allocentric head direction (Fisher et al., 2019; Kim et al., 2019). Once formed, this map enables directional sensory cues to dictate the bump's angular position in the EB.

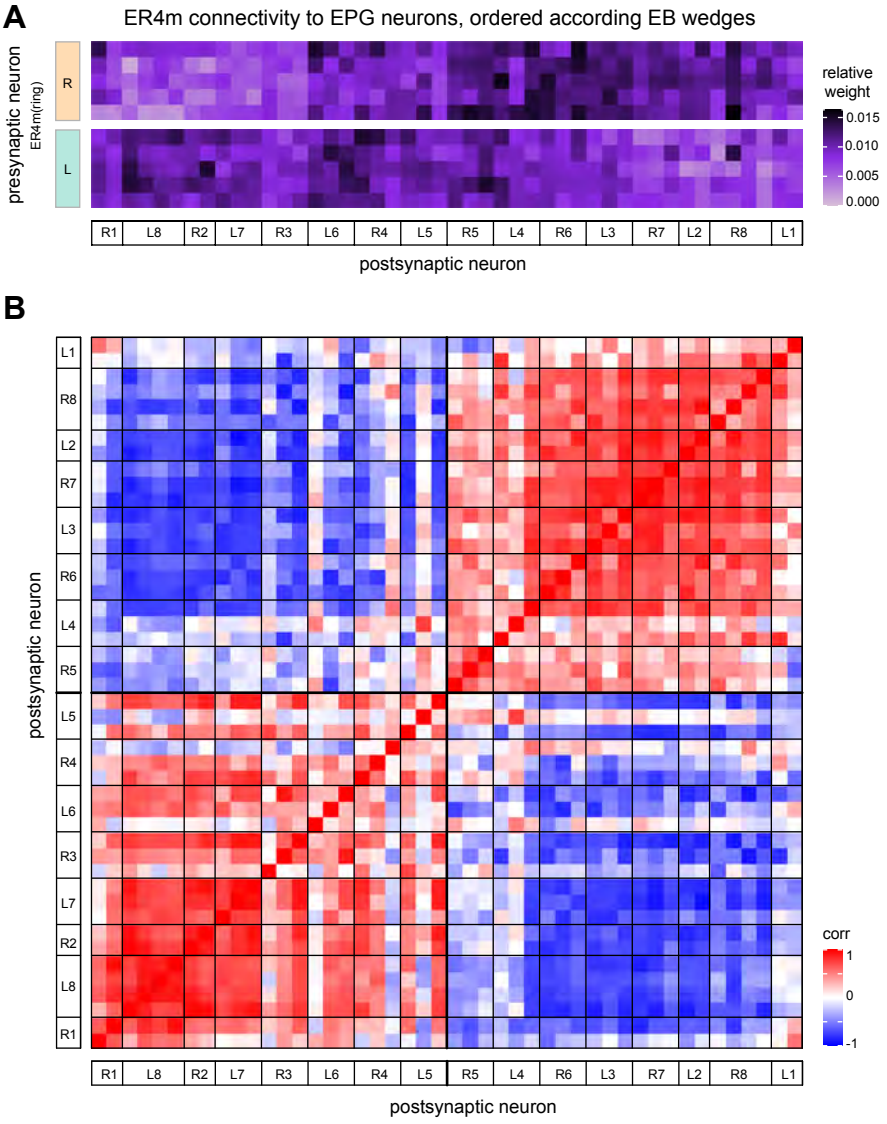
(**Related to data in Figure 11 and citations above**).

B) Hypothetical competition (blue) and transformation (pink) (**Bi**) that could occur through interactions between ring neuron types (green) conveying distinct directional information (**Bii**). In the former case, one sensory cue—in this case, celestial visual cues—could dominate at the expect of other, less-reliable cues. In the latter case, complementary directional cues could be combined to estimate the fly's head direction.

C) Schematic of ring neurons that respond to local features in a visual scene (**Ci**). Plasticity between these ring neurons and EPG neurons (**Cii**) ensure that the compass reliably tethers to the visual scene.

(Conceptual models related to data in Figure 11)

Figure 67: Disambiguating directional information from polarized light sensors



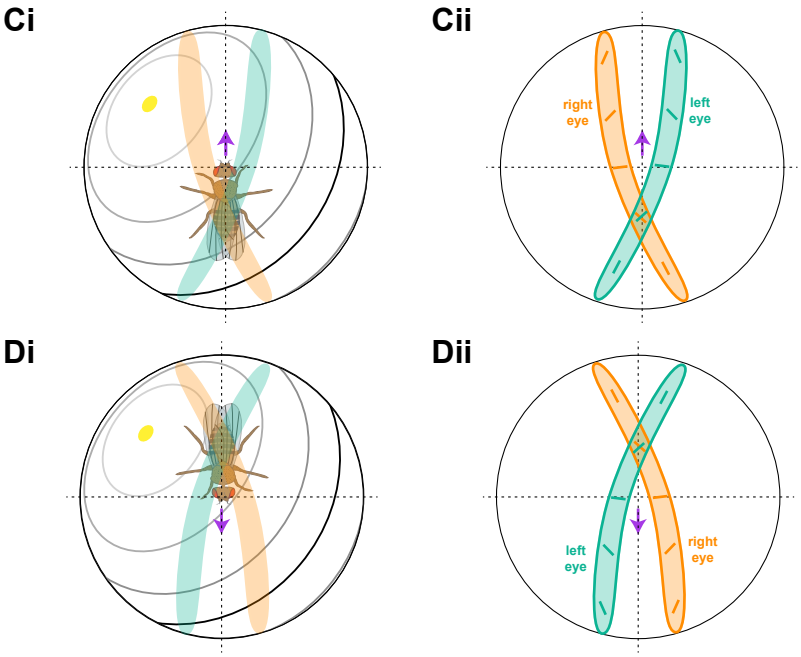


Figure 67: Disambiguating directional information from polarized light sensors

A) Connectivity matrix of ER4m inputs to EPG neurons in the EB. EPG neurons are sorted and averaged according to the EB wedge they innervate.

B) Schematic of the polarization pattern and the sun position of the sky. Under most conditions the two eyes and thus the left and right polarization sensitive dorsal rim area are expected to receive different input.

Figure 68: Conveying and transforming the head direction representation from the EB to the FB

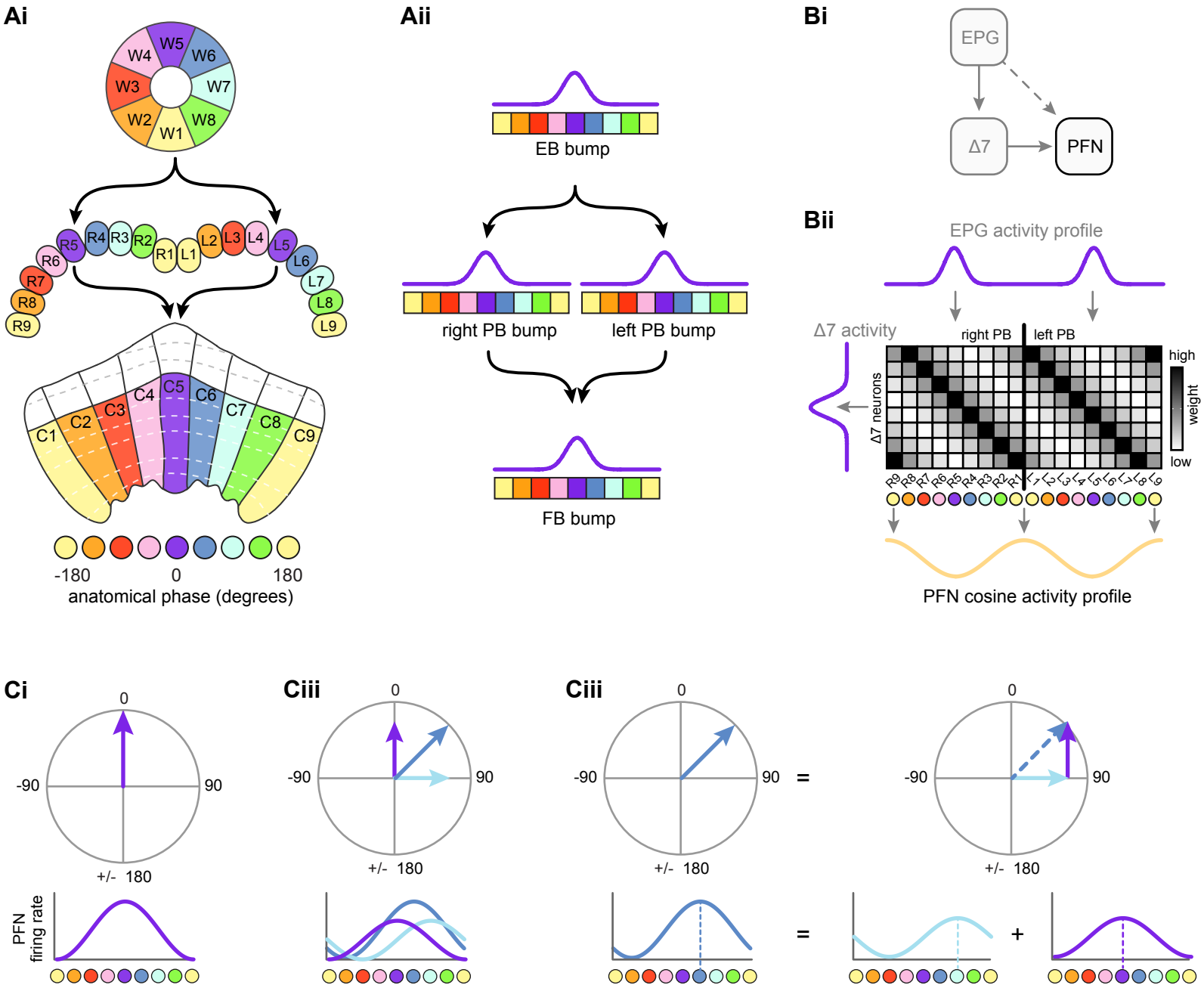


Figure 68: Conveying and transforming the head direction representation from the EB to the FB

A) Schematic showing how a bump of activity gets conveyed from the EB to the left and right PB before converging on the FB. EB wedges, PB glomeruli, and FB columns are colored by their functional column phase, indicating corresponding bump locations in each region, which assumes no anatomical offset in the projection between regions (**Ai**). For example, a bump of activity at EB W5 (purple) gets conveyed to PB R5 and L5 before converging on FB C5 (**Aii**).

(**Based on data from Figure 16, Figure 20, Figure 20—figure supplement 1, Figure 30**)

B) Δ7 neurons in the PB transform any EPG activity profile into a sinusoidal activity profile that gets inherited by FB columnar neurons. PFN neurons receive the EB bump directly from EPG neurons (dashed arrow) as well as through Δ7 neurons (solid arrow) (**Bi**). Schematic showing how the connectivity of Δ7 neurons might transform any EPG activity profile into a sinusoidal activity profile (illustrated for PFN neurons; yellow curve) (**Bii**).

(Based on data from Figure 20)

C) Phasor representation of a sinusoidal activity profile. Any sinusoidal activity bump can be represented as a vector whose angle encodes bump phase and whose magnitude encodes bump amplitude. Schematic of hypothetical sinusoidal activity bump (purple line), centered at 0 degrees, encoded by a population of neurons that function as a sinusoidal array, and phase representation (purple arrow) of the same activity (**Ci**). Vector addition can easily be implemented by sinusoidal arrays carrying different activity bumps (purple, blue, light blue) (**Cii**). Two vectors (purple and light blue) can be summed to generate a new vector (blue) (**Ciii**).

(**Based on data from Figure 20**)

Figure 69: Conceptual model showing that PFN offsets, when combined with differential NO input, could produce +/- 45 degree bump shifts between the PB and FB

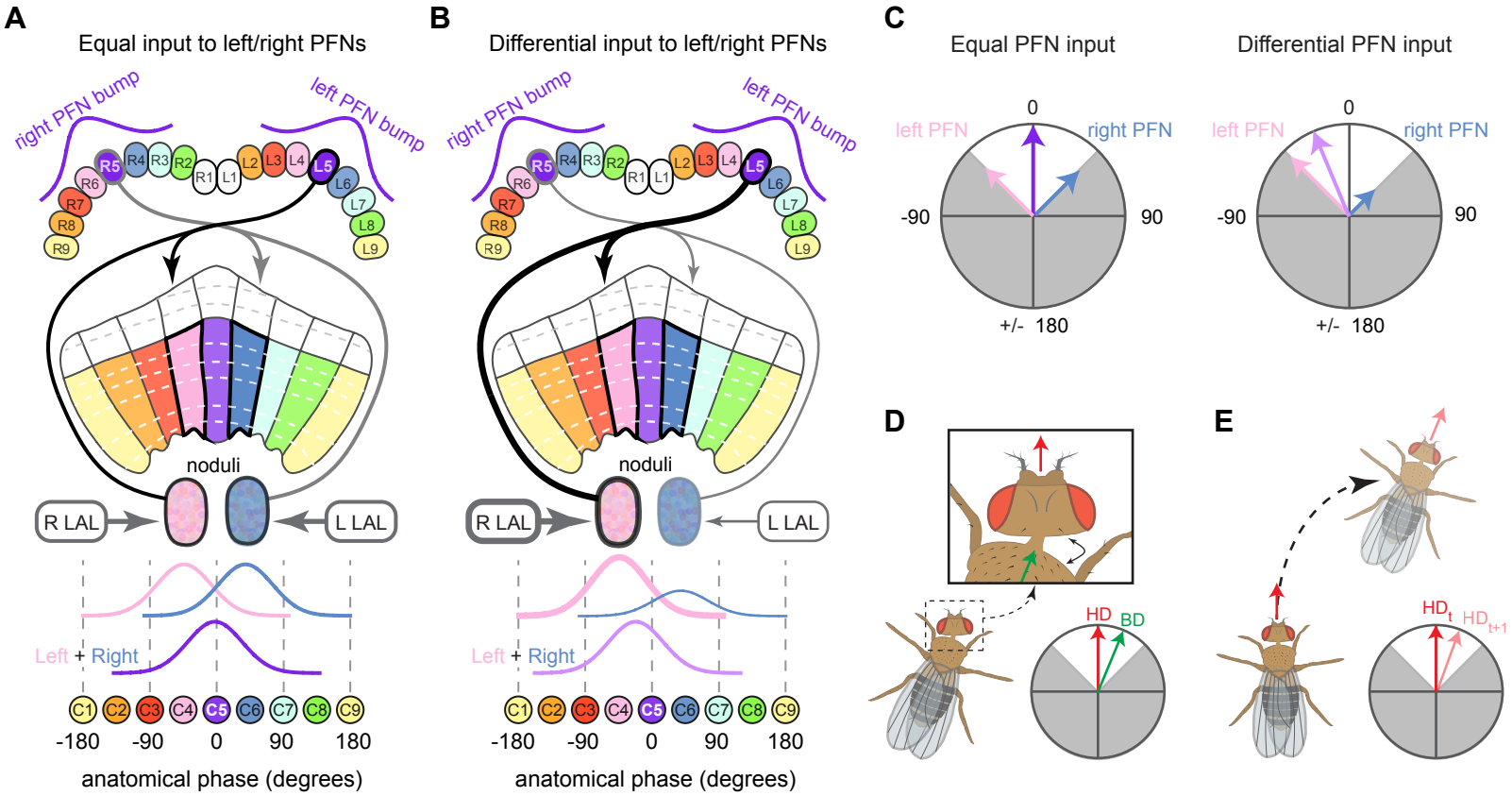


Figure 69: Conceptual model showing that PFN offsets, when combined with differential NO input, could produce +/- 45 degree bump shifts between the PB and FB.

A) Schematic of a PB-to-FB projection pattern with a 1-column contralateral offset. PB glomeruli and FB columns are colored according to anatomical phase (from –180 to 180), which indicates matching bump locations. PFN neurons innervating the right PB project to the left NO, where they receive input from LAL-NO neurons carrying self-motion information from the left LAL. Similarly, PFN neurons innervating the left PB project to the right NO and receive self-motion inputs from LAL-NO neurons innervating the right LAL. As shown in the bottom panel, when these two LAL inputs are equal, the spatially-offset bumps from the left and right PB sum to generate a new bump located halfway between the two.

B) Same as in A, but with differential noduli input. In this case, as shown in the bottom panel, the R LAL neurons increase the bump amplitude of the left PB population (pink bump), and the L LAL neurons decrease the bump amplitude of the right PB population (blue bump). The sum of these two bumps will end up closer to -90, the location of the left PB bump, due to the difference in bump amplitudes.

C) Phasor diagram interpretations of the scenarios from A and B. In the left panel, with equal NO input, the left PFN and right PFN bumps sum to produce the purple vector located at 0. In the right panel, with differential noduli input, the left PFN bump becomes bigger than the right PFN bump (as in B), and therefore, their sum is closer to the left PFN bump. This effectively shifts the bump by a phase that depends on the difference in amplitude between the left and right PFN populations. Importantly, the PFN neurons' 1-column ipsilateral offset limits such bump shifts to +/-45 degrees from the PB bump. Phases shifts outside this area (marked in gray) cannot be produced.

D) Illustration of a head direction to body direction coordinate transformation.

E) Illustration of a forward model of head direction.

(Figure describes conceptual models based on data from Figure 30)

Figure 70: Conceptual model showing how two PFN populations, when combined with differential NO input, could form a four vector basis set whose summation could produce any vector

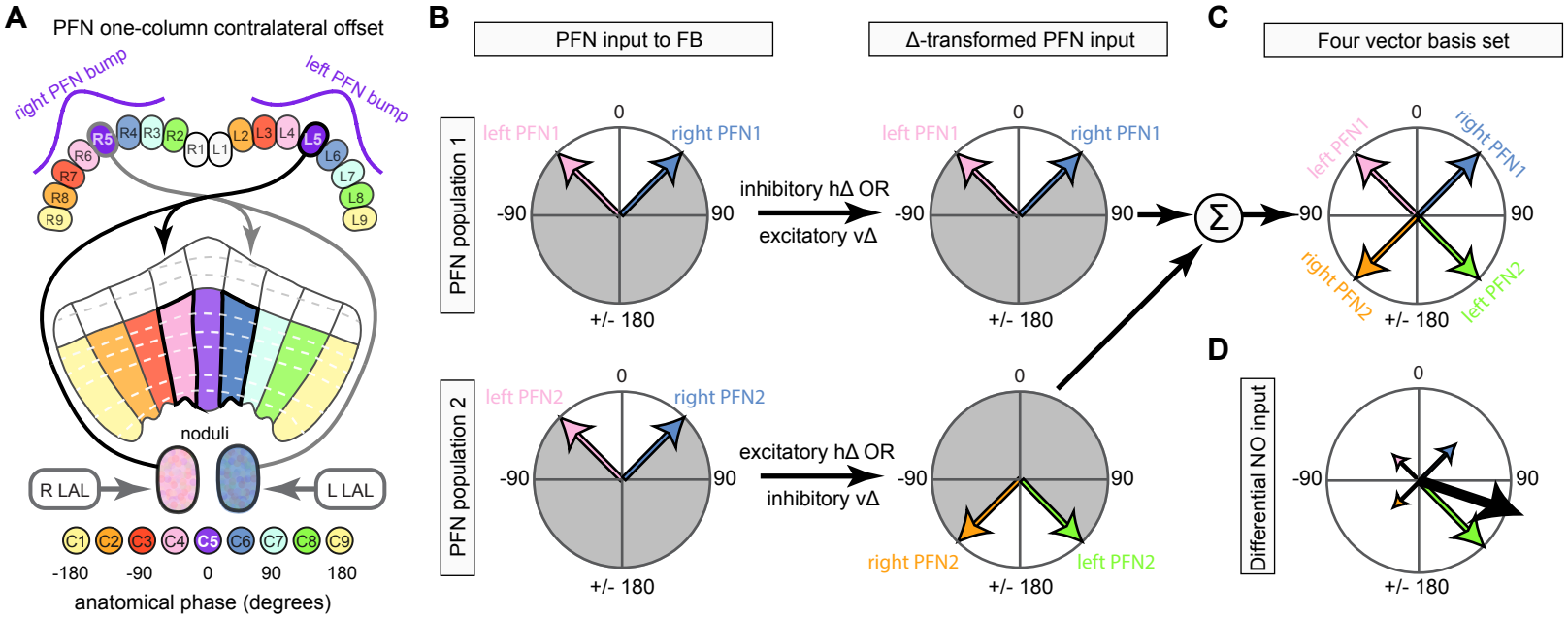


Figure 70: Conceptual model showing how two PFN populations, when combined with differential noduli input, could form a four vector basis set whose summation could produce any vector.

A) Schematic of a PB-to-FB projection pattern with a 1-column contralateral offset.

B) Phasor diagram interpretation showing how hΔ and vΔ motifs could be used to transform PFN bumps. For the first PFN population (top panels), an inhibitory hΔ or an excitatory vΔ could pass the PFN1 bump along while maintaining is phase. In contrast, for the second PFN population (bottom panels), an excitatory hΔ or and inhibitory vΔ could shift the PFN2 bump by 180 degrees. For both PFN population 1 and 2, differential NO input could shift their summed FB bump location by +/- 45 degrees, but not outside this region (marked by gray portions of each circle).

C) Phasor diagram of a hypothetical downstream neuron which sums the input from the PFN1 and PFN2 populations. In this case, the downstream neuron's population activity would be the sum of four independent bumps, located 90 degrees apart from one another and whose amplitudes are modulated by independent noduli input. These four bumps could act as an orthogonal basis for computing an arbitrary vector, thus freeing the resulting bump from the +/- 45-degree range.

D) Phasor diagram showing how modulating the amplitude of the left and right PFN1 and PFN2 populations could be used to compute an arbitrary vector (shown in black).

(Figure describes a conceptual model based partially on data from Figure 30, Figure 31, Figure 33, Figure 34, and Figure 37)

Figure 70—figure supplement 1: Dynamic updating of the four-vector basis set

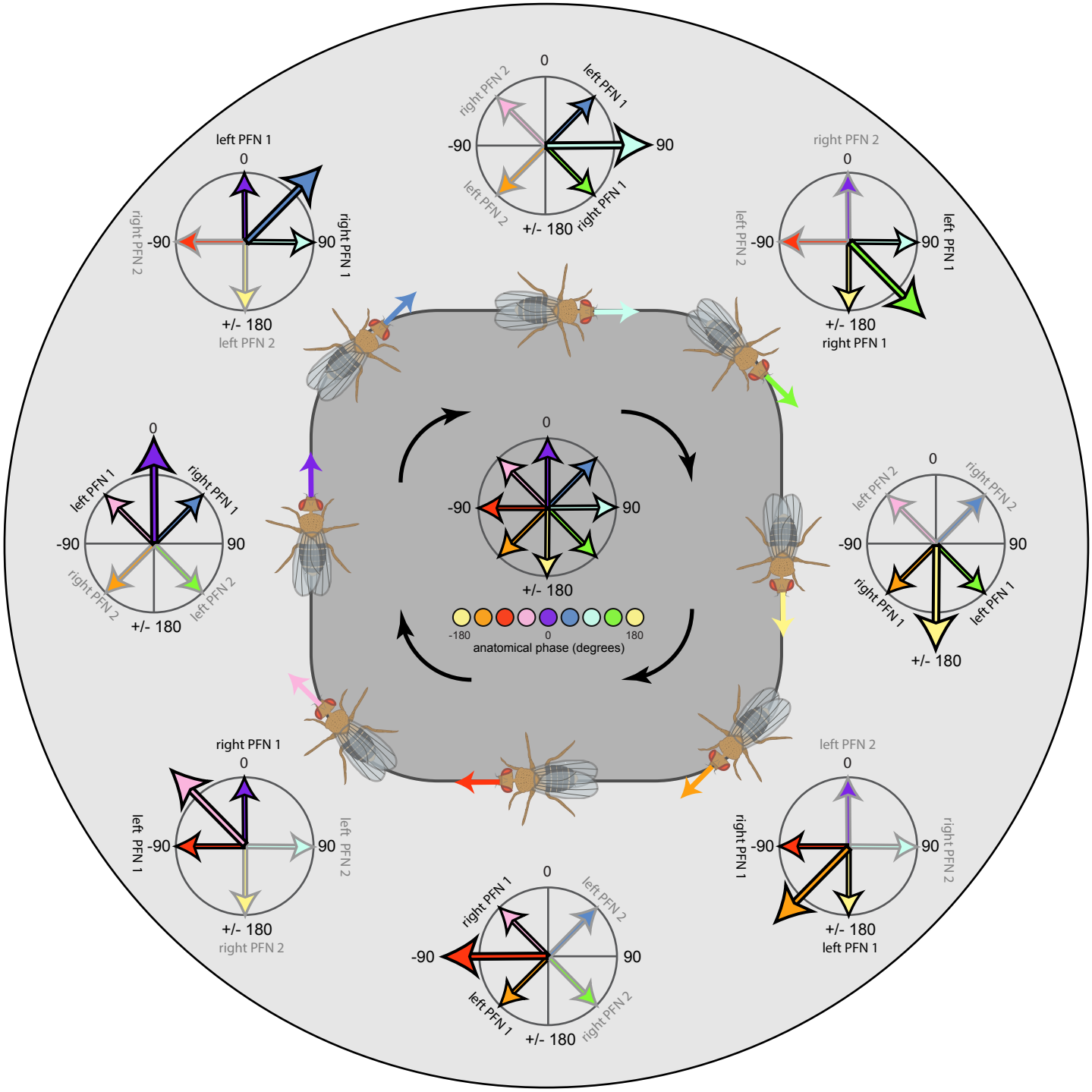


Figure 70—figure supplement 1: Dynamic updating of the four-vector basis set

Schematic showing how the four-vector basis set gets updated as the fly's head direction changes. Here a fly is shown walking in a loop, with phasor plots showing the state of the FB network's four vector basis set at eight different head directions. The fly's head direction is marked by the large arrow in each phase diagram. As the fly's head direction changes, so too do the bump positions of the PFN populations that form the four-vector basis set, though the relative positions across the four populations are maintained. For example, the Right PFN1 population always has a bump located 45-degrees clockwise from the fly's head direction vector. In this view, at every moment in time, the FB network has access to four vectors pointing in four distinct allocentric directions. Modulating the amplitude of each vector according to self-motion cues may allow for various navigational computations, as described in later figures.

(Figure describes a conceptual model)

Figure 70—figure supplement 2: The FB network has the necessary connectivity and depth to form a basis set: bump propagation using simulated activity through actual FB connectivity

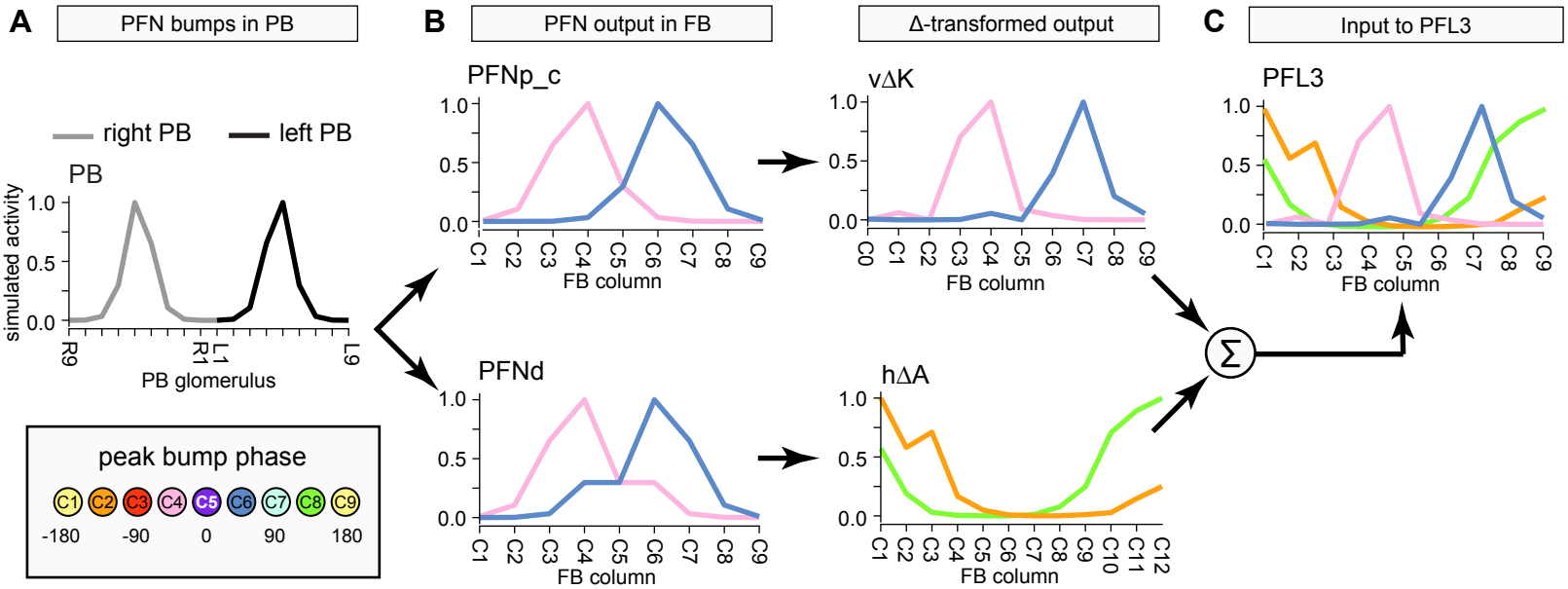


Figure 70—figure supplement 2: The FB network has the necessary connectivity and depth to form a basis set: bump propagation using simulated activity through actual FB connectivity.

A) Two PFN types (PFNp_c and PFNd) receive compass input that generates two simulated activity bumps in the left and right PB, centered at R5 and L5.

B) In FB column space, the two simulated activity bumps end up 90-degrees apart owing to the PFN 1-column contralateral offset. In particular, the activity bump at PB R5 is centered at FB C6 (blue bump), and the activity bump at PB L5 is centered at FB C4 (pink bump).

C) An excitatory vΔK inherits the PFNp_c activity bump and moves it to downstream types while maintaining bump phase. In contrast, an excitatory hΔA receives the PFNd activity and shifts it by 180 degrees before passing it on to downstream types.

D) PFL3 neurons, a major CX output neuron type, receive input from both vΔK and hΔA, which could potentially instantiate a four vector basis set since PFL3. Note that this figure keeps the left and right PFN bumps separate as they propagate through the network, each layer would represent the summation of their inputs as a single bump. While we assume both vΔK and hΔA are excitatory, this currently remains unknown. However, many similar pathways exist and an excitatory vΔ type could function similar to an inhibitory hΔ type.

(Figure shows a simple model that uses actual connectivity to simulate bump propagation through the FB network. See Materials & Methods for details.)

Figure 71: A conceptual model that computes an allocentric translational velocity vector using head-centered optic flow sensors during flight

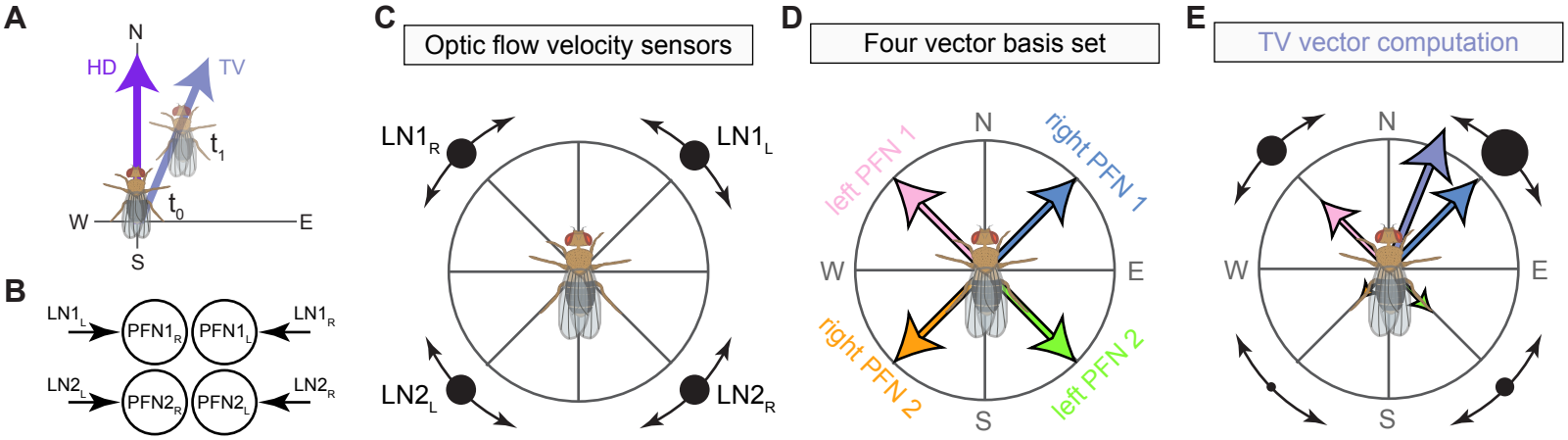


Figure 71: A conceptual model that computes an allocentric translational velocity vector using head-centered optic flow sensors during flight

A) Illustration of a fly whose head and body direction are pointed north and whose translational velocity vector is 22.5 degrees east of north.

B) Schematic of noduli circuity, showing that the left and right PFN1 and PFN2 populations receive input from right and left LN1 and LN2 neuron types, respectively.

C) LN1 and LN2 neuron types are those described by **Stone et al.**, which function as optic flow-based velocity sensors with preferred expansions points spaced at 45 degree intervals around the fly's head.

D) Schematic of a four vector basis set. Importantly, note that each PFN vector points in the same direction as its upstream LN neuron's preferred optic flow direction.

E) Schematic showing how the four vector basis set, whose vectors are amplitude-modulated by the LN velocity sensors, can compute the fly's translational velocity vector. In this case, because the fly is moving just east of north, $LN1_L$ is driven most strongly which increases the amplitude of right PFN1 population (blue vector). When properly calibrate, summing the amplitude-modulated PFN vectors compute the fly's translational velocity vector.

(Figure describes a conceptual model based on previous work (Stone et al., 2017))

Figure 72: A conceptual model that computes an allocentric translational velocity vector using body-centered velocity estimates during walking

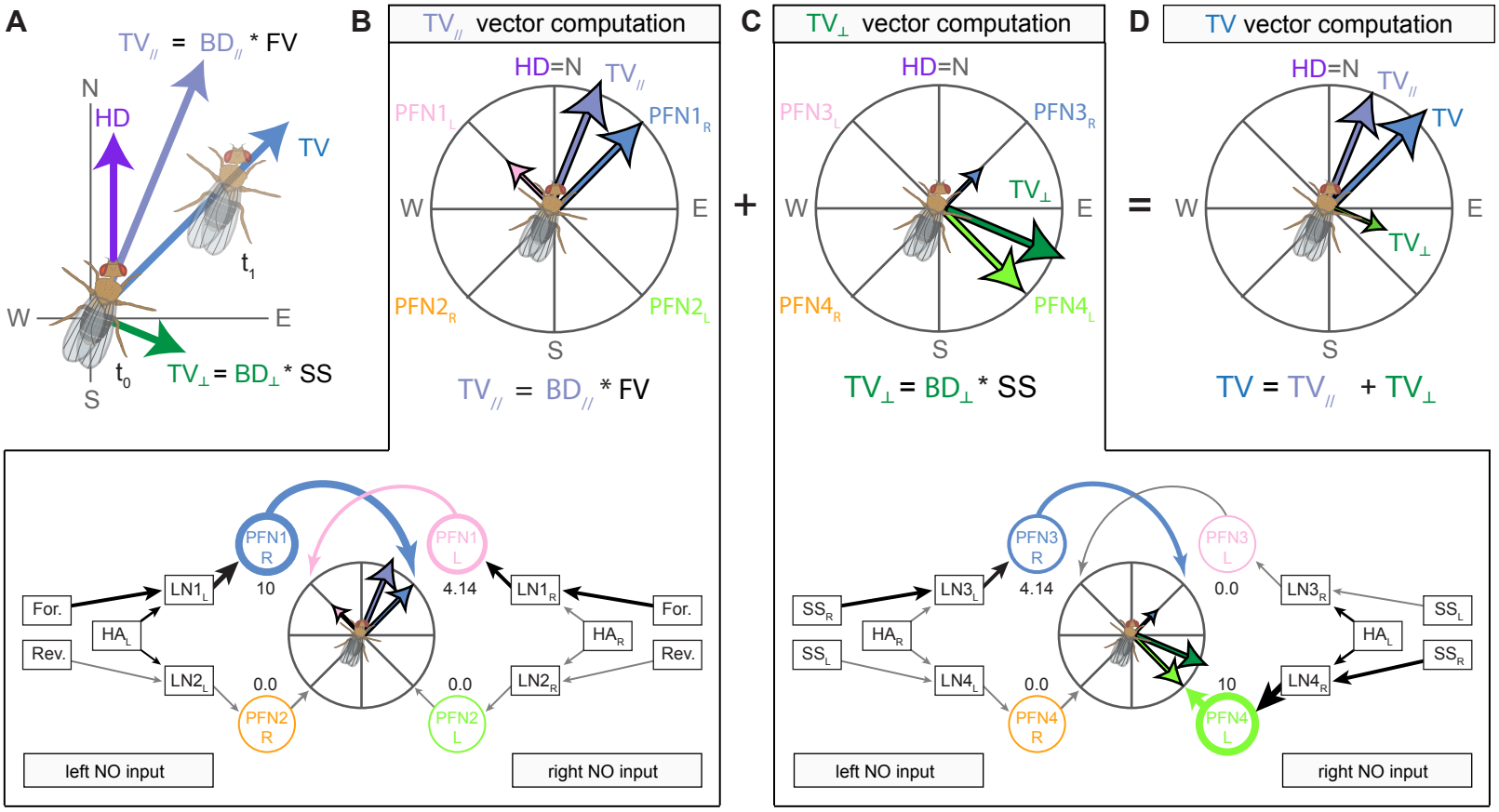


Figure 72: A conceptual model that computes an allocentric translational velocity vector using body-centered velocity estimates during walking.

A) Illustration of a walking fly whose head direction, body direction (BD), and translational velocity (TV) direction are all different. The fly's head is pointing north, its body 22.5 degrees east of north, and it's walking northeast (i.e. 45 degrees east of north). The fly's translational velocity vector can be computed by summing the component of its movement parallel to its body axis ($TV_{//}$) with the component of its movement perpendicular to its body axis ($TV_{\perp}$). Circuits for computing these quantities are shown in B and C, respectively.

B) Circuit for computing the component of the TV vector parallel to the fly's body axis. As shown in the bottom panel, this circuit uses a four vector basis set whose PFN vector amplitudes are modulated by LAL-NO inputs that encode whether the head is left (HA_L) or right (HA_R) of the fly's body axis as well as either a forward (For.) or reverse (Rev.) velocity signal. The firing rate of each PFN population is noted below each PFN node. Arrow width is proportional to firing rate. Gray arrows indicate neurons that are silent. Note that head angle input alone is insufficient to bring the LN neurons to threshold, but it can boost PFN firing when combined with a velocity input. In this case, $LN2_L$ remains silent despite receiving a head angle input from HA_L, and $LN1_L$ is strongly driven by both the forward velocity signal and HA_L. $LN1_R$, meanwhile, is moderately driven by the forward velocity signal alone. This conditional effect of the head angle input could be achieved in other ways, but the core conceptual model would remain the same. In all cases, the circuit would require proper calibration for the vector summation to accurately compute the fly's $TV_{//}$ vector.

C) Circuit for computing the component of the TV vector perpendicular to the fly's body axis. The circuit shown in the bottom panel operates similar to that described in B, but the forward and reverse velocity signals have been replaced by left (SS_L) and right (SS_R) sideslip velocity signals. As in B, a head angle input alone is insufficient to bring LN neurons to threshold. Note that these circuits function regardless of which direction the fly's head is facing and which direction the fly is moving, as detailed for four other examples in **Figure 72—figure supplement 1**.

D) Phasor diagram showing how summing the output from the circuits in B and C yields an exact TV vector whose integration would compute the path integration vector.

(Figure describes a conceptual model)

Figure 72—figure supplement 1: The circuit for computing $TV_{\perp}$ operates independent of the fly's head-body angle and direction of sideslip

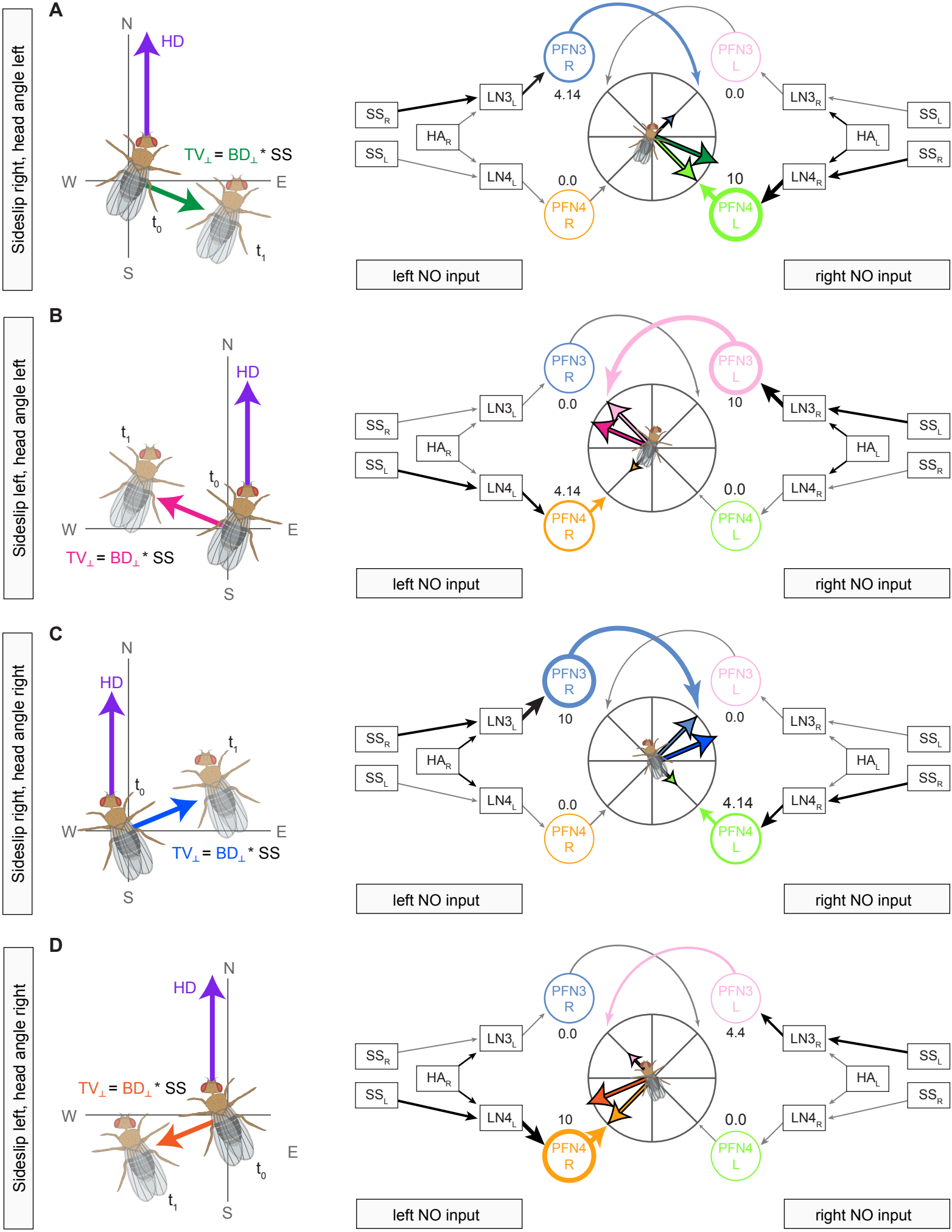


Figure 72—figure supplement 1: The circuit for computing $TV_{\perp}$ operates independent of the fly's head-body angle and which direction the sideslip component is towards.

A) Left panel shows an example of a fly whose head direction is north, body direction is 22.5 degrees east of north, and whose $TV_{\perp}$ vector is 22.5 degrees south of east, which is towards the fly's right (green array). The circuit on the right is the same as in **Figure 72B**, and uses a four vector basis set and head-angle and sideslip velocity to compute $TV_{\perp}$.

B) Same as in **A** but for a fly that is sideslipping towards its left.

C) Example showing a fly whose head direction is north, whose body direction is now 22.5 west of north, and whose translational velocity vector has a component 22.5 degrees north of east, which is towards the fly's right.

D) Same as in **C**, but for a fly that sideslipping towards its left.

Figure 73: PFL neurons could generate egocentric motor commands by comparing the fly's allocentric head direction to an allocentric goal vector.

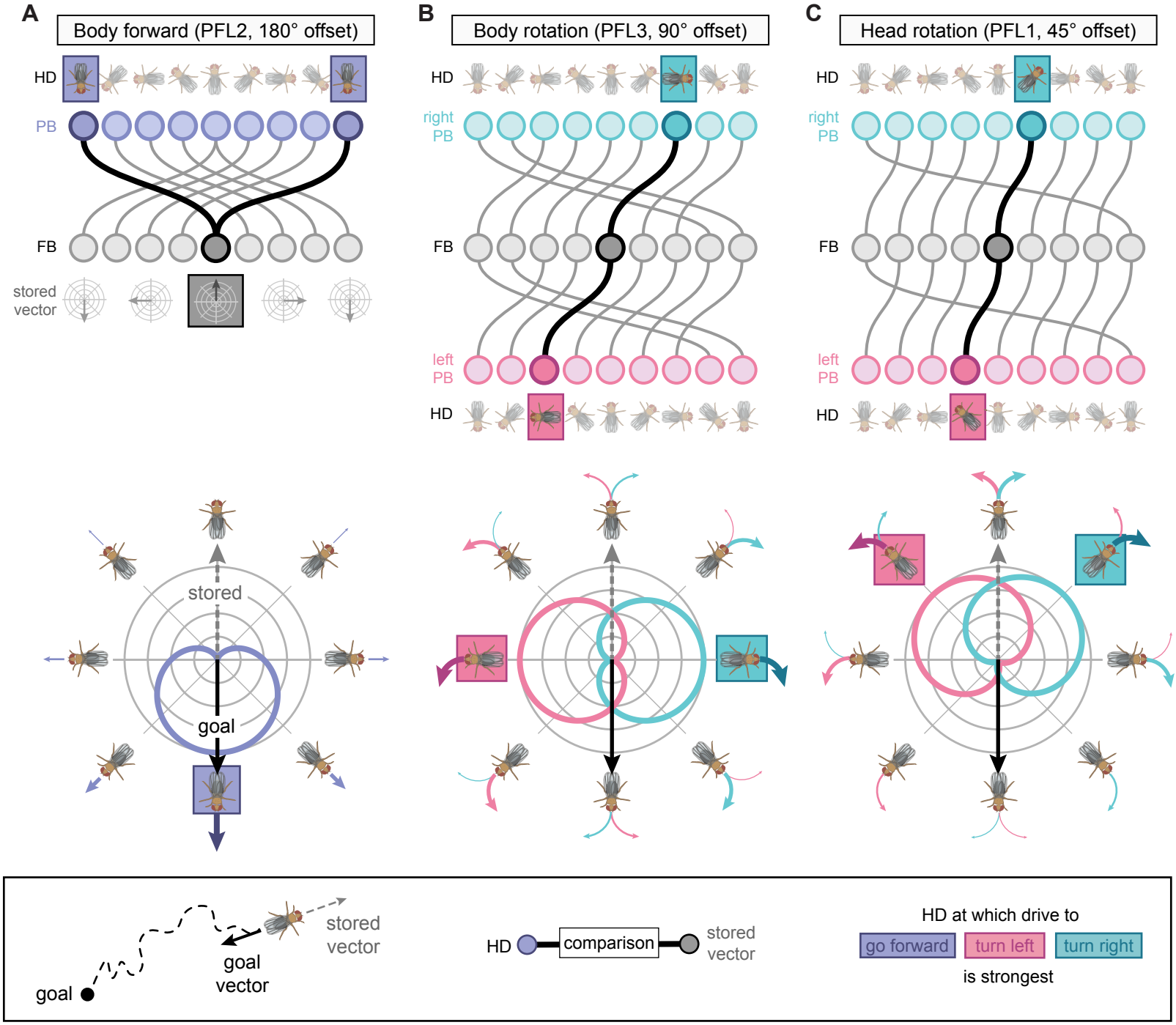


Figure 73: PFL neurons could generate egocentric motor commands by comparing the fly's allocentric head direction to an allocentric vector stored in the FB.

A) PFL2 neurons could use their 180-degree PB-FB phase shift to generate a forward velocity signal that is largest when the fly is oriented towards the 'goal vector', which in our formulation is away from the 'stored vector' (see bottom panel and Discussion text). PFL2 neurons sample a single bump in the PB and individual PFL2 neurons project to both the left and right LAL, consistent with a bilateral velocity signal like forward walking. Top panel shows a schematic of the PFL2 180-degree phase shift between PB glomeruli (top row) and FB columns (bottom row). In this example, the stored vector points due north. To return to the goal location, PFL neurons compare the fly's instantaneous head direction to the stored vector. The 180-degree phase shift ensures that PFL2 output will be largest when the fly is oriented towards the goal direction (and opposite the stored vector).

B) Similar to **A**, but for the PFL3 neuron type and its 90-degree PB-FB phase shift. Unlike PFL2 neurons, PFL3 and PFL1 neurons (**C**) sample head direction bumps from the left and right PB, and individual neurons project to either the left or right LAL, consistent with motor commands with a left/right asymmetry, such as turning. In the case of PFL3 neurons, the 90-degree phase shift ensures that the left PFL3 population will be most active when the fly is 90-degrees to the right of the goal direction. Similarly, the right PFL3 population will be most active when the fly is 90-degrees to the left of the goal direction. If we assume that the right PFL3 neurons generate right turns and left PFL3 neurons generate left turns, then the motor command would act to align the fly's heading with that of the goal direction.

C) Same as in B, but for PFL1 neurons and their 45-degree PB-FB phase shift.

Note that in all cases, the PB-FB phase shifts are an idealized version of those from **Figure 39**. The actual PFL phase shifts are not as stereotyped, since the phase shifts are continuous in anatomical space, unlike the discrete mapping schematized here.

(Based on data from Figure 39).

Figure 73—figure supplement 1

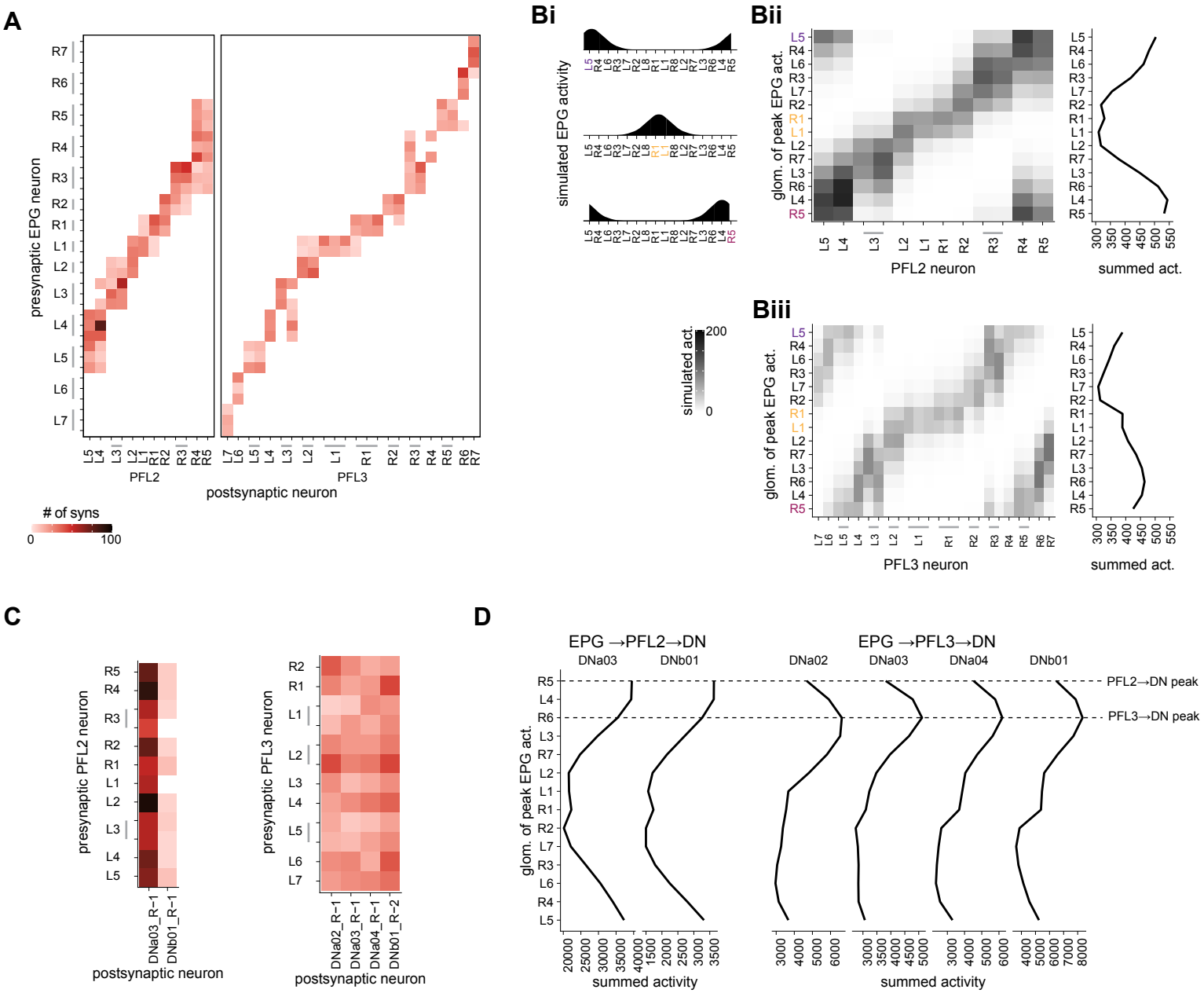


Figure 73—figure supplement 1: Numerosity and systematic asymmetries in synapse counts across columns may set up a potential 'default goal vector' through the PFL neurons.

A) Connectivity between EPG and PFL2 and PFL3 neurons shows systematic columnar variation in synapse counts and across-column spread, beyond those expected from differences in proofreading.

B) Simulated EPG activity in the PB (**Bi**) propagated across connectivity matrix in **A** would evoke differential activity in the population of PFL2 (**Bii**) and PFL3 (**Biii**) neurons in the PB depending on their columnar identity. Note that this ignores any influence that the $\Delta 7$ neurons may have on activity propagation between the EPG and PFL populations.

C) Connectivity matrix of PFL2 and PFL3 neurons to DNs.

D) Resulting summated DN activity based on propagating the activity of PFL neurons across the DNs for different positions of the EPG bump in the PB. The activity propagation shown explicitly excludes any influence on PFL activity from their many inputs in the FB. Under these assumptions, DN activity would peak at different positions (phase-shifted by 45 degrees) for the two DNs, based on whether they were activated by the bilaterally projecting (and likely forward-movement modulating) PFL2 neurons or the unilaterally projecting (and likely turn-modulating) PFL3 neurons. For reasons spelled out in **Figure 72**, this could, in principle, create a default 'goal' that could be moved in the FB. A scheme with some similarities to this, and also relying on somewhat different inhomogeneities in synaptic weights onto PFL3 neurons and modulation of activity in the FB has been proposed by (Rayshubskiy et al., 2020). Importantly, synaptic count inhomogeneities in the PB are not required for the FB-driven framework conceptualized in **Figure 72**.

Figure 74: A summary of outputs from the CX

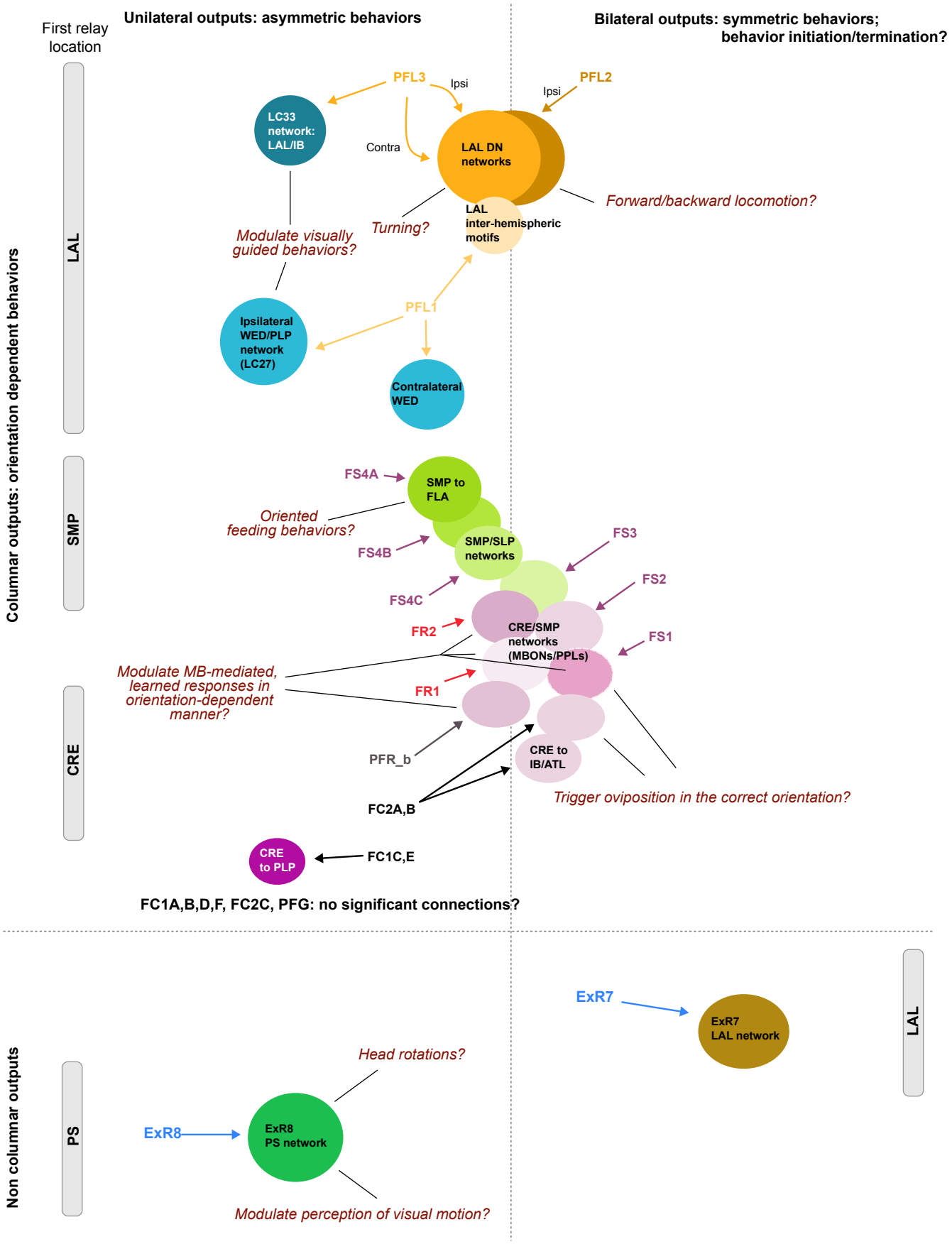


Figure 74: Summary of output networks

Schematic representation of the contributions of CX output neurons to various subnetworks and their potential functions. Outputs are divided between unilateral (like PFL3) versus bilateral (like PFL2), as those are likely to control different types of behavior (asymmetric vs symmetric), and between columnar and non-columnar, likely distinguishing between orientation-dependent and orientation-independent action selection.

Figure 74—figure supplement 1

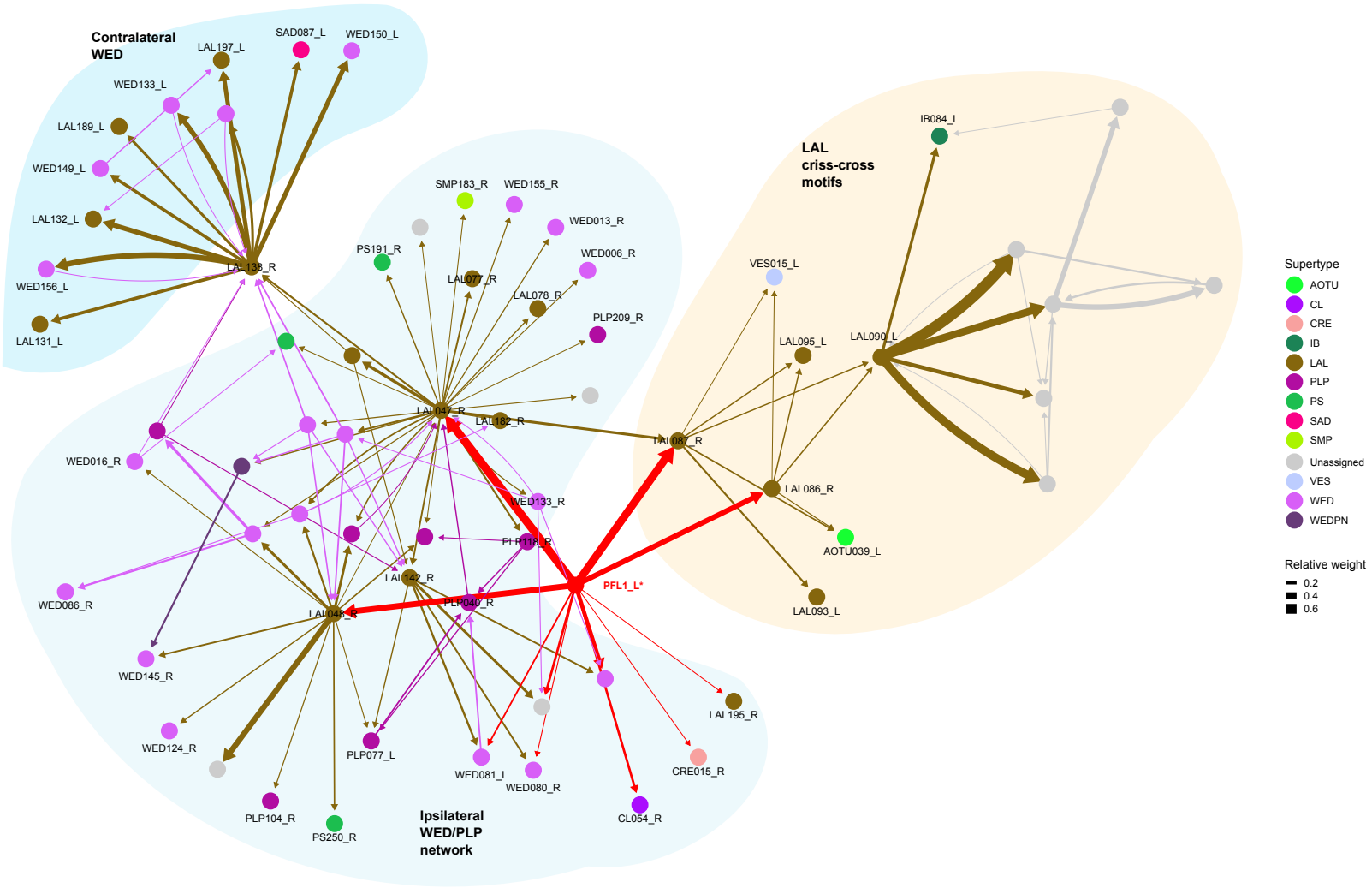


Figure 74—figure supplement 1: PFL1 subnetworks, rationale behind Figure 74

Network diagram of all targets of PFL1 receiving at least 0.5% of pathway weight from PFL1. We divide the network into three domains:

- Projections to the ipsilateral WED and PLP

- Projections to the contralateral WED through a single neuron, LAL138.

- Criss-crossing motifs: a set of LAL neurons that cross the midline back and forth.

We analyzed these networks and the morphology of the neurons that constitute them to construct **Figure 74**.

Figure 75: A deep recurrent neural network with a difference

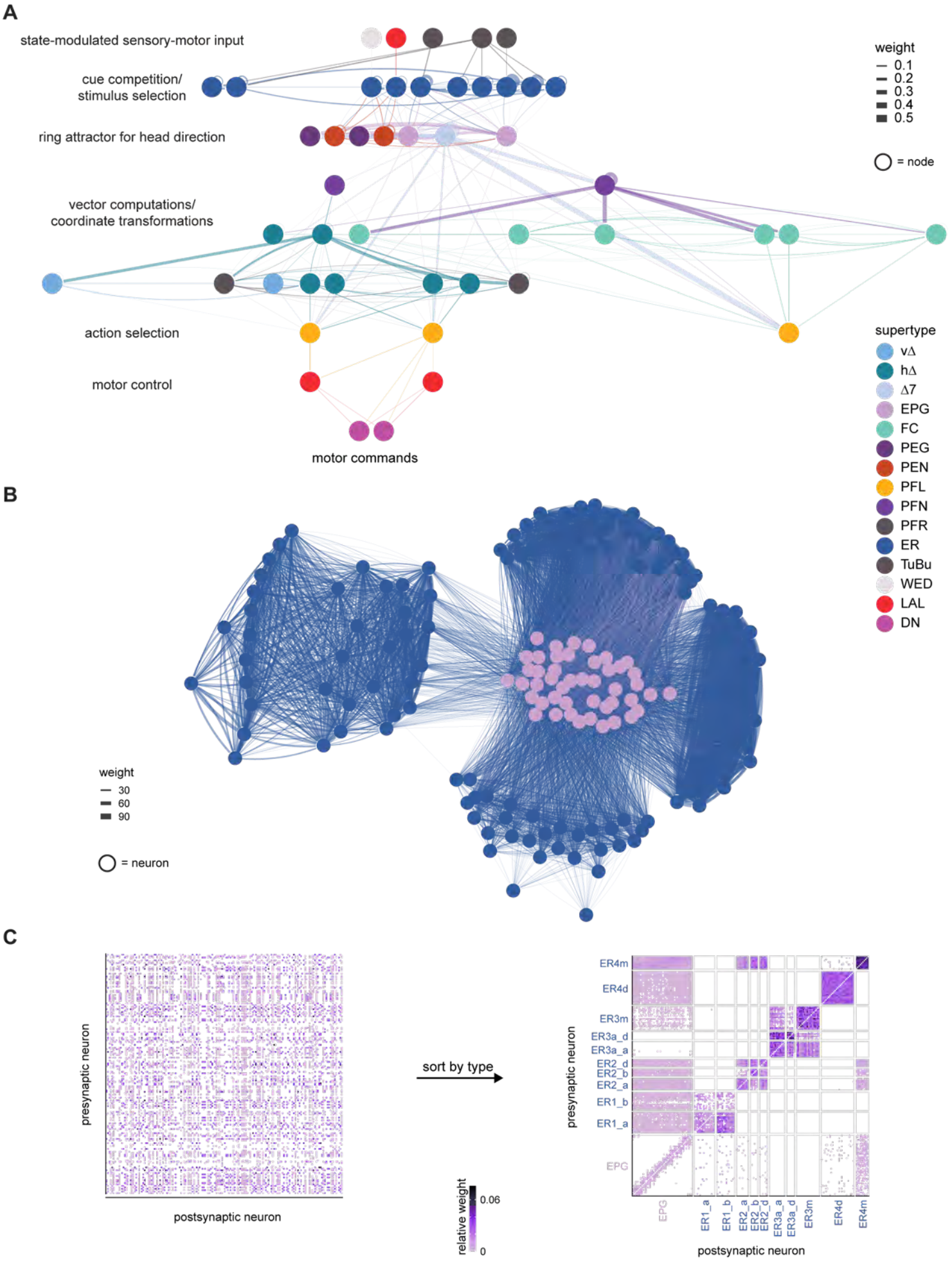


Figure 75: The CX seen as a deep recurrent neural network for navigation

A) A layered representation of the connectivity of a selection of neuron types in the CX, with a bias towards those involved in navigation. Layers have been labeled by their putative computational roles in a navigational context.

B) The connectivity of ER4m, ER3a_a,d, ER3m, ER4d, ER2_a,b,d, ER1_a,b, and EPG neurons is densely recurrent. However, different neuron types have specific roles in circuit function. The ER types plotted here are also the types plotted in layer 2 (cue competition/stimulus selection) in **A**.

C) If neurons in **B** were unsorted, the structure in their connectivity would be difficult to recognize (left). When properly sorted by types, the structure in the network connectivity becomes clear (right). The neuron names were randomly shuffled to generate the unsorted plot at left.

Figure 75—figure supplement 1: The structure in the FB connectivity becomes clear when neurons are sorted by type

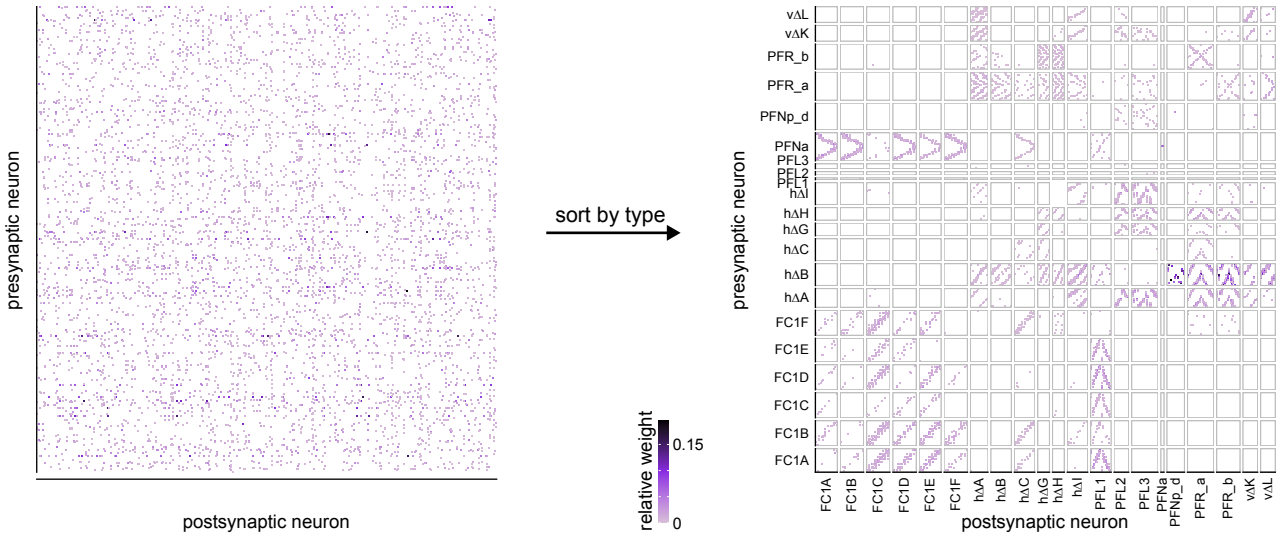
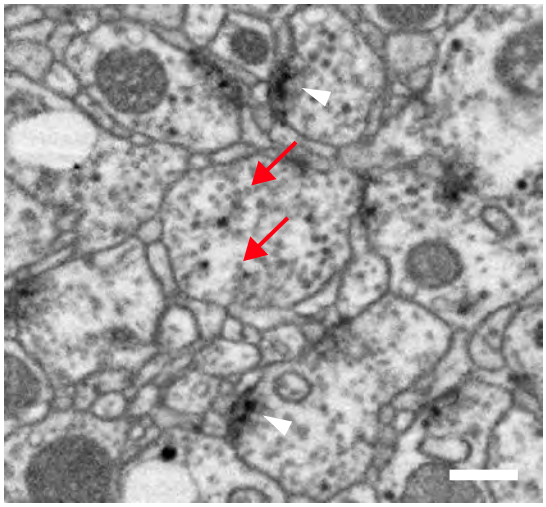


Figure 75—figure supplement 1: The structure in the FB connectivity becomes clear when neurons are sorted by type

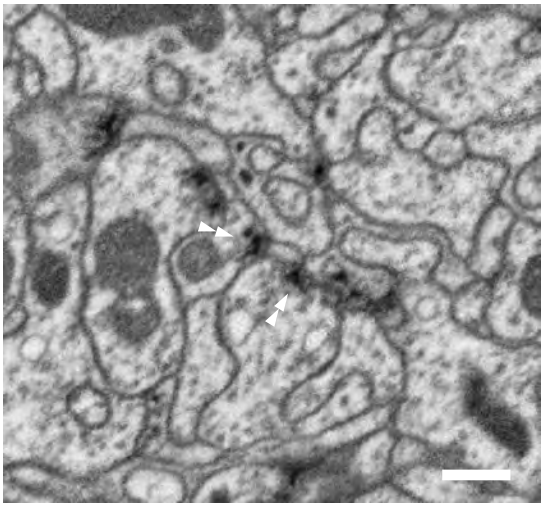
As in **Figure 75 C**, but now for the neurons in layers 4-7 (vector computations/coordinate transformations, action selection) of **Figure 75 A**.

Methods Figure 1: Regular and convergent synapses in the CX

A



B



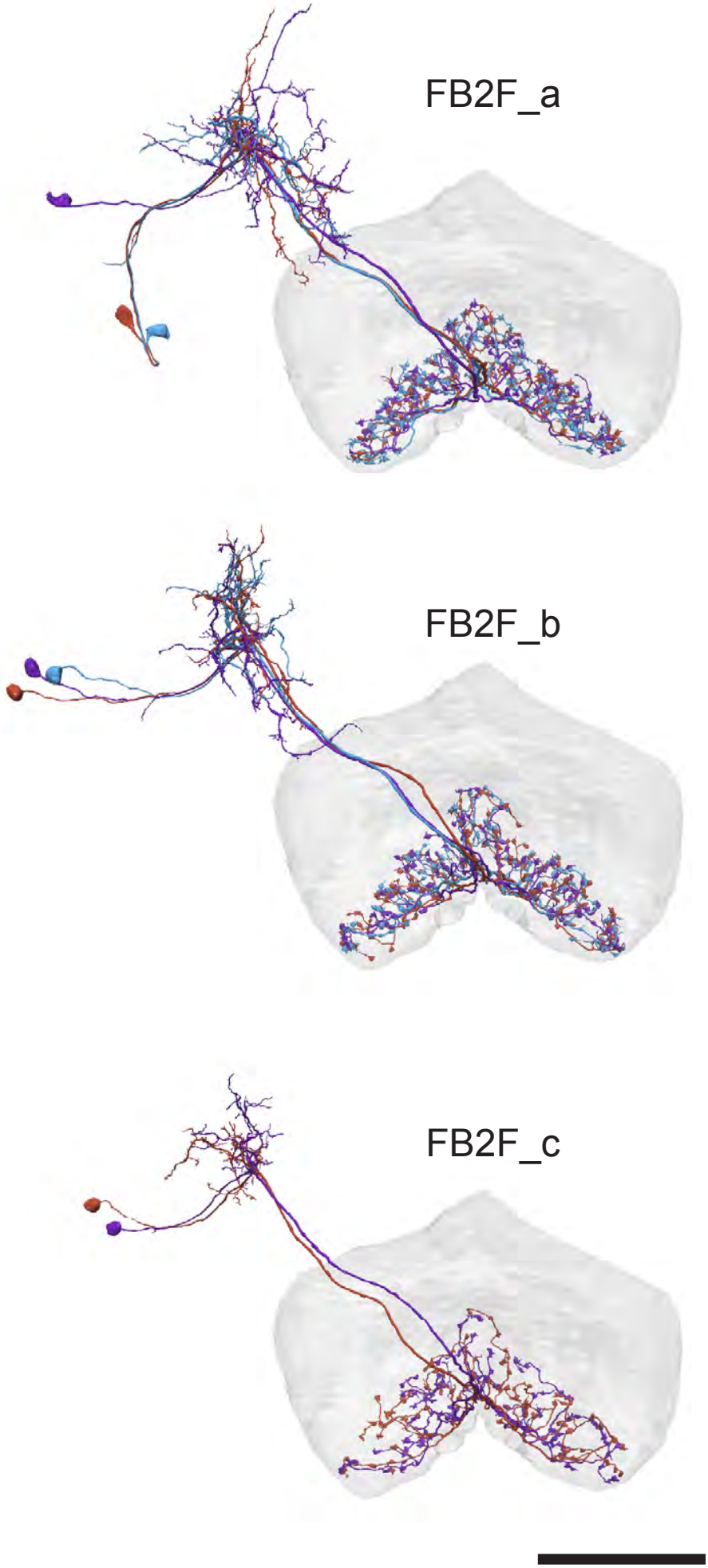
Methods Figure 1: Regular and convergent synapses in the CX

EM micrographs from the CX. Scale bars: 200 nm.

A) Typical polyadic synapses (in FB, arrowheads), and synaptic vesicles (red arrows).

B) Convergent synapses found in EB (double arrowheads).

Methods Figure 2: An example of connectivity subtypes within a single morphology type.

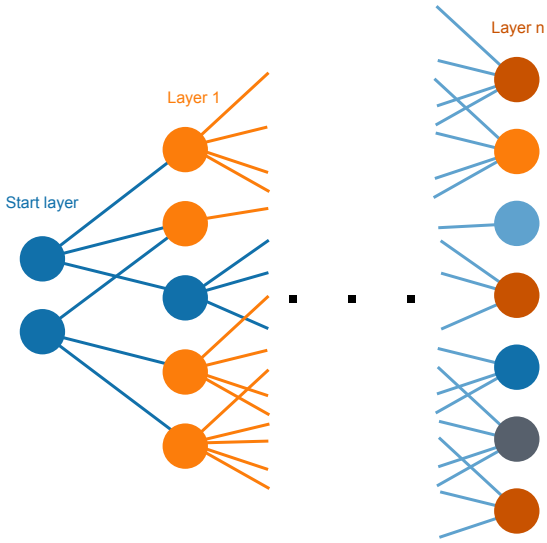


Methods Figure 2: An example of connectivity subtypes within a single morphology type. All these neurons were classified as type 'FB2F' but subdivided into three connectivity types. Scale bar: 50 μm.

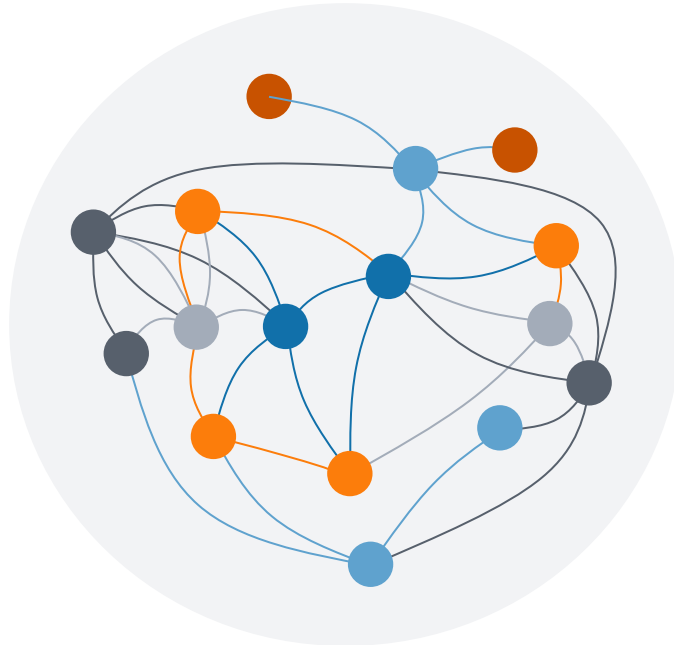
Methods Figure 3: Computing pathway weights

A

Walking down n layers

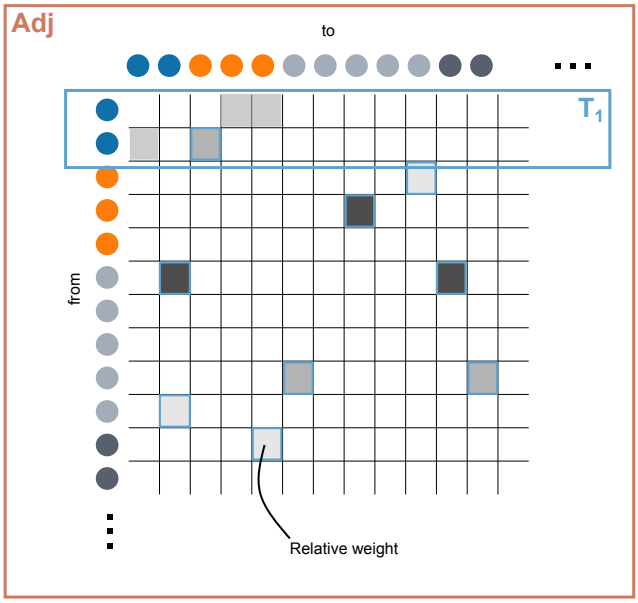


... yields an interconnected graph

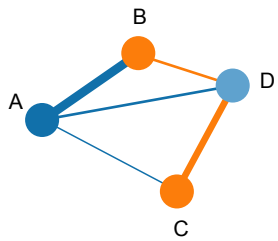


Bi

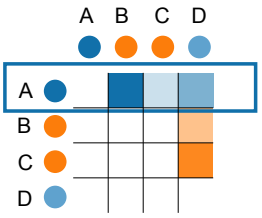
... with an adjacency matrix Adj



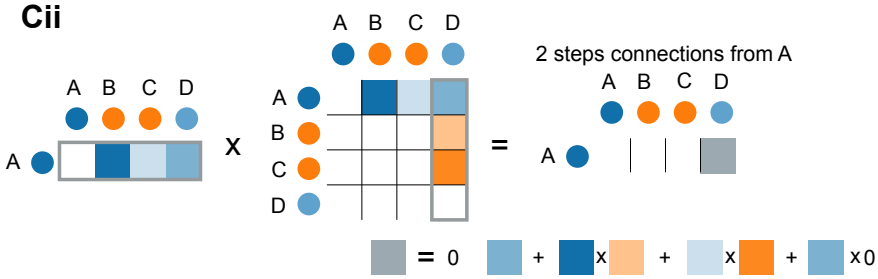
Ci



Direct connections from A



Cii



Bii

T_1 = connectivity matrix of direct connections from the source neurons

$T_2 = T_1^* * Adj$, connectivity matrix of connections of length 2 from the source neurons

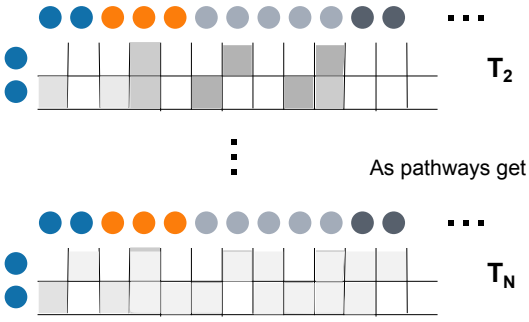
...

$T_{N+1} = T_N^* * Adj$, connectivity matrix of connections of length N+1 from the source neurons

Pathway weight = $\sum T_N$

(TN* is TN where the connections from source to source are set to zero)

D



As pathways get longer, the matrix gets denser but its norm converges to zero as weights are less than 1

Methods Figure 3: Graphical methods for pathway tracing and computation of pathway weights.

A) By walking n layers (5 in the case of the outputs network) downstream from a starting layer (here, potential CX output neurons), one obtains a complex interconnected graph.

B) Computing pathway weights:
Bi The graph obtained in A yields an adjacency matrix Adj of relative weights. T_1 is the connectivity matrix of direct connections from the source neurons.
Bii Formulas used to compute the pathway weight. The full pathway weight can be obtained by summing the powers of the adjacency matrix.

C) Toy example for a network with four neurons.
Ci Network graph and associated adjacency matrix. The first line of the matrix is the output connectivity vector of neuron A.
Cii Multiplying the first line of the matrix by the full adjacency matrix yields the two step connectivity vector from neuron A (this would be T_2 in Bii). In this case only A to D is non zero. The 2-steps weight from A to D is obtained by multiplying weights along paths and summing across path as shown in the schematic formula below.

D) Since the metrics used are between zero and one, the norm of the connectivity matrix of connections of length n converges to zero as n grows. Intuitively, when considering long paths, connectivity gets very weak and diffuse. As a consequence the pathway weights matrix, which is the sum of T_N converges to a stable value.

Since the metrics used for weights are between zero and one, the norm of the connectivity matrices of connection of length n T_N tend to zero as n grows. Intuitively, as the paths considered get longer, the connectivity gets weak and diffuse. As a consequence, the matrix of pathway weights, which is the sum over n of T_N converges to a stable value.