Supplemental File 1. Methods

We used the following fly stocks: R15A05^{AD}R28H10^{DBD} (NB1-2), R70D06^{AD}R28H10^{DBD} (NB2-1), Ac^{AD} Gsb^{DBD}, 25A05^{kz} (NB7-1), *R19B03^{AD} R18F07^{DBD} (NB7-4)*, *castor-gal4* (Technau lab), *hs-Flp.G5.PEST.Opt*(FBti0161061), *13xlexAop2(FRT.stop)myr:smGfP-Flag*(FBti0169275), *13xlexAop2(FRT.stop)myr:smGfP-V5(*FBti0169272), *13xlexAop2(FRT.stop)myr:smGfP-HA*(FBti0169269), and *hb-T2A-LexA.GADfl.* Transgenic lines were made by BestGene (Chino Hills, CA) or Genetivision (Houston, TX).

Immunostaining and imaging

Standard confocal microscopy, immunocytochemistry and MCFO methods were performed as previously described for larvae (1-3) or adults (4, 5). Primary antibodies used recognize: GFP or Venus (rabbit, 1:500, ThermoFisher, Waltham, MA; chicken 1:1000, Abcam13970, Eugene, OR), GFP or Citrine (Camelid sdAB direct labeled with AbberiorStar635P, 1:1000, NanoTab Biotech., Gottingen, Germany), GABA (rabbit, 1:1000, Sigma, St. Louis, MO), mCherry (rabbit, 1:1000, Novus, Littleton, CO), Corazonin (rabbit, 1:2000, J. Veenstra, Univ Bordeaux), FasII (mouse, 1:100, Developmental Studies Hybridoma Bank, Iowa City, IA), HA (mouse, 1:200, Cell signaling, Danvers, MA), or V5 (rabbit, 1:400, Rockland, Atlanta, GA), Flag (rabbit, 1:200, Rockland, Atlanta, GA). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA) and used according to manufacturer's instructions. Confocal image stacks were acquired on Zeiss 700, 710, or 800 microscopes. Images were processed in Fiji (https://imagej.net/Fiji), Adobe Photoshop (Adobe, San Jose, CA), and Adobe Illustrator (Adobe, San Jose, CA). When adjustments to brightness and contrast were needed, they were applied to the entire image uniformly. Mosaic images to show different focal planes were assembled in Fiji or Photoshop.

Clone generation and lineage identification

The clones were generated with the following flies: *hs-Flp.G5.PEST.Opt(FBti0161061), 13xlexAop2(FRT.stop)myr:smGfP-Flag* (FBti0169275), *13xlexAop2(FRT.stop)myr:smGfP-V5*(FBti0169272), *13xlexAop2(FRT.stop)myr:smGfP-HA*(FBti0169269), and *hb-T2A-LexA.GADfl* (see below). The embryos were collected for 7 hours in 25 ˚C, submerged in 32 ˚C water bath for 15-min heat shock, and then incubated at 25 ˚C until larvae hatched. The CNS of newly hatched larvae was dissected, stained and mounted as previously described (1-3). Antibodies used were: Dylight™405-conjugated rabbit anti-HA (Rockland), Dylight™488-conjugated rabbit anti-Flag (Rockland), and Dylight™549-conjugated rabbit anti-V5 (Rockland), and the neuropil was stained with Alexa Fluor™ 647 Phalloidin (ThermoFisher) by following manufacturer's protocol. The images were collected with Zeiss710 and processed with Imaris.

hb-T2A-LexA.GADfl was generated by in-frame fusion of T2A-LexA.GADfl to the C-terminus of the hb open reading frame with CRISPR-Cas9 gene editing. The ds-DNA donor vector for homology-directed repair was composed of left homologous arm (1098bp), T2A(6), LexA.GADfl (7), and the right homologous arm (799bp); the fragments were amplified with PCR and then assembled in pHD-DsRed (addgene #51434) with NEBuilder (New England BioLabs). The gRNAs were generated from the vector pCFD4-U6:1_U6:3tandemgRNAs (8)containing target sequence GAAACTTAGGTCTAGAATTAG and GGACGCCGTCGAACTGGCAC. The ds-DNA donor vectors and gRNA vectors were co-injected into *yw;nos-Cas9* (9) flies by BestGene. The selection marker 3xP3-DsRed was then removed in transgenic flies by hs-Cre (FBti0012692).

Lineages were identified in the EM volume by finding neurons with morphologies that matched the clonal morphology, and then identifying their neuropil entry point. We then examined every neuron which entered the neuropil in the same fascicle. In most cases, every neuron in the fascicle had a morphology that matched the clonal morphology. In a small number of cases, the fascicles diverged

slightly before the neuropil entry point. We verified the number of neurons by looking at fasciculating cell populations from at least two hemisegments (A1L and A1R). In some cases, we were able to identify a stereotyped number of cells across as many as four hemisegments, suggesting that fasciculation is stereotyped and reliable.

Morphological analysis of lineages

Morphological analysis was done using NBLAST and the NAT package (10), and analysis and figure generation was done using R. Neurons were preprocessed by pruning the most distal twigs (Strahler order 4), converting neurons to dot-props, and running an all-by-all NBLAST. For individual lineages, clusters were set using a cutoff of 3.0. In the case of NB2-1, where nearly every neuron shares a very similar morphology, we first confirmed the presence of a hemilineage using anti-Hey staining. After confirmation of a hemilineage, we next removed A02o and A02l since we could not find any clones that contained either an anterior projection (A02o) or a second contralateral projection (A02l). We reasoned that the hemilineages would represent the next largest morphological division (Fig. S1).

Synaptic distributions and density analysis

Synapse distribution plots and density contours were generated using MATLAB. Neuron synaptic and skeleton information was imported to MATLAB using pymaid (11). Cross sectional synapse distribution plots were made by taking all synapse positions between the T3 and A2 segments as positional information becomes lost due to changes in brain shape beyond these bounds. Synapse distribution plots are 1D kernel density estimates. Sensory and motor density maps were made by taking the synapse positions of all sensory neurons entering the A1 nerve, and all motor neurons exiting the A1 nerve as well as all neurons with at least 3 synapses connected to one of these neurons. For sensory and motor maps as well as individual hemilineages, density plots are 2D kernel density estimates of all synapse positions across the neuropil. A cutoff of 60% was used to set the outermost contour. For lineage maps (Figure 4F/G) , we used 80% as a cutoff. Polyadic synapses were counted as many times as they have targets. For synapse distribution plots, polyadic synapses are scaled by their number of targets.

Temporal Cohort assignment

Cortex neurite length was calculated by converting the skeletonized neuronal arbor into a directed graph away from the soma, and performing a depth-first-search of all vertices. The neuropil borders were defined by a previously created neuropil volume object (10). The neuropil entry point was defined as the first vertex within the neuropil volume object. Cortex neurite length was then the path length between the soma and the neuropil entry point. Neurons were binned into 5 groups with 6μm edges to define temporal cohorts.

Synapse similarity clustering

Synapse similarity was calculated as described previously (11):

$$f(is,jk) = e^{\frac{-d_{sk}^2}{2\sigma^2}} e^{\frac{|n_{is}-n_{jk}|}{n_{is}+n_{jk}}}$$

where $f(is,jk)$ is the mean synapse similarity between all synapses of neuron i and neuron j. d_{sk} is the Euclidean distance between synapses s and k such that synapse k is the closest synapse of neuron j to synapse s of neuron i. σ is a bandwidth term that determines what is considered close. n_{is} and n_{jk} are the fraction of synapses for neuron i and neuron j that are within ω of synapse s and synapse k respectively. We used parameters $\omega = \sigma = 4000$nm. Clusters for dendrograms were created based on the mean distance between elements of each cluster using the average linkage clustering method.

Electron microscopy and CATMAID

We reconstructed neurons in CATMAID as previously described (12-14).

Figures

Figures were generated using Matlab, R, CATMAID, and FIJI, and edited in either Adobe Illustrator or Photoshop.

Statistical analysis

Statistical significance is denoted by asterisks: ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05; n.s., not significant. All statistical analysis was done in MATLAB. When comparing two groups of quantitative data, an unpaired t-test was performed if data was normally distributed (determined using a one-sample Kolmogorov-Smirnov test) and Wilcoxon rank sum test if the data was not normally distributed. Linear models were generated in MATLAB using lmfit.

Methods citations

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