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Article

Clonal Development and Organization of the Adult *Drosophila* Central Brain

Hung-Hsiang Yu,[1,3,5] Takeshi Awasaki,[1,3,6] Mark David Schroeder,[1,4] Fuhui Long,[1,4] Jacob S. Yang,[1,4] Yisheng He,[2] Peng Ding,[2] Jui-Chun Kao,[2] Gloria Yueh-Yi Wu,[1] Hanchuan Peng,[1] Gene Myers,[1] and Tzumin Lee[1,2,*]

[1]Howard Hughes Medical Institute, Janelia Farm Research Campus, 19700 Helix Drive, Ashburn, VA 20147, USA

[2]Department of Neurobiology, University of Massachusetts, 364 Plantation Street, Worcester, MA 01605, USA

Summary

Background: The insect brain can be divided into neuropils that are formed by neurites of both local and remote origin. The complexity of the interconnections obscures how these neuropils are established and interconnected through development. The *Drosophila* central brain develops from a fixed number of neuroblasts (NBs) that deposit neurons in regional clusters.

Results: By determining individual NB clones and pursuing their projections into specific neuropils, we unravel the regional development of the brain neural network. Exhaustive clonal analysis revealed 95 stereotyped neuronal lineages with characteristic cell-body locations and neurite trajectories. Most clones show complex projection patterns, but despite the complexity, neighboring clones often coinnervate the same local neuropil or neuropils and further target a restricted set of distant neuropils.

Conclusions: These observations argue for regional clonal development of both neuropils and neuropil connectivity throughout the *Drosophila* central brain.

Introduction

In the adult brain of *Drosophila melanogaster*, we define neuropils as distinct synapse-dense areas arising due to denser local interconnectivity between neurites within one region compared to the adjacent region. These anatomical features are thereby a convenient anatomical proxy for decomposing brain circuitry into distinct subcircuits. The sets of neurons derived from the same neural stem cell progenitor, or neuroblast (NB), represent one of the few levels of organization operating at this same scale between individual neurons and gross anatomy, motivating the analysis of how NBs generate neuropils and wire them together. Given the lack of active migration of neurons outside the optic lobes (OLs), the NB lineages are expected to build regional neuropils through a series of clonal units. One common convention is to classify neurons relative to the neuropil they innervate, with a primary distinction between local interneurons (LNs), which elaborate solely within a single neuropil, and projection neurons (PNs), which project between neuropils, thereby connecting them together.

The most studied neuropils in the *Drosophila* central brain are those with a striking morphology and clear boundaries. These include the antennal lobe (AL), the mushroom body (MB), and the components of the central complex (CX), which include the protocerebral bridge (PB), the fan-shaped body (FB), the ellipsoid body (EB), and the paired noduli (NO). All of these neuropils are composed of anatomically distinct subregions, such as the glomeruli of the AL [1] and the input calyx and output lobes of the MB [2], as well as array-like structures within the components of the CX [3]. These cases make clear that the subdivisibility of the brain into neuropils represents a convenient idealization, but the substructure within neuropils and the superstructures that span them indicate that the level where the division is drawn is somewhat arbitrary. Recently, the Insect Brain Name Working Group has generated a standardized set of 33 neuropils, building off previous efforts at generating a standard brain nomenclature [4, 5] (K. Ito, personal communication). Although the choice of neuropil boundaries that best reflect the underlying circuitry can be debated, the effort at standardization makes the standardized 33 a good set to analyze, based on current knowledge and common terminology.

Most NBs in the central nervous system (CNS) have similar proliferation patterns, where repeated asymmetric divisions generate a series of ganglion mother cells (GMCs) that divide once to produce a Notch-high A sibling and Notch-low B sibling [6, 7]. Serially produced neurons that share an A or B fate tend to be of the same neuronal class, such as LN versus PN, and this has led to the concept of "hemilineages" [8]. More recently, the posterior asense-negative (PAN), or type II NBs [9, 10], have been found, which generate a series of intermediate neural progenitors (INPs) through asymmetric divisions that then produce a relatively short series of GMCs. Most NBs undergo two periods of proliferation: one during embryogenesis that generates the larval nervous system and a second during larval development that generates the adult nervous system [11–13]. The only exceptions are the MB NBs and the lateral lineage of the AL (lAL), which skip the quiescent period beginning in late embryogenesis to generate many more neurons than most lineages [11]. Another exception to this pattern is the NB precursors of the OL, which form a separate neuroepithelium that proliferates to generate many NBs before producing migratory neurons that do not maintain cell-body clustering [14].

Technau and coworkers have identified 106 uniquely identifiable NBs that delaminate in a stereotyped spatiotemporal pattern within the procephalic neurogenic region of early *Drosophila* embryos [15, 16]. The procephalic region plus the OL primordium form the supraesophageal ganglion (SPG), which is fused with the subesophageal ganglion (SEG), and these together form the adult fly brain. Because the OL has a more complex clonal structure, we focus on the central portion of the SPG, which is termed the cerebrum (K. Ito, personal communication), to analyze the relationship between NBs and brain anatomy. In both the brain and thoracic

[3]These authors contributed equally to this work
[4]These authors contributed equally to this work
[5]Present address: Institute of Cellular and Organismic Biology, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan
[6]Present address: School of Medicine, Kyorin University, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan
*Correspondence: leetz@janelia.hhmi.org

ganglion of larvae, NB clones containing immature larval-born neurons generate just one or two well-defined tracts [12, 17], but it is unclear if this simplicity persists in the adult brain.

Through clonal analysis of larval-born neurons labeled with ubiquitous drivers, we identified 95 stereotyped neuronal lineages in the adult *Drosophila* cerebrum. The clones show lineage-characteristic features in clone size, cell-body distribution, neurite projection, and neuropil innervation. Most clones show immediate neurite elaborations in specific local neuropils before targeting other brain regions. Using these clones aligned to a preselected brain [4, 18], we outline the strongest clonal trajectories between neuropils. In sum, this exhaustive clonal study has uncovered the majority of the developmental building blocks of the adult *Drosophila* cerebrum, paving the way for single-cell lineage mapping of brain development and circuitry to ultimately build a complete cellular and developmental fly brain map.

Results

Identification of 95 NB Lineages

In order to clearly visualize all the progeny present in one NB clone, it is necessary to generate sparse clones with ubiquitous drivers. However, the lack of cell-type specificity in ubiquitous drivers requires a genetic strategy that can label one to two NBs out of the hundreds of NBs and the even larger pool of GMCs dividing during larval development. In order to achieve the necessary specificity, we utilized "flip-out MARCM," [19] a composite strategy where mitotic MARCM clones [20] and flip-out of a stop cassette blocking GAL4 expression were both under control of the FLP recombinase driven by a heat shock-inducible promoter. Notably, flip-out MARCM significantly increased the specificity of clone induction, as evidenced by a drastic reduction of background clones, presumably due to the requirement of two distinct FLP-mediated events.

Using flip-out MARCM, we collected confocal stacks for over 1,500 of our most sparsely labeled NB clones in the cerebrum, generated upon larval hatching. Such NB clones lack embryonic-born primary neurons. We grouped clones based on cell-body cluster position followed by neurite projection pattern and found stereotypy in cell-body distribution and neurite elaboration among members of the same group (e.g., Figure 1; see Table S1 available online). This stereotypy facilitated identification of neuronal lineages and is consistent with previous work where individual precursors generate defined sets of progeny neurons. For example, clones of the SLPa&l1 lineage (see below for the naming convention) consistently contain two cell-body clusters located near the anterior dorsolateral corner of the cerebrum (Figures 1A and 1B). Their neurites selectively innervate the lateral domain of the SLP, the Clamp (CL; SCL/ICL) surrounding the MB peduncle, the LAL, the WED, and the LO in the OL (see Figure 3 for a neuropil schematic). Distinct lineages exhibit different characteristic morphologies, such as the neurite entry bundle where the primary neurites from a cell-body cluster enter the neuropil, allowing unambiguous separation of nearby clones into different lineage groups. For instance, the neighboring LHl1 lineage carries only one cell-body cluster and specifically targets the lateral horn (LH), SLP, and superior intermediate protocerebrum (SIP), plus the laterally located AVLP and PVLP, in addition to the LO of the OL (Figures 1C and 1D).

Through manual annotation of the detailed neuropil innervation patterns, we established the presence of 92 stereotyped clones (Figure 2; Table S1, and see Figure S2 for larger images of the clones). Given four "equivalent" copies of the MB lineage that yield indistinguishable neurons during postembryonic development [21], we have identified 95 neuronal lineages per cerebral hemisphere. The difficulty in distinguishing the four MB lineages based on adult clone morphology suggests our catalog of clones may actually cover more than 95 distinct NB lineages. Given the large range in clone frequency (Table S1), it is not possible to use this sort of information to guess if certain NBs are indistinguishable duplicates, as in the MB case. The fusion of segments in the brain and integration of adjacent neuropils complicates assignment of NB clones bordering the OL and SEG, such that a few may have inadvertently been missorted. Because there is currently no suitable technique for reliably tracking NBs between the embryo and adult, position and lack of neuronal migration were used as criteria to focus on the cerebral NB clones.

Lineage-Characteristic Clone Sizes and Morphologies

Distinct lineages differ greatly in clone size, and some lineages carry two clusters of cell bodies with independent neurite tracts. Counts of cell bodies demonstrated that each lineage produced a characteristic number of offspring that survived into the adult stage (Table S1). Although most lineages generated 30–150 neurons, the MB, ALl, and PAN lineages generate ~200 or more neurons, due to their special proliferation patterns. There were also two notably small lineages, the FLAa1 and PBp1 lineages, that contain only nine neurons (Table S1 and Figure S1A). Such ultrashort lineages might result from premature NB death [22].

To assess the fates of the first larval neurons derived from a given NB, we repeated the clonal analysis with twin-spot MARCM, which allows differential labeling of sister clones and thus the detection of NB clones with their paired GMC (or INP) progeny (Figures 1E–1H) [23]. We obtained NB clones for 73 of the 95 cerebral lineages and saw that each had a characteristic first progeny. Distinct NB clones associated with different numbers of postmitotic neurons (Table S1). First, we validated seven PAN lineages because their NB clones consistently pair with six to nine mature neurons. However, the much smaller eighth PAN NB clone (the DL2 lineage) paired with only one adult neuron, requiring other strategies to confirm its PAN NB origin (our unpublished data). The six dorsomedial PAN NB clones correspond to the known larval DM1-6 lineages, whereas the two dorsolateral PAN NB clones are referred to as the DL1 and DL2 lineages, respectively. Second, other NB clones paired with no more than two neurons, consistent with their generating GMCs, as in other lineages. In cases where NB clones have two cell-body clusters and paired with two viable neurons, we consistently observed presence of one first larval-born neuron in each cell-body assembly (Figures 1G and 1H) [17]. As implicated from the ensembles' morphologies, these sister neurons show distinct cluster-specific neurite projections, indicating a sister hemilineage relationship between the separate ensembles from the same lineage. In addition, we have detected in different individuals not only the same cell number (Table S1) but also undistinguishable morphology for the first-born larval offspring, supporting the notion that diverse neurons arise from each neural progenitor in a stereotyped sequence.

Notably, two-thirds of single-cell paired NB clones carry fewer than 80 neurons. By contrast, two-thirds of two-cell paired NB clones contain more than 100 neurons. These

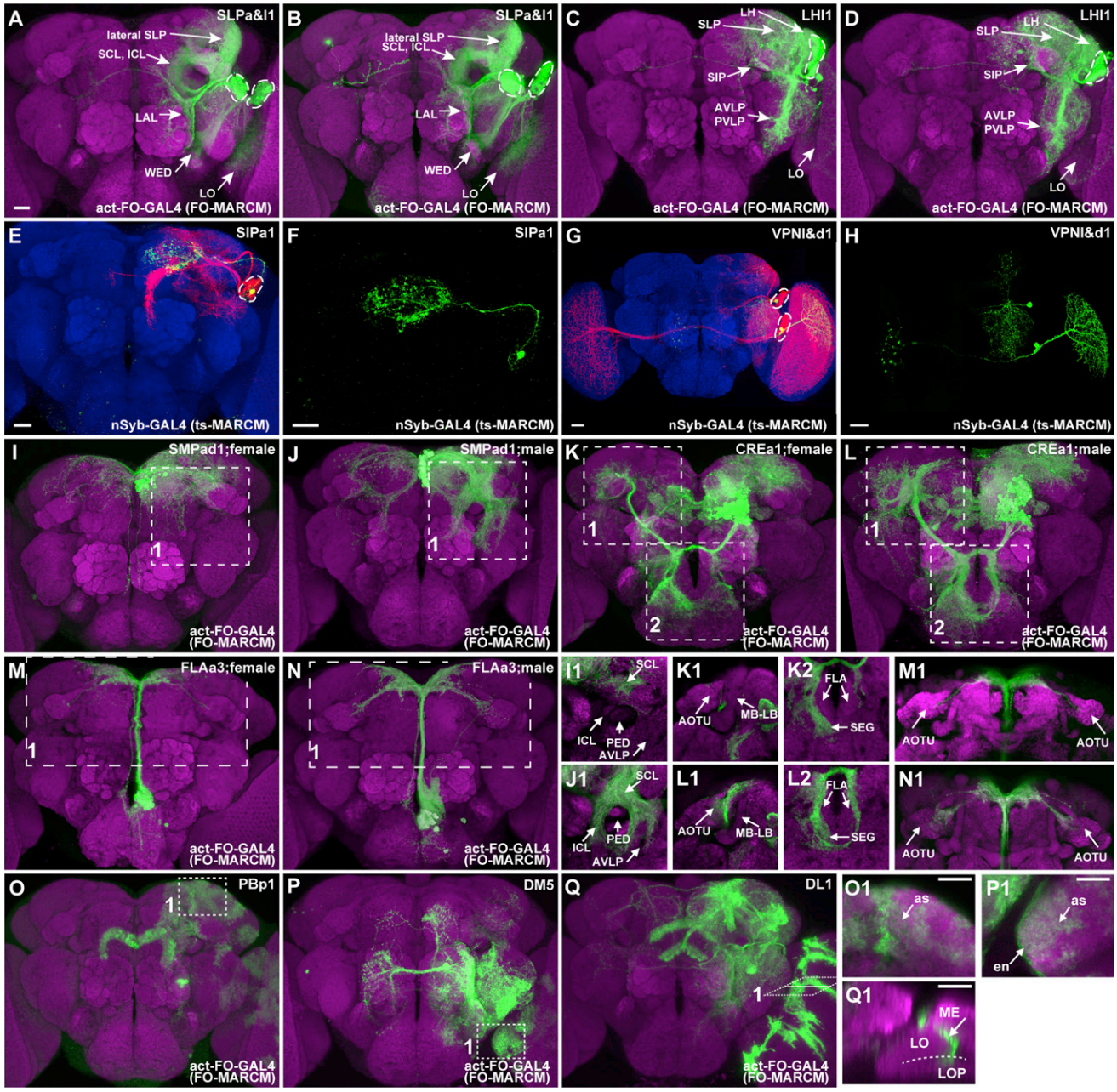


Figure 1. Representative NB Clones with Stereotyped Morphologies

(A–D) Merged confocal images of MARCM clones (green) in nc82-counterstained adult *Drosophila* brains (magenta). The same NB clone (SLPa&l1) was hit in (A) and (B); a neighboring but distinct clone (LHl1) was identified in (C) and (D). Note their possession of two versus one cluster of cell bodies (dashed circles) and the innervation of distinct sets of neuropils (arrows).

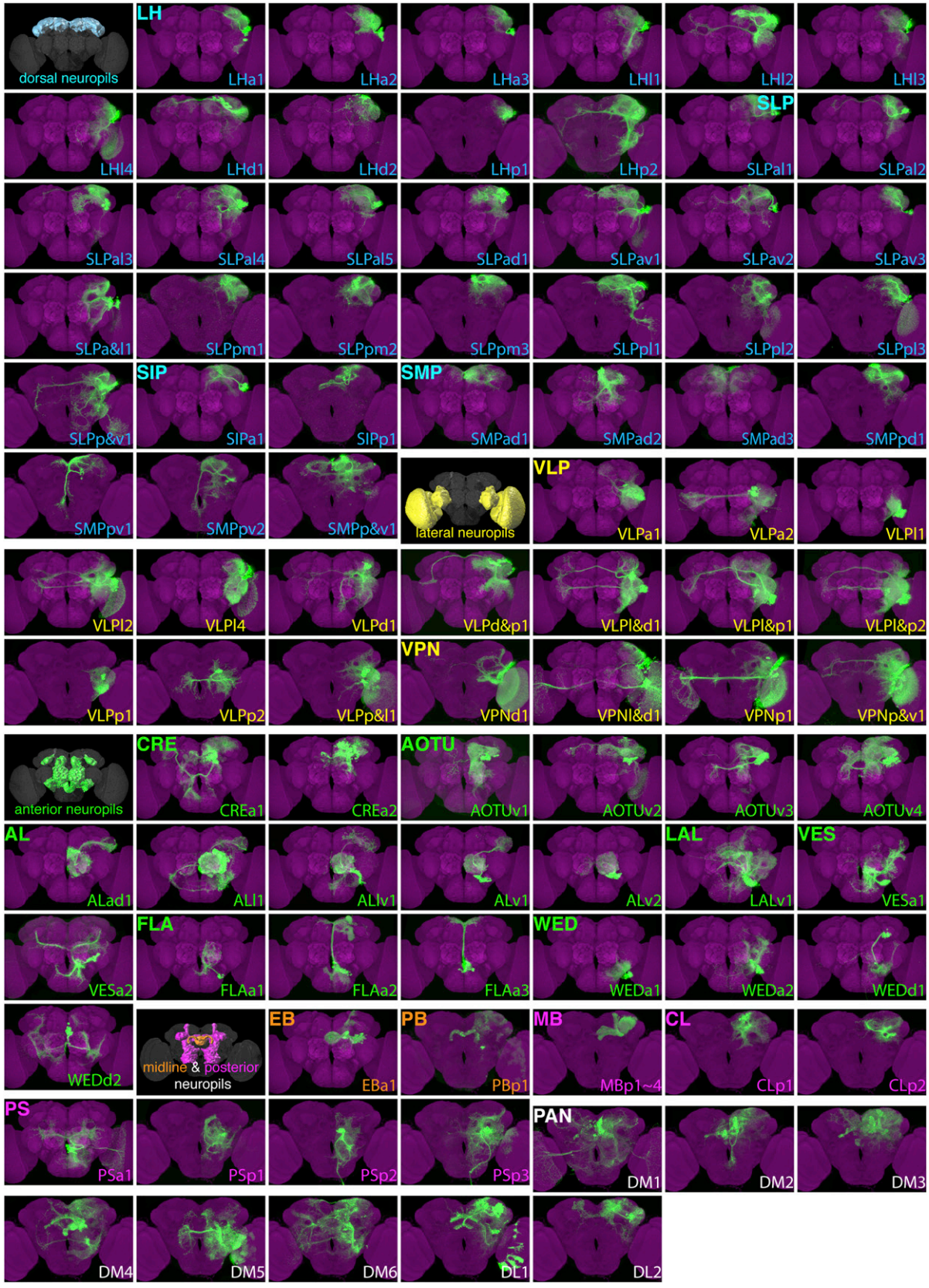
(E–H) Adult SIPa1 (E and F) and VPNl&d1 (G and H) clones, induced shortly after larval hatching, were labeled with twin-spot MARCM. As revealed from the paired GMC clones (green), the first larval-born GMC yielded only one viable neuron in the SIPa1 lineage but produced two neurons in the VPNl&d1 lineage. Note the segregation of the twin VPNl&d1 neurons with distinct projections (H) into each of the two cell-body clusters (dashed circles) present in the NB clone (G).

(I–N) SMPad1, CREa1, and FLAa3 clones exhibit gender-specific neurite elaborations, as pointed out with arrows in the subpanels of (I)–(N).

(O–Q) PBp1, DM5, and DL1 clones show glia-like elaborations. Close-up views (insets) reveal astrocyte-like (as) glia in the PBp1 clone, both ensheathing (en) and astrocyte-like (as) glia in the DM5 clone, and optic lobe glia separating the medulla (ME) and lobule/lobule plate (LO/LOP) in the DL1 clone.

Scale bars represent 20 μm. Spatially segregated background clones were removed in some cases.

observations support the interpretation that hemilineage-dependent cell death plays an important role in NB clone size differences (Figure S1B). In clones with two distinct cell clusters, the smaller cluster contains roughly 40% of the neurons in the larger cluster (Table S1), suggesting that a significant amount of temporally controlled cell death may also occur. The LHa1 and WEDd2 lineages exhibit an extreme example of this phenomenon, with one separate neuron with



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a unique projection paired with the main cell-body cluster (Figure 2). Differential apoptosis may also explain why the DM5 and DL2 PAN NBs produce many fewer neurons than the other PAN clones [24].

Few NB Clones Show Obvious Sexual Dimorphism or Glia-like Elaboration

Three lineages, including SMPad1, CREa1, and FLAa3, exhibit obvious gender-dependent dimorphic phenotypes (Figures 1I–1N; Table S1). Notably, the male clones of all three lineages carry more cells and elaborate more exuberantly than their female counterparts. The SMPad1 lineage shows male-specific elaborations around the MB peduncle in both brain hemispheres, especially within the ipsilateral superior clamp (SCL), inferior clamp (ICL), and AVLP (Figures 1I and 1J). The CREa1 lineage differentially innervates the contralateral anterior optic tubercle (AOTU)/SLP/mushroom body lobe (MB-LB) and the ipsilateral flange (FLA)/SEG in male versus female brains (Figures 1K and 1L). The FLAa3 lineage, by contrast, acquires bilaterally symmetric neurite trajectories that prominently extend into the AOTUs in the male brain only (Figures 1M and 1N). These sexually dimorphic cerebral lineages probably contain the aSP-a/aSP2, aDT-b/mAL/aDT2, and aDT-h/mcALa/aDT6 clusters of Fru-expressing neurons, respectively [25–27]. The sexual dimorphism of the CREa1 clones has been shown to result from programmed cell death of neurons with male-characteristic projections in the female brain lacking male-specific Fru, again highlighting cell death in shaping lineage composition [28].

Three lineages, including PBp1 and two PAN lineages, make glia-like progenies occupying large brain territories (Figures 1O–1Q). The PB lineage yields about nine neurons that exclusively innervate the PB, but it produces many astrocyte-like cells that scatter in the dorsal and lateral cerebrum and further into the OL. By contrast, the DM5 PAN lineage deposits heterogeneous populations of neurons and glia. We detected ensheathing as well as astrocyte-like glia in DM5 NB clones. Notably, the astrocyte-like glia of the PB and DM5 clones show a complementary distribution. In both the cerebrum and the OL, the glial cells of PBp1 lineage populate more dorsolateral areas than the analogous DM5-derived glia. The origin-dependent production of specific glial subsets is also evident in the DL1 PAN lineage, which makes elongated OL glia that align at the interface between LO/lobula plate (LOP) and ME.

There should be additional sexually dimorphic lineages, including possibly male-only lineages, that carry Fru-positive neurons [25–27], and most, if not all, of the PAN lineages yield neurons plus Repo-positive glial offspring [29, 30]. We tentatively mapped major Fru-positive clusters onto those NB clones whose sexual dimorphism could be masked by the gross complexity (Table S1), but it is difficult to map *fru* clones containing only a few neurons. To unveil all sexually dimorphic or glia-producing neuronal lineages requires more detailed morphological analysis of not only entire clones but also their specific constituents.

Regional Clustering of Neuropil-Characteristic Neuronal Lineages

Notably, almost all cerebral clones have an entry bundle that leads to one or more local neuropils, suggesting that regional establishment of neuropils may be a general phenomenon. Our clones further group well based on the defined neuropil regions and roughly support the current neuropil definitions. We therefore use a regional naming convention based on the main adjacent neuropil, except for the eight PAN NBs, which have complex projection patterns and are more simply designated by cell-body locations.

Eighty-four stereotyped clones were individually assigned a "home" neuropil, based on prominent proximal neurite elaboration, and were subsequently grouped into 18 neuropil-oriented families. They were then named following the pattern NPp#, where NP is the neuropil, p is the cell body position, and # is the number that together with the other elements yields a unique name. Our collection and the independent effort by Ito et al. [31], also published in this issue of *Current Biology*, have been coordinated to follow the same naming convention and clone assignment. For example, the two collections have jointly uncovered 12 non-PAN clones that show prominent immediate neurite elaboration in the LH and are thus placed into the LH lineage family. The cell bodies of these LH clones lie anterior (a), lateral (l), dorsal (d), or posterior (p) to the LH and are therefore called LHa1–4, LHl1–4, LHd1–2, and LHp1–2, respectively. Ten of the twelve LH lineages, excluding LHa4 (unique in the Tokyo collection) and LHd2 (unique in the Janelia collection), were dually identified (Table S1). For known lineages, such as adPN or lAL, we renamed them ALad1 and ALl1, which essentially maintains the original naming. In common use, we expect people to give the full name prior to simplifying to the minimal unique length such as ALad. In this way if additional clones are found in a group, uniqueness will be maintained, but shorter names can be used in practice. In addition to the eight PAN lineages, we identified 11 LH lineages, 17 SLP lineages, 2 SIP lineages, 7 SMP lineages, 13 ventrolateral protocerebrum (VLP) lineages, 4 visual projection neuron (VPN) lineages (with the OL as the "home neuropil"), 2 CRE lineages, 4 AOTU lineages, 5 AL lineages, 1 LAL lineage, 2 VES lineages, 3 FLA lineages, 4 WED lineages, 2 CL lineages, 4 posterior slope (PS) lineages, 1 PB lineage, 1 EB lineage, and 4 MB lineages (Figure 2).

Some neuropils host many distinct lineages, but certain neuropils are pioneered by just a few lineages. For instance, the 17 SLP lineages occupy different subregions within the SLP and further relay the zone-specific information to distinct brain domains. This could reflect the relatively large size or substructural complexity of the SLP and its possible function as a regional hub in the neural circuitry of the cerebrum. By contrast, the CRE is mainly patterned by two lineages with analogous CRE-related neurite elaboration and projection patterns. Interestingly, the most obvious difference between the CREa1 and CREa2 clones is the extra, sexually dimorphic, neurite tract system that primarily innervates regions outside the CRE. We did not find lineages selectively dedicated to eight neuropils: the anterior PRW/SAD, the central FB/NO,

Figure 2. Catalog of Cerebral NB Clones

Ninety-two stereotyped NB clones (green) are shown individually after warping into an nc82-counterstained adult *Drosophila* brain (magenta). They are named according to their primary immediate neuropil targets, referred to as home neuropils, and cataloged by grouping home neuropils into dorsal, lateral, anterior, and midline/posterior neuropil sets. Note the brains are shown with the anterior or posterior surface up, depending on the location of the clone cell bodies. Spatially segregated background clones were removed in some cases. Note that the PSa1 clone originates from anterior brain surface. See the same images at higher resolution in Figure S2 and the 3D images in Virtual Fly Brain: www.virtualflybrain.org.

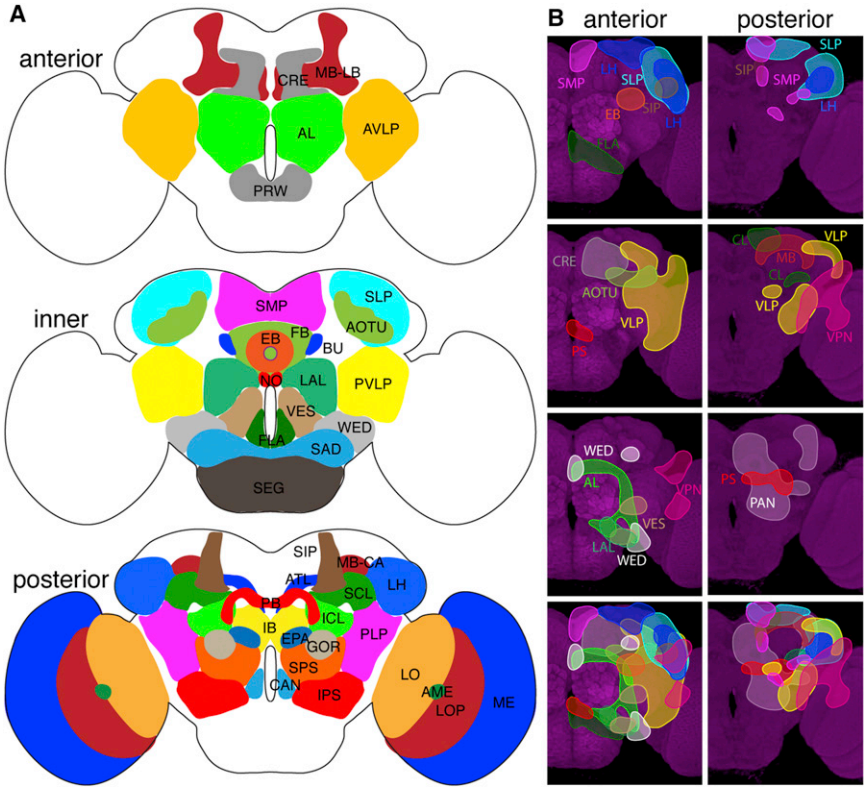

Figure 3. Cell-Body Distribution of Neuropil-Characteristic Neuronal Lineages

(A) Illustration of neuropils that have been arbitrarily assigned to three cross-sections in the adult *Drosophila* brain.

Anterior neuropils: AL, antennal lobe; AVLP, anterior ventrolateral protocerebrum; CRE, crepine; MB-LB, mushroom body lobe; PRW, prow.

Inner neuropils: AOTU, anterior optic tubercle; BU, bulb; EB, ellipsoid body; FB, fan-shaped body; FLA, flange; LAL, lateral accessory lobe; NO, noduli; PVLP, posterior ventrolateral protocerebrum; SAD, saddle; SEG, subesophageal ganglion; SLP, superior lateral protocerebrum; SMP, superior medial protocerebrum; VES, vest; WED, wedge.

Posterior neuropils: AME, accessory medulla; ATL, antler; CAN, cantle; EPA, epaulette; GOR, gorget; IB, inferior bridge; ICL, inferior clamp; IPS, inferior posterior slope; LH, lateral horn; LO, lobula; LOP, lobula plate; MB-CA, mushroom body calyx; ME, medulla; PB, protocerebral bridge; PLP, posterior lateral protocerebrum; SCL, superior clamp; SIP, superior intermediate protocerebrum; SPS, superior posterior slope.

(B) Illustration of neuropil-characteristic clonal cell-body distributions on the anterior or posterior brain surface. Clonal cell-body loci for various neuropils shown in different colors in the top panels are superimposed in the bottom panel to reveal the overall coverage by the identified clones.

and the posterior ATL/IB/EPA/GOR/CAN neuropils. The lack of obvious neuropil-specific lineages could result from derivation from the SEG (e.g., PRW, SAD, and CAN), the PAN lineages (e.g., FB, NO, ATL, and IB), or assignment of the clone to adjacent neuropils where the NB elaborates more extensively (e.g., SAD, ATL, EPA, and GOR). The ALl lineage produces non-AL neurons based on a fate switch in the PN hemilineage leading to innervation of other target neuropils, including the SAD and SEG [32]. This indicates that anatomical neuropils and developmental origin are related, but not necessarily in a one-to-one fashion.

Given the minimal migration of postmitotic neurons, the clonal cell body positions should roughly reflect the responsible progenitors' relative developmental loci in the brain primordium. Mapping the cell body distribution for clones that coinnervate a given neuropil should therefore hint where the neuropil arises. Notably, the clonal-unit families of dorsal neuropils (LH/SLP/SIP/SMP) as well as lateral neuropils (VLP/OL) consist of clones whose cell bodies may reside anterior, posterior, dorsal, or lateral to the involved neuropils that jointly cover the dorsal and lateral periphery of the cerebrum (Figure 3B). By contrast, centrally located neuropils originate largely from either anteriorly or posteriorly situated clones (Figure 3B).

Neuropil Connectivity Deduced from Clonal Innervation

It has been proposed that the neural circuits of the *Drosophila* brain are generated in a modular fashion from a series of clonal units [12, 17, 33]. However, only seven lineages have restricted elaboration in five or fewer neuropils, and over 40 lineages target 15 or more brain compartments, indicating that the situation is more complex (Figure S1C). The innervation of multiple neuropils by a given clone is likely to reflect neuronal diversity with overlapping projection patterns complicating determination of neuropil connectivity. Given that our clones cover 90% of the neurons in the cerebrum and that there are clear trends in neuropil connectivity, we combined manual analysis of projection patterns in neuropils generated by two or fewer clones with computational analysis of preferred distal targets shared by the majority of clones in the larger families. By analyzing "anatomical connectivity" between neuropils, rather than connectivity at the neuronal level, we avoid the difficulties of determining neuronal connections at the light level. Although this approach may not be optimal, it is straightforward and provides a map of the major NB-based connections that can be refined by future single-cell lineage studies.

Toward this aim, we first aligned 95 clean clones (representing 92 distinct lineages plus three male clones showing sexual dimorphism) with a fly brain template containing 33 segmented neuropil regions in each hemisphere [4, 18]. This allowed us to compute the fractional innervation of each neuropil by each NB, which can be conveniently visualized as a heat map (Figure 4). To use the clonal innervation to generate directional connectivity, we made the simplifying assumption that the proximal home neuropil was input and any other neuropil with 20% or higher voxel coverage by more than half of the family members was a target neuropil or output neuropil (Figure 5). The threshold of 20% was simply set to minimize issues with alignment and neuropil boundaries and to emphasize strong connections. Although the approach may not be optimal, the results are reasonable upon inspection, and it fulfills the goal of providing a rough guide to connectivity without attempting to overinterpret our data. Due to their complex innervation patterns and lack of home neuropil, the PAN lineages were not included in this analysis. For families that consist of no more than two lineages, we identified the distal targets by manually following any neurite fascicles that could be reliably tracked in individual clones. In addition, we

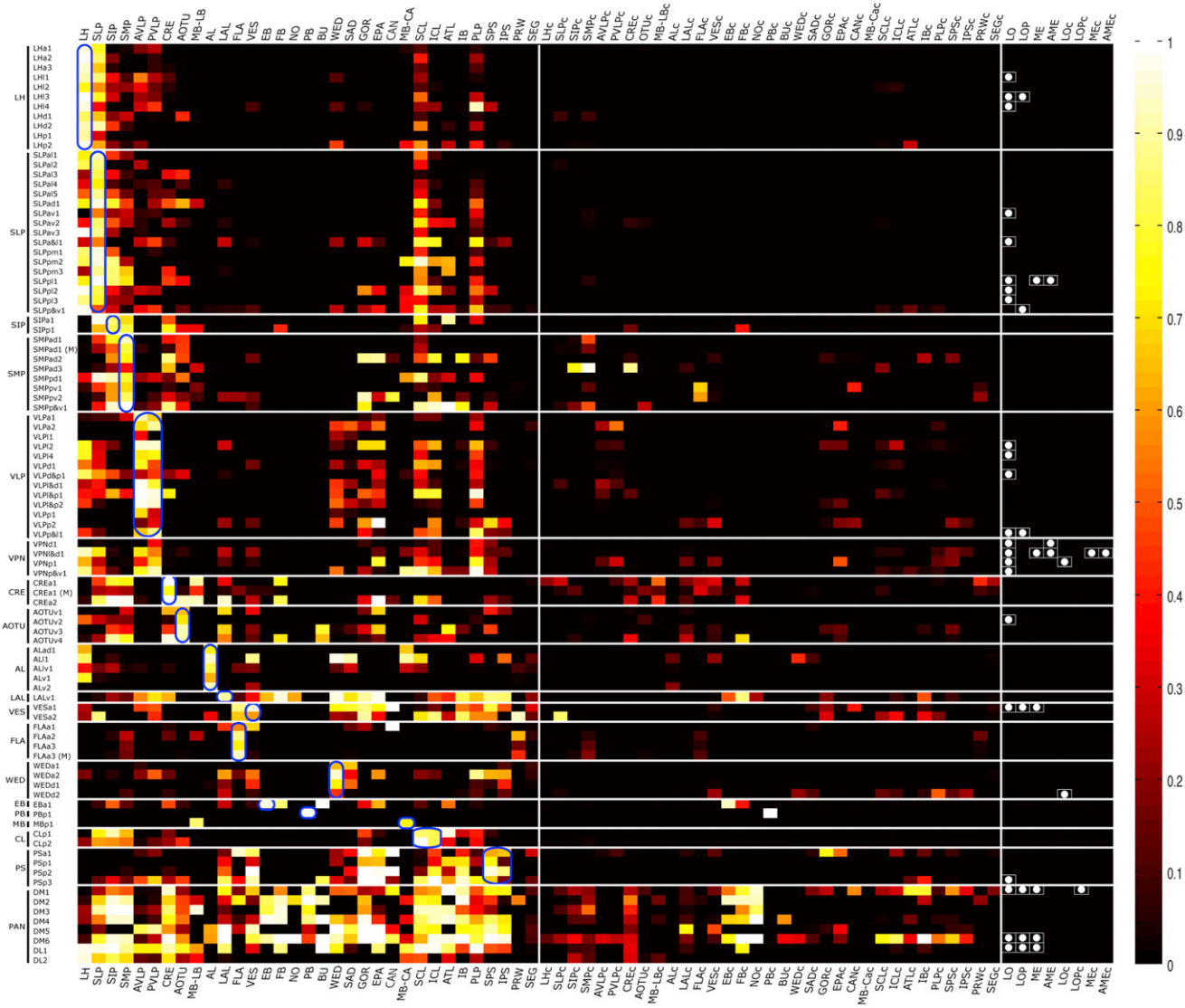

Figure 4. Heatmap of Neuropil Innervations by Various NB Clones

Degrees of voxel coverage in distinct neuropils in the ipsilateral as well as contralateral hemisphere by 95 representative NB clones, including one female sample from each of the 92 stereotyped lineages plus three male clones showing obvious sexual dimorphism. Note the neurite elaborations in various optic lobe neuropils are simply indicated with white dots. Blue circles indicate home neuropils.

included the FLA-to-SMP and the SMP-to-contralateral FLA projections, as evidenced in the relatively simple FLAa3 and SMPpv1 clones, which create some circular connections among the bilateral FLAs and SMPs. We combined these multiple lines of information to derive a neuropil connectivity matrix between the identified home neuropils and the entire set of brain neuropils (Figure 5B). One can thus predict the inputs as well as outputs for most neuropils. However, we provide no insight into the output from neuropils that lack obvious founding lineages (e.g., SAD) or are founded by lineages that do not project out of the neuropil (e.g., MB and PB). The complexity of the PAN lineages severely limits inference of connectivity but may relate to the integrated but modular composition of the CX. Together with other data, this connectivity will be analyzed elsewhere (our unpublished data).

The connectivity matrix correctly identified the LH and MB calyx as the main targets of the AL lineage family, which process the olfactory information downstream of the AL. This motivated us to use the neuropil connectivity matrix to build a rough map of anatomical connectivity for less-studied brain regions (Figure 5B). Tracing the circuit downstream of the AL reveals that the olfactory information processed by the LH [34, 35] may enter the SLP and SCL, which can further communicate with various neuropils, including the SIP, SMP, CRE, AOTU, ATL, and PLP (Figures 5C and 5E). By contrast, the visual information can be processed through the AOTU [36] into the SMP, CRE, and LAL (the CX input/output center) or computed via the VLP [36, 37] into the SCL/ICL (CL), LH, PLP, EPA, and WED (Figures 5D and 5F). Notably, the CL may relay the VLP-processed visual information back to the AOTU. In addition, reciprocal connections exist broadly among higher brain centers (bidirectional arrows in Figure 5B). For example, extensive mutual innervations are evident among neighboring neuropils in the dorsal brain domain and between the dorsal neuropils and CRE/CL.

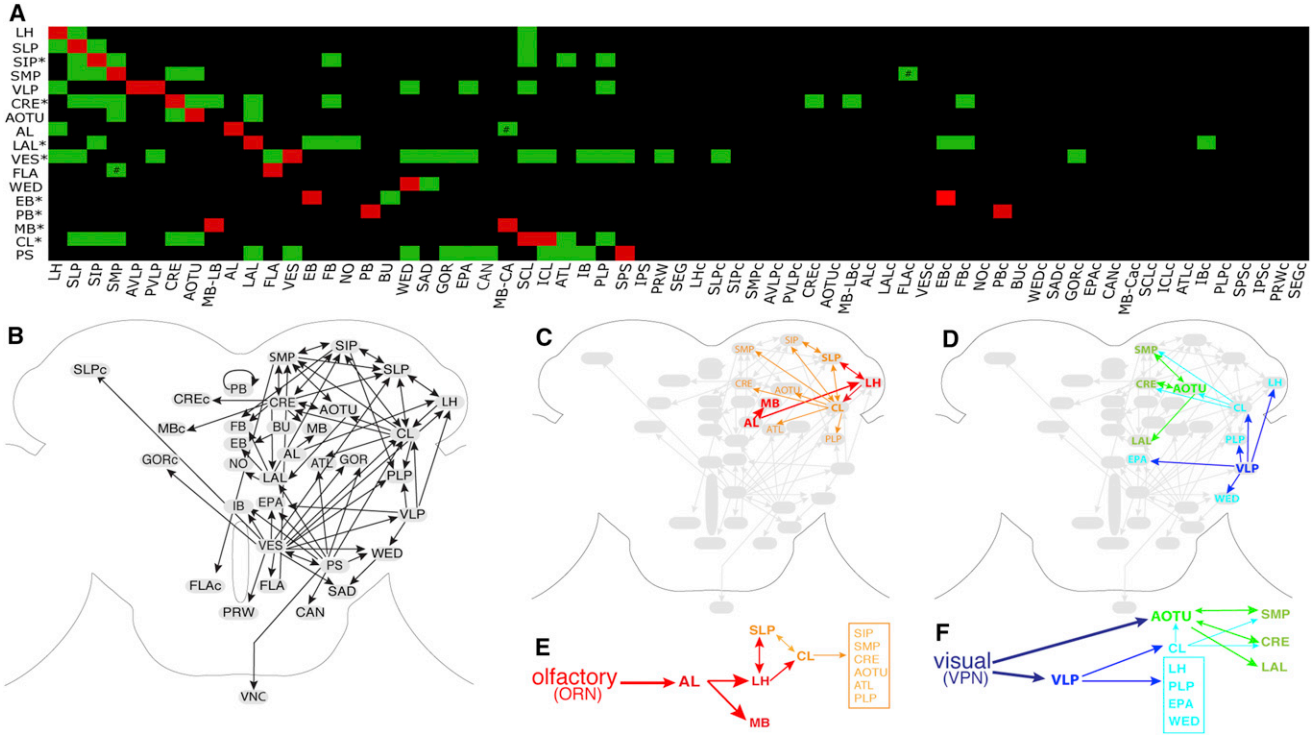

Figure 5. Putative Clonal-Level Neuropil Connectome

(A) The likely connections between various home neuropils (listed on the y axis) and other neuropil regions (arranged along the x axis) are indicated with green boxes. This putative neuropil connectome was deduced primarily through the determination of the major distal targets shared by most of the NB clones cofounding a home neuropil, as revealed from the heatmap of clonal neuropil innervation patterns (Figure 4). For those home neuropils founded by no more than two lineages (*), their possible distal targets were identified by manually tracking the readily traceable neurite fascicles in individual clones. The reciprocal connections between the SMP and FLA (#) are evident in the relatively simple FLPa3 and SMPpv1 clones; the AL-to-MB-CA connection (#) is known.

(B–F) Diagrams of possible information flow across distinct neuropils in the *Drosophila* cerebrum, as judged from the neuropil connection matrix shown in (A). And neural activities presumably propagate from home neuropils to their connected neuropils, except that the EB lies distal to the BU in the EBa1 clone, the sole EB founding lineage. The putative subnetworks that process olfactory or visual information are illustrated in additional diagrams.

In our analysis, the primary cerebral output appears to pass through the PS (SPS/IPS) to the ventral ganglion. Interestingly, this neuropil has projections to a number of neuropils including the LAL, VES, WED, GOR, EPA, CAN, ATL, CL, and IB. This may suggest that the current output is relayed throughout the brain. Interestingly, all PAN lineages innervate either the SPS or IPS, except for DL2. Given the role of the PAN lineages in establishing the CX and the role of the CX in navigation and locomotion, just such a connection from the CX to an output region like the PS would be expected. We did not uncover the SEG as a major distal target for any cerebral home neuropil, although its intermediate position makes it a potential output region. However, some neuropils such as the FLA, PRW, and CAN are not well separated from the SEG in neuropil staining and may play such an intermediate role through clones like FLAa2 and PSp3 that elaborate rather broadly in the SEG.

Discussion

The stereotyped nature of NB clones indicates extensive lineage-intrinsic neural development, where the unique fate of each NB at delamination programs it to make a specific set of distinct neurons [38]. Distinct siblings probably arise in an invariant sequence, given that the first larval-born GMCs reproducibly generate clones with the same morphology. Such birth-order/time-dependent neuron fate specification is evident even in the complex PAN lineages that consistently produce the same sets of offspring from the same INPs (unpublished data). Although the length of lineages and the rate of NB divisions could affect final clone sizes, patterned apoptosis governed by hemilineage identity and neuronal temporal fate seems to account for much of the difference in lineage cell number [6, 17, 39].

Likely Coverage of the 106 NBs of the Procephalic Origin

Urbach et al. have identified 106 unique NBs, which are formed in a stereotyped spatiotemporal pattern on either side of the procephalic neurogenic region of early *Drosophila* embryos, that underlie the formation of the *Drosophila* cerebrum [15, 16]. Given the increased interest in *Drosophila* behavior and brain anatomy, we have determined the morphology of 95 NB clones, about 90% of the expected 106, and examined how they contribute to diverse neuropils. We believe that a similar number of NBs generate the adult nervous system, based on counts of large Dpn-positive cells during larval neurogenesis (data not shown). The 95 lineages we outline here should generate roughly 10,100 neurons with an average (not including the PAN, MB, or ALl lineages) of about 70 neurons surviving in the adult, suggesting that all 106 lineages should generate about 10,800 larval-born neurons. These numbers begin to provide increasingly detailed target information for refining our knowledge of the composition of the *Drosophila*

brain. Most of the uncertainty in determining our coverage is that the extensive fusion of neuropils clouds the developmental origin of clones at the boundary of our region of focus. However, the difficulty in comprehensively tracing NBs from embryonic to adult stages makes this an unavoidable issue. Given this fusion, our coverage of NBs contributing to the cerebrum is fairly robust and largely a developmental accounting issue from an anatomical standpoint. In our opinion, the greater issue is developing future strategies that permit comprehensive analysis of the SEG and OL NBs.

Independent efforts by Ito et al. at Tokyo University [31] have recovered a comparable number of NB clones in the adult *Drosophila* cerebrum. Cross-comparison based on 3D images revealed 77 clones present in both collections, leaving 19 Tokyo clones (including two two-cell clones with unclear lineage identity) and 18 Janelia clones unmatched. This yields a combined set of 114 NB clones. There are 13 VPN clones at the cerebrum/OL border, including nine Tokyo-unique VPN clones. Some of them may derive from the OL and had been excluded from our collection because of variation in twin clone size suggesting a more complex proliferation pattern (data not shown). Together, the two collections jointly cover 114 lineages and are likely to cover almost all of the 106 NB clones of the procephalic origin plus some OL-derived VPN clones.

Complex Interrelation between Lineages and Neuropils

The adult *Drosophila* cerebrum appears as an indivisible structure, showing no anatomical evidence for its development from three procephalic neuromeres, namely the tritocerebrum, deutocerebrum, and protocerebrum. Similarly, the fusion of the SEG with the central brain is another example of the trend toward greater integration of distinct segmental circuitry over the course of evolution. This trend from local circuitry toward more integrated circuits has occurred throughout the various metazoan kingdoms [40]. The complex interrelation between lineages and neuropils could partially result from the fusion of segments and other neuropil units to integrate information across the brain to generate coherent behavior from diverse circuitry.

However, despite omitting highly diverse primary neurons, many clones have extended neurites along discrete tracts and apparently contributed to distinct circuits. The existence of clones innervating multiple separate neuropils may be an indication that they contain greater diversity in neuronal class than the hemilineage-based fating mechanisms inherent in the GMC program. In support of this interpretation, the derivation of PN classes of various sensory modalities from a single lAL hemilineage has recently been shown to involve Notch signaling to accomplish a binary fate switch within a hemilineage that is analogous to GMC sister fating [32]. This blurs the distinction of neuron class specification based on hemilineage origin versus neural type specification through GMC birth order.

NB Lineages as Modules for Evolution

The existence of four highly similar yet distinct MB lineages [41] suggests that expansion of brain anatomy during insect evolution has involved reuse of existing NB fating programs [42]. Such mechanisms may explain the derivation of neurons of the same class from hemilineages of distinct NB origin, such as uniglomerular PNs from the ALl and ALad lineages. If the set of NB lineages laid out a simple set of modular evolutionary building blocks, their determination would lay out a clear road map of brain anatomy, but this is unfortunately not the case. The NB mode of specification appears to have arisen within crustacea and likely involved packaging of preexisting neuronal cell types into a modular developmental unit derived from a single precursor [43]. The complexity of the sets of neurons in a NB suggests that there may be just such a lack of modularity in the ancestral set of neurons. If selection acted to expand a certain neural class through duplication of a NB, the differential requirement for distinct subtypes might be expected to lead to selection for developmentally programmed cell death to remove unnecessary neurons—a prominent feature in our data.

Anatomical Brain Connectivity

As to the detailed neural network, the existence of few clones with simple tracts and obvious polarity limits our ability to interpret this information. The LH and VLP/AOTU family of clones suggest convergence of olfactory and visual information in the SCL and CRE (Figure 5). For those interested in inputs into a specific neuropil, our map provides potential clues as to input pathways. Notably, the neuropils already implicated in specific sensory modalities (e.g., VLP and SAD) receive inputs from limited sources, whereas most deep neuropils are targeted by diverse clones and possibly integrate diverse information. By analyzing the inputs and outputs together, we find that certain neuropils appear to cluster into functional networks, such as the SMP, SIP, and CRE or the SIP, SLP, and CL.

However, single-cell analysis is essential to validate these hypothetical circuits and refine our understanding of anatomical connectivity. In contrast to the existing fly circuit map [44] that generated single neuron clones from broad neuronal drivers, our study lacks the same resolution. However, our approach allows determination of coverage, giving significant advantages in gauging whether important anatomical connections are missing based on driver choice or other technical considerations. Though Chiang et al. [44] emphasize the importance of single-cell patterns in defining neuropil boundaries, it is difficult to judge the meaning of neuropil units defined from incomplete single-neuron sampling. Until a more agreed-upon set of neuropil boundaries is determined, lack of a standard neuropil set makes cross comparison difficult. However, their study found few connections with the caudalmedial protocerebrum (CMP), which corresponds to the PS region and appears to be an important output neuropil, suggesting their sampling may miss important regions. Although our NB connectivity likely covers 90% of the neurons in the cerebrum, our strategy to filter out the strongest connections reduces our sensitivity to detect minor connections. We plan to utilize and extend our NB atlas to systematically map the lineages of the *Drosophila* brain at single-cell resolution to achieve the best coverage possible.

Experimental Procedures

Induction of *Drosophila* NB Clones

Flip-out MARCM clones were generated by heat shocking newly hatched larvae with the following genotype: *hs-FLP^{1}/+; FRT^{G13}, UAS-mCD8::GFP/FRT^{G13}, tubP-GAL80; actin5cP-FRT-stop-FRT-GAL4/+* or *hs-FLP^{1}/actin5cP-FRT-stop-FRT-GAL4; FRT^{G13}, UAS-mCD8::GFP/FRT^{G13}, tubP-GAL80; +*, for 15–25 min at 38°C. We utilized independent actin5cP-FRT-stop-FRT-GAL4 transgenes to exclude positional effects on the clone coverage, though only female brains carried clones during use of the X chromosome driver, resulting in a 3:1 female-dominant ratio. Twin-spot MARCM clones were induced by heat shocking newly hatched larvae with the following genotype: *hs-FLP1/+; FRT^{40A}, UAS-mCD8::GFP, UAS-rCD2-Mir/FRT^{40A}, UAS-rCD2::RFP, UAS-gfp-Mir; nSyb-GAL4/+*, for 10 min at 38°C.

The clones of six lineages, including LHd2, SLPpm1, SIPp1, SMPad3, SMPpv2, and WEDd1, were obtained via embryonic NB-specific stochastic excision of the STOP cassette from *actin5cP-loxP-stop-loxP-lexA::p65* (our unpublished data).

Immunostaining and Confocal Imaging

Brains were dissected, fixed, and processed as described previously [20, 23]. Antibodies used in this study include rabbit anti-GFP (1:1,000, Invitrogen), rat monoclonal anti-mCD8 (1:100, Invitrogen), rabbit anti-RFP (1:1,000, Clontech), mouse monoclonal anti-Bruchpilot, nc82 (1:50, Developmental Studies Hybridoma Bank, Iowa City, IA), Alexa 488 (Invitrogen), Cy3 or Cy5 (Jackson ImmunoResearch) conjugated anti-mouse, anti-rabbit, and anti-rat antibody (1:300). Fluorescent signals of whole-mount adult fly brains and cell bodies for counting cell number of each lineage were collected by confocal serial scanning at 0.8 or 1.0 μm intervals, using LSM710 microscope (Carl Zeiss).

Manual Analysis of Neuropil Innervation Patterns and Clone Sizes

For manual analysis of neuropil innervation patterns, a maximum intensity projection of each confocal z stack was generated. Similar patterns of 2D confocal images of NB clones were initially sorted into the same groups. Confocal image stacks of all collected NB clones were then carefully reexamined to refine grouping accuracy. Neuropil innervation patterns were annotated based on clear staining within nc82-identifiable neuropils. The three highest-quality samples of each lineage were then used to count cell number with a Fiji [45] macro written by Arnim Jenett (available at http://janelia.org/janelia-technology/available-technology).

Heatmap Generation

Confocal stacks of each clonal lineage were registered using BrainAligner [18] to a preselected brain with predefined neuropil boundaries [4, 18]. For each aligned stack, we extracted GFP signals, using adaptive thresholding, and visually confirmed that the threshold appropriately detected the signal from the clone. The fraction of voxels above the threshold was computed for each neuropil with cases of extremely low voxel coverage not included. The manual annotation of neuropil innervation was compared against the automated annotation, and discrepancies (e.g., confirmation of missed neuropils and removal of false positives adjacent to the strong signal in cell body regions) were resolved.

Supplemental Information

Supplemental Information includes two figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.02.057.

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