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Diverse neuronal lineages make stereotyped contributions to the *Drosophila* locomotor control center, the central complex

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Summary

The *Drosophila* central brain develops from a fixed number of neuroblasts. Each neuroblast makes a clone of neurons that exhibit common trajectories. Here we identified 15 distinct clones that carry larval-born neurons innervating the *Drosophila* central complex (CX), which consists of four midline structures including the protocerebral bridge (PB), fan-shape body (FB), ellipsoid body (EB), and noduli (NO). Clonal analysis revealed that the small-field CX neurons, which establish intricate projections across different CX substructures, exist in four isomorphic groups that respectively derive from four complex posterior asense-negative lineages. About the region-characteristic large-field CX neurons, we found that two lineages make PB neurons, ten lineages produce FB neurons, three lineages generate EB neurons, and two lineages yield NO neurons. The diverse FB developmental origins reflect the discrete input pathways for different FB subcompartments. Clonal analysis enlightens both development and anatomy of the insect locomotor control center.

Keywords

adult *Drosophila* brain; central complex neurons; clonal analysis; neuroblasts; type II neuroblast lineages

Introduction

An insect brain consists of multiple neuropil regions where information flows through intricate networks of local interneurons (LNs) and across synapses from the incoming neurites to the outgoing projection neurons (PNs). To have a basic understanding of any neuropil region requires identification of its resident LNs as well as the associated PNs. Whereas single-neuron sampling reveals the basic units of projection, clonal labeling of the entire repertoire of LNs and PNs generated from a single developmental progenitor visualizes all potential trajectories.

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Conflict of interest statement

The authors declare that they have no competing interests.

The *Drosophila* central nervous system (CNS), including its central brain, originates from a stereotyped set of neural progenitors, called neuroblasts (NBs) (Doe, 1992; Ito and Hotta, 1992; Truman and Bate, 1988; Urbach et al., 2003). Each NB deposits a characteristic pool of neurons that contribute to specific neural circuits (Booker and Truman, 1987; Prokop and Technau, 1991; Truman and Bate, 1988). Neurons derived from the same NB remain largely clustered through development. Sibling neurons further share common trajectories: their primary neurites are packed into bundles that extend along specific paths to innervate certain neuropils (Dumstreit et al., 2003; Truman et al., 2004). The entire *Drosophila* central brain can thus be viewed as a composite of multiple independent blocks of neurons that are partitioned into various local circuits (Ito and Awasaki, 2007). Labeling neurons in clones based on their lineage origins should not only reveal how the *Drosophila* brain develops, but also shed light on the cellular organization of the brain.

The *Drosophila* central complex (CX) possesses essential roles for locomotor control (Liu et al., 2006; Poeck et al., 2008; Strauss, 2002; Strauss and Heisenberg, 1993; Triphan et al., 2010) and consists of four interconnecting midline neuropils, including the protocerebral bridge (PB), the fan-shape body (FB), the ellipsoid body (EB), and the paired noduli (NO) (Hanesch et al., 1989; Young and Armstrong, 2010) (Fig. 1). Each of these CX substructures exhibits a regular array of subcompartments, as revealed by both silver staining of fiber tracks and Golgi staining of single neurons (Hanesch et al., 1989). The PB lies near the dorso-posterior cell body-neuropil interface, and exists as a curved rod composed of a linear array of 16 glomeruli. The FB is a saucer-shaped structure; its convex side points dorso-posteriorly toward the PB while its concave anterior surface partially encloses the doughnut-like EB. The FB and EB are both structured into three-dimensional matrices. The FB shows a rectangular array of horizontal layers and vertical segments, while the EB ring can be divided into multiple concentric zones and several radial sectors. Underneath the FB/EB lie the paired noduli, which are roughly spherical and contain multiple subdomains as well. Besides, two adjacent neuropil regions in each brain lobe, the bulb (BU) and the lateral accessory lobe (LAL), reside lateral and latero-ventral to the FB/EB, respectively, and intimately associate with the CX.

Multiple sets of isomorphic neurons wire these four CX substructures together in intricate periodic patterns (Hanesch et al., 1989; Ito and Awasaki, 2007; Young and Armstrong, 2010). These neurons, belonging to the class of small-field CX neurons, target small domains of substructures. Neurons of the same isomorphic type innervate the same subset of CX substructures in an identical pattern to wire subdomains of different substructures into an array. Such small-field CX neurons originate from the dorso-posterior cell body region and mostly extend from one of the 16 PB glomeruli to specific subdomains of various combinations of FB, EB and NO (e.g. the magenta and green neurons in Fig. 1B). There are also small-field neurons that elaborate among FB, EB and NO only. In addition, there exist FB-intrinsic small-field neurons that wire specific subdomains of the FB together (e.g. the cyan neuron in Fig. 1B). These CX-confined small-field neurons constitute an intricate network of multiple arrayed circuits that allows processing of common information in distinct local circuits across different substructures of the multi-domain CX. Additional subsets of small-field neurons extend neurites into the LAL or BU and may relay the CX activities back to where most of the CX inputs originate (see the description of large-field CX neurons below).

By contrast, most CX-extrinsic neurons send neurites from the surrounding into only one of the four CX substructures. These cells are called large-field CX neurons because they elaborate broadly through entire layers or possibly the whole neuropil. The best known large-field CX neurons link the BU and LAL to the EB and FB respectively, making the BU and LAL as the main hubs for CX inputs and possibly outputs as well (Hanesch et al., 1989;

Li et al., 2009; Young and Armstrong, 2010) (e.g. the orange and blue neurons in Fig. 1B). Beyond extrinsic neurons with a role in input/output some large-field neurons are LNs that only innervate a single substructure within the CX.

Despite the extensive characterization of CX structure and circuitry, we are far from identifying all CX neurons to deduce a complete CX map. We also know very little about the development of the already identified CX neurons (Ito and Awasaki, 2007). The recently identified posterior *asense*-negative (PAN) lineages, which originate near the midline and dorso-posterior brain surface and generate transit-amplifying precursors with similarity to vertebrate neurogenesis, have been implicated in generating CX neurons (Bayraktar et al., 2010; Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009; Jiang and Reichert, 2012). Here we tried to determine all the neuronal lineages that make CX neurons, as an initial step toward building a complete cellular and developmental map for the insect locomotor control center. We found that seven out of eight PAN lineages make diverse CX neurons. The DM1 to DM4 lineages each produce morphologically similar isomorphic neurons that populate a unique quadrant of the EB and jointly constitute a specific repertoire of small-field CX neurons. The DM5, DM6 and DL1 lineages plus eight simpler lineages yield diverse types of large-field CX neurons. Distinct lineages make different characteristic sets of CX neurons, as evidenced by their innervation of different substructures in distinct lineage-specific patterns. Notably, many more lineages yield large-field neurons targeting specific layers of the FB than innervating the PB, EB or NO. They potentially link diverse brain regions with the FB and underlie the layer-specific FB inputs. Taken together, clonal analysis reveals complex yet stereotyped development of the *Drosophila* central complex. It lays the essential foundation for identifying individual CX neurons systematically by cell lineage analysis to reconstruct the CX circuitry and development.

Materials and Methods

Fly Strains and transgenes

Following fly stocks were used in this study; (1) *hs-FLP¹; FRT^{G13}, tubP-GAL80/CyO* and (2) *Actin-FRT-stop-FRT-GAL4; FRT^{G13}, UAS-mCD8::GFP/CyO* or (3) *FRT^{G13}, UAS-mCD8::GFP/CyO; Actin-FRT-stop-FRT-GAL4* for flip-out MARCM; (4) *hs-FLP¹; FRT^{40A}, UAS-rCD2::RFP, UAS-gfp-Mir/CyO; nSyb-GAL4* and (5) *FRT^{40A}, UAS-mCD8::GFP, UAS-rCD2-Mir/CyO; nSyb-GAL4* for twin-spot MARCM. *nSyb-GAL4* is a kind gift from Julie Simpson. Other transgenes have been described in previous studies (Lee and Luo, 1999; Yu et al., 2009).

Clonal analysis with flip-out MARCM and twin-Spot MARCM

Details of MARCM and twin-spot MARCM have been described elsewhere (Lee and Luo, 1999; Yu et al., 2009). To induce MARCM clones, mitotic recombination was induced in newly hatched larvae by heat-shock at 38°C for 60 min and 10 min for flip-out and twin-spot MARCM, respectively. Newly hatched larvae were collected for a 4-hour interval and cultured at the density of 60–80 larvae per vial. Excluding SIPp1 lineage, all lineages were identified by flip-out and twin-spot MARCM analysis. The SIPp1 lineage was identified by the neuroblast specific flip-out labeling with embryonic heat-shock. Detail of this method will be described elsewhere. All lineages shown in this study have been identified more than five times by MARCM or flip-out labeling.

Immunohistochemistry

Brains were dissected, fixed, and processed according to previously studies (Lee and Luo, 1999; Yu et al., 2009). Fly brains were dissected in PBS (phosphate-buffered saline, pH

7.4), fixed in PBS with 4% formaldehyde at room temperature for 20 min, washed by PBT (1× PBS with 0.75% Triton X-100) three times for 15 min each, and incubated in Blocking solution (PBT with 0.5% normal goat serum) for 30 min before incubation with primary antibodies at 4°C one to two overnights. Following day, the brains were washed in PBT three times for 15 min each before incubated with secondary antibodies at 4°C one to two overnights. Following day, the brains were washed with PBT for 15 min for three times and mounted with SlowFade gold anti-fade reagent (Invitrogen, Carlsbad, CA). Antibodies used in this study include rabbit anti-GFP (1:1000, Invitrogen), rat monoclonal anti-mCD8 (1:150, Invitrogen), rabbit anti-DsRed (1:500, Clontech, Mountain View, CA), mouse monoclonal anti-Bruchpilot, nc82 (1:30, DSHB; Developmental Studies Hybridoma Bank, Iowa City, IA)(Wagh et al., 2006), Alexa-488 (Invitrogen), Cy3 (JIR; Jackson ImmunoResearch, West Grove, PA) or Cy5 (JIR) conjugated anti-mouse, rabbit and rat antibody (1:250 – 1:500).

Antibody characterization—Please see Table 1 for a list of all primary antibodies used in this study.

Rabbit anti-GFP is a polyclonal IgG produced against green fluorescent protein (GFP) from *Aequorea victoria* and. Anti-GFP antibody was used to enhance signal of mCD8::GFP that was exogenously expressed by GAL4 drivers.

Rat anti-mCD8 is a monoclonal Ig-G produced against the α -chain of mouse CD8. This antibody was also used to enhance the signal of mCD8::GFP driven by GAL4 drivers.

Rabbit anti-DsRed is a polyclonal antibody produced against *Discosoma* red fluorescent protein (DsRed) and used to enhance signal of rCD2::RFP, which was exogenously expressed by GAL4 drivers.

Monoclonal mouse anti-Bruchpilot (nc82) is an Ig-G produced by a hybridoma clone from a large library generated against *Drosophila* head homogenate. The nc82 antibody recognizes Bruchpilot, a ubiquitously expressed active zone protein (Wagh et al., 2006). The immunoreactivity of this antibody disappears when *bruchpilot* gene is knocked down by panneuronal expression of *bruchpilot* RNAi (Wagh et al., 2006). The nc82 antibody was used for counterstaining of brain neuropils.

Anti-GFP, mCD8 and DsRed antibodies have no nonspecific staining in the adult fly brain. These antibodies showed no immunolabeling in brains unless the GAL4 drove expression of *UAS-mCD8::GFP* or *UAS-rCD2::RFP*. Both anti-GFP and mCD8 antibodies have no cross-reactivity with rCD2::RFP and anti-DsRed antibody also has no cross-reactivity with mCD8::GFP.

Microscopic observation and image data processing

Confocal serial scanning images were obtained at 0.8 or 1.0 μ m intervals using LSM710 microscope (Carl Zeiss, Oberkochen, Germany). To reconstruct the stack of images, serial sections were projected using LSM image Browser V3.1 (Carl Zeiss), Vaa3D (Peng et al., 2010) or FIJI (NIH). The brightness, contrast, size and resolution of the resulting images were processed with Photoshop CS software (Adobe System, San Jose, CA). In addition, signals derived from irrelevant background clones were removed by Photoshop when they were obviously traceable. Note, serial-section images of flip-out MARCM clones in nc82-counterstained brains (Fig. 2 and 5) were aligned onto one standard target brain using BrainAligner program (Peng et al., 2011).

Neural projections into neuropils were determined based on neuropil staining with nc82. Neuropils are named according to Virtual Fly Brain, <http://www.virtualflybrain.org/site/stacks/index.htm>, FLYBRAIN Neuron Database, <http://flybrain-ndb.iam.u-tokyo.ac.jp/>, and the coordinated nomenclature system by the insect brain name working group (Ito et al., submitted).

AOTU: optic tubercle. BU: bulb. CRE: crepine. EB: ellipsoid body. FB: fan-shaped body. FLA: flange. IB: inferior bridge. ICL: inferior clamp. IPS: inferior posterior slope. LAL: lateral accessory lobe. LO: lobula. MB: mushroom body. ME: medulla. NO: nodulus. PLP: posterior lateral protocerebrum. PRW: prow. PVLP: posterior ventrolateral protocerebrum. SCL: superior clamp. SEG: subesophageal ganglion. SIP: superior intermediate protocerebrum. SLP: superior lateral protocerebrum. SMP: superior medial protocerebrum. SEG: subesophageal ganglion. SPS: superior posterior slope. VLP: ventrolateral protocerebrum.

Results

Identification of PAN lineages that yield CX neurons

Typical NBs in the developing *Drosophila* CNS are positive for *asense* (Brand et al., 1993). They undergo asymmetric cell divisions to produce ganglion mother cells (GMCs) that divide once and generate two post-mitotic neurons following each self-renewal division of a NB (Doe, 2008; Knoblich, 2008). By contrast, eight posterior *asense*-negative (PAN) neural progenitors in each brain lobe deposit post-mitotic neurons through serial production of *asense*-positive transit amplifying precursors (TAPs) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009). Each TAP can bud off several GMCs that divide once to yield multiple pairs of post-mitotic cells after each PAN NB self-renewal. The PAN (or type II) lineages, when examined by the wandering larval stage, have more progeny and exhibit more complex primary trajectories than conventional type I neural lineages. Tracing of the larval trajectories of the combined pattern generated by six dorsomedial PAN lineages by the group of Heinrich Reichert (Izergina et al., 2009) has revealed their possible innervation of diverse brain structures, including the CX. However, given the complexity of individual clones, we analyzed each PAN lineage independently to determine the contribution of each lineage to the CX.

Two independent approaches were taken to uncover the PAN lineages in adult *Drosophila* brains. First, we labeled the progeny of a single NB in an isolated clone using flip-out MARCM. Clonal labeling in flip-out MARCM requires activation of GAL4 via flip-out and loss of GAL80 after mitotic recombination. Both events are controlled by heat-shock induction of FLP. When they were employed separately, we noticed occurrence of heat shock-independent background clones. Such low-frequency incidents generate a large number of contaminating clones when lineages are non-selectively labeled using a ubiquitous GAL4 driver. Incorporating flip-out into MARCM drastically reduced the background presumably because their primary sources of background clones are distinct. Moreover, use of a ubiquitous driver that is silent until flip-out should suppress the toxicity of constitutive GAL4 expression. In fact, many NB clones appear more exuberant when labeled with the homozygous viable *actin>stop>GAL4*, compared to the homozygous semi-lethal *tubP-GAL4*, in MARCM. We generated clones with multiple independent *actin>stop>GAL4s* to avoid biases in labeling due to positional effects. Second, we labeled NB clones by twin-spot MARCM using *nSyb-GAL4*, which appears to label all mature neurons without harm. Although background clones were frequently encountered as explained above, twin-spot MARCM permits labeling of the paired sister clones derived from the same mitotic recombination in different colors. It reveals the progeny of the just budded-out intermediate precursor for any labeled NB clone. Given that GMCs divide once

but one TAP can make multiple GMCs, a PAN NB clone should be accompanied by a multi-cellular sister clone rather than a two-cell or single-cell clone.

Through analysis of thousands of adult NB clones induced shortly after larval hatching and thus lacking embryonic-born primary neurons, we found seven NB clones that, when labeled with twin-spot MARCM, consistently associate with a multi-cellular sister clone carrying six to nine neurons, which were derived from TAPs of PAN lineages (Fig. 2). These PAN clones exhibit stereotyped morphologies and are named based on cell body positions. The cell bodies of DM1 to DM6, probably correspond to the larval DM1 to DM6 lineages (Jiang and Reichert, 2012; Peraanu and Hartenstein, 2006), and occupy a contiguous region on the posterior brain surface from the dorsal midline to more ventrolateral regions. By contrast, DL1 cell bodies reside near the dorsolateral brain edge. These PAN cell bodies, including the ones constituting the accompanying TAP clones, are further clustered in lineage-characteristic patterns (Fig. 2). For instance, the cell bodies of DM4 and DL1 are consistently partitioned into two discrete clusters (Fig. 2J–L and 2S–U). Moreover, the TAP clones accompanying various PAN NB clones show different fixed cell counts, ranging from six to nine, arguing for the stereotyped TAP sublineages (Fig. 2).

As to clone projections, we observe complex yet stereotyped patterns of neurite elaboration. They acquire multiple, often fine, neurite tracks that innervate specific neuropils in defined patterns as well as many additional brain regions loosely without obvious subpatterns. This is in contrast with other NB clones recovered from the same screen that mostly extend out no more than two primary tracks which elaborate locally and then innervate a limited number of distal targets (e.g. Fig. 8 and 9; Yu et al., submitted). The broad intricate patterns of elaboration unique to PAN NB clones are consistent with the notion that TAP-containing lineages yield a large diversity of neuron types. Additionally, some PAN lineages have been shown to produce Repo-positive glia that can be difficult to distinguish from elaborate neuronal processes when labeled together (Izergina et al., 2009). However, glial-like processes are evident in DL1 NB clones as they reside in the ipsilateral optic lobe (bracket in Fig. 2S) and thereby spatially segregated from the remaining clone including the domains of neuronal cell bodies (arrows in Fig. 2S). In the absence of our more specific labeling strategy it would be difficult to distinguish such cells from background clones. Despite the extraordinary complexity that extends throughout the brain (which will be detailed in a separate paper by Wang et al., in preparation), we are able to determine the innervation of specific CX substructures by the DM1 to DM6 and DL1 clones. Notably, each PAN lineages exhibits a unique characteristic pattern of CX innervation (see below), demonstrating that even the more complex PAN lineages follow a stereotyped developmental program in generating the repetitively organized CX.

DM1–4 lineages make isomorphic small-field CX neurons of diverse types

DM1 to DM4 lineages send neurites into all the four CX substructures. Individual clones innervate discrete subregions of PB, FB and EB, but elaborate throughout the contralateral NO (Fig. 3). Each of the eight bilaterally paired lineages innervates two nearby PB glomeruli (white arrows in Fig. 3A, E, I and M), and they occupy the PB with minimal overlap. In the FB, DM1–4 lineages of the same brain hemisphere individually occupy distinct, partially overlapping sets of longitudinal segments and may redundantly cover the entire substructure with the contralateral DM1–4 lineages (brackets in Fig. 3B, F, J and N). Within the EB, the DM1–4 lineages show interdigitated patterns of innervation with each lineage occupying two radial sectors separated by sectors of neurites derived from the opposite side (solid arrowheads in Fig. 3C, G, K and O). Their innervation of discrete but equivalent subdomains in multiple CX substructures implicates production of isomorphic small-field CX neurons by the DM1–4 lineages (Fig. 3D, 3H, 3L and 3P). Each of the bilaterally paired DM1–4 lineages apparently yields one eighth of all varieties of small-field

CX neurons. Except this commonality, DM1–4 lineages acquire very distinct morphologies (Fig. 2A–L).

Given the clonal arrangement of neurite elaboration, the small-field CX neurons of DM1 may project among the ipsilateral medial glomeruli of PB, the contralateral lateral and ipsilateral medial domains of FB, the contralateral ventral sectors of EB, and the contralateral NO (Fig. 3A–D). Compared with the contralateral FB innervation, the ipsilateral one is narrow and small (Fig. 3B). The equivalent domains of innervation in DM2 shift laterally in the ipsilateral half of PB and FB, but move toward the midline of the contralateral FB and dorsally in the contralateral crescent of EB, while staying in the contralateral NO (Fig. 3E–H). As to the more laterally situated DM3 and DM4, their CX descendents innervate the next equivalent domains of PB, FB and EB besides the contralateral NO (Fig. 3I–P). As a result, the FB and EB receive bilateral innervations whereas the PB and NO are exclusively innervated by the ipsilateral and contralateral small-field CX neurons, respectively. In addition to the isomorphic pattern of neurite elaboration; we observe some minor innervations in the EB that may spill from the FB in DM1–3 lineages (open arrowheads in Fig. 3). In the DM4 lineage, there is weak innervation in the upper two layer of FB. Such layer-wide labeling would derive from large-field neurons rather than small-field neurons (Table 2 and data not shown), arguing that, unlike the DM1–3 lineages, the DM4 lineage might generate a few large-field neurons. Taken together, the bilaterally paired DM1–4 lineages contribute almost evenly to and jointly make most, if not all, types of isomorphic small-field CX neurons.

DM5, DM6 AND DL7 lineages produce specific types of large-field CX neurons

By contrast, DM5, DM6 and DL7 clones exhibit individually unique patterns of neurite elaboration in the CX (Fig. 4). And distinct from the DM1–4 lineages, they innervate certain CX substructures broadly rather than occupy small domains across all CX substructures. These features of CX innervation imply that DM5, DM6 and DL7 lineages yield large-field CX neurons of diverse types.

Among them, DM6 shows the most complex pattern of CX innervation (Fig. 4D–F). It selectively innervates the ipsilateral most lateral glomerulus of the PB (open arrowhead in Fig. 4D), but elaborates broadly in stereotyped patterns within the FB, EB and NO (Fig. 4E and F). Counterstaining of the brain with the nc82 monoclonal antibody that recognizes the presynaptic protein, Bruchpilot (Wagh et al., 2006), allows subdivision of the FB into 7 horizontal layers (Fig. 4J). Three regions of strong nc82 staining (with strongest labeling in the upper and lower domains) are sandwiched by four weak zones; we number these domains in a ventral-to-dorsal order (Fig. 4J). Complementary to the counterstaining pattern, the DM6 neurite arbors in the FB are distributed in reverse densities: they aggregate more in the nc82-weak zones and only lightly decorate the nc82-strong layers (Fig. 4E and Table 2). In the EB, we observe a ring-like elaboration that does not fill the entire EB (Fig. 4F). Superimposed on the ring lies a separate elaboration that occupies the two most ventral sectors of the ipsilateral EB crescent (bracket in Fig. 4F). As to the NO, three subcompartments, including one dorsal, one middle-anterior, and one ventral-posterior domain, could be recognized in the nc82-counterstained brain (Fig. 4K–L). And DM6 arbors sparsely innervate all three NO subcompartments (arrowheads in Fig. 4E, and Table 3).

Compared to the DM6's involvement in all CX substructures, DM5 and DL1 exclusively innervate the PB and FB, respectively (Fig. 4A and H). DM5 may send only one neurite that arborizes in various densities along the curved PB (Fig. 4A). Except the glomeruli next to the midline and the lateral end (arrowheads in Fig. 4A), the ipsilateral side is more densely populated than the contralateral side; and within a given side, the lateral half of elaboration further stands out (bracket in Fig. 4A). In DL1, one thick neurite bundle enters the FB from

the superior brain region and gives rise to two broad layers of dense elaboration (Fig. 4H). With respect to the nc82 staining, the upper elaboration occupies the top two layers and the lower elaboration covers the bottom two layers of the FB (Fig. 4H and Table 2). It appears that DL1 is the major contributor to the most prominent layers of nc82 staining in the FB. These layer innervations can be traced back to the SMP/SIP region, suggesting that these large-field neurons project from SIP/SMP to the FB layers.

Taken together, DM5, DM6 and DL7 progenitors yield lineage-specific CX neurons: DM6 produces substructure-characteristic CX neurons for all CX substructures whereas DM5 and DL1 make PB- and FB-specific CX neurons, respectively. Without single-cell labeling it is challenging, especially in the complex PAN lineages, to determine if the CX innervating progeny are composed of substructure-specific LNs and/or PNs. Nonetheless, the neurite trajectories of DL1 CX neurons are relatively simple and mimic those of layer-specific large-field FB neurons that provide various inputs into distinct horizontal layers of the FB.

Identification of eight additional lineages that make large-field CX neurons

Beside the DM5, DM6 and DL1 lineages, we found eight conventional lineages that make large-field CX neurons of additional types (Fig. 5 and 6). These include the known EBa1 lineage (Wang et al., 2002), the LALv1 lineage whose first seven larval-born CX neurons have been determined (Yu et al., 2009), a documented sexually dimorphic Fru-positive lineage (Kimura et al., 2005), and five novel lineages. Among them, one ultra-short lineage consistently makes PB neurons (Fig. 5S) whereas the LALv1 lineage produces CX neurons for all CX substructures except the PB (Fig. 5D–F). The remaining six lineages, including the EBa1 lineage previously thought to exclusively make the R1–4 types of large-field EB neurons, yield various layer-specific FB neurons (Table 2). Notably, the first neuron born from after larval hatching is the only neuron innervating the CX from the SMPad2 lineage, as both flip-out MARCM clones born slightly later and the NB side of twin-spot MARCM clones are devoid of CX innervation (Fig. 6G and 7G).

The EBa1 lineage has been shown to produce large-field EB neurons that branch in the bulb (BU) and subsequently terminate in discrete patterns within specific concentric zones of the EB (Fig. 5A–B). They apparently relay the information from the BU to the EB. Notably, there is an additional pattern of neurite elaboration spreading over the LAL/SMP/SIP/AOTU as well as the contralateral SMP/AOTU (Fig. 8A–C). Close inspection further reveals innervation of two internal nc82-weak layers of the FB (arrowheads in Fig. 5C and 7B). Interestingly, the upper FB innervation exclusively derives from the 1st larval-born neuron of the EBa1 lineage (yellow arrowhead in Fig. 7B). Tracing its neurite trajectory reveals proximal branches extending into LAL and terminal elaborations in both FB and SMP/SIP (Fig. 8B).

The LALv1 lineage consists of two distinct hemilineages, as evidenced by the pairing of NB clones with two discrete sister neurons (Fig. 6B). The first seven larval-born neurons in the LAL-CX hemilineage have been determined; and they elaborate in the LAL before innervating various subcompartments of the NO, the EB in characteristic ring morphologies, or specific layers of the FB (Yu et al., 2010). These neurons should have major contributions to the EB, NO and FB projections of the LALv1 lineage (Fig. 5D–F and 7E). The same hemilineage possibly produce neurons that project into the contralateral NO or CRE/SMP/SIP at later time points (Fig. 7E and 8E). Besides, there exist two additional track systems: the lateral track extends into VLP whereas the posterior track projects into IB, SPS, IPS, and posterior SEG as well as LO and ME (Fig. 8D–F). They probably derive from neurons made by the other hemilineage, as the sister neuron of the first larval-born LAL-CX neuron has followed the posterior path into IB, SPS and IPS (Fig. 8F).

The AOTUv4 lineage shows elaboration in upper layers of the FB (Fig. 5I and 7H, Table 2). This lineage exhibits two track systems that probably derive from distinct sister hemilineages, as the first larval-born GMC clone has consistently carried two neurons that project along each of the two neurite tracks (Fig. 6C and 8G–I). One track extends from AOTU to SMP/SIP and bifurcates into BU and further into LAL/SCL/ICL and contralateral LAL and SIP. The other track goes straight into SMP/SIP. About its innervation of multiple upper layers of FB, we could only track the neurites back into the co-targeted SMP/SIP region. It requires single-cell labeling to determine the path(s) and the possible input region(s) of these large-field FB neurons.

The CREa1 lineage again consists of two distinct hemilineages that could be separated with scrutiny (Fig. 8J–L). The Fru-positive lower cluster projects ventromedially and crosses the midline above the esophagus passage to innervate FLA/PRWc/SEG bilaterally though asymmetrically. It further extends dorsolaterally to innervate the contralateral SLP/SIP/PLP/PVLP. By contrast, the upper cluster elaborates in AOTU/SLP/SMP/SIP as well as within the MB lobes, the FB and the CRE. It selectively targets the lower nc82-strong layer of the FB (Fig. 5L and 7K, Table 2). But at the lineage level, we could not determine the trajectory of the large-field FB neuron(s).

The CREa2 lineage shows dense elaboration in the superior protocerebrum, including SMP/SIP/SLP (Fig. 9A–C). Other primary innervations cover AOTU/CRE/LAL/SCL/ICL and MB lobes. But it selectively targets the lower nc82-strong layer of the FB as in the CREa1 lineage (Fig. 5O and 7N, Table 2). In addition, despite the presence of over 120 neuronal cell bodies in the NB clone generated upon larval neurogenesis, the first larval-born GMC consistently yields only one viable neuron that elaborates in CRE/SIP/SMP and further arborizes in LAL/SCL/ICL (Fig. 6E, and 9J). Depicting the trajectory of the FB neuron(s) from the compact NB clone is not possible.

The PB lineage is extraordinarily small, consisting of only 10 larval-born neurons that exclusively innervate the PB (Fig. 5S–T and 6F). They arborize along the entire PB but in a heterogeneous pattern. Close inspection shows characteristic spots of dense elaboration in irregular shapes within a given glomerulus or across multiple glomeruli of the PB (Fig. 7R). And the first larval-born pan-PB neuron consistently innervates the ipsilateral 5th and contralateral 4th glomeruli densely while spreading through the entire PB (yellow arrowheads in Fig. 7R). The SIPp1 lineage reveals proximal innervation in SIP and terminates in upper three layers of FB (Fig. 5P–R and Table 2). Neurons of this lineage also innervate SMP/CRE (Fig. 9G–I). They could be FB large-field neurons collecting inputs from SIP and SMP/CRE.

The SMPad2 lineage, which elaborates from SMP/SIP to IB as well as via CRE/SCL/ICL to SPS, shows no CX innervation after the birth of its first larval-born sister-neuron pair (Fig. 6G and 9D–F). Only one of the paired sister neurons targets the CX (Fig. 9K). Unlike typical large-field CX neurons with elaborations restricted to only one CX substructure, this layer-specific FB neuron innervates the paired NO bilaterally as well (Fig. 7T and Table 2). However, it is not possible to trace the neurite back to its cell body, as the other non-CX neuron arborizes extensively in SMP/SIP as well as within CRE/SCL/ICL (Fig. 9K).

Taken together, the large-field CX neurons arise from diverse yet specific neural progenitors. Seven of the eight conventional lineages, which include the EBa1 lineage, which predominantly innervates the EB, but not the ultra-short PBp1 lineage, produce various layer-specific FB neurons in addition to many more non-FB or non-CX offspring. The existence of FB-targeting neurons from diverse lineage origins is probably due in large part to the layer-characteristic inputs from a variety of brain regions, as distinct lineages

acquire different neurite elaboration patterns outside the FB (Fig. 10). By the same token, the EBa1 and LALv1 lineages make EB neurons that receive inputs from the BU and the LAL, respectively. The pure PBp1 lineage is very unique in its extremely compartment-specific innervation pattern, which lacks elaboration outside the PB.

Discussion

Exhaustive clonal analysis has led to the identification of 15 discrete neuronal lineages that make neurons innervating specific CX substructures. Twelve of them innervate many additional brain regions with broad complex patterns of elaborations, showing involvement of non-CX neurons. As to the three CX-neuron-dominant lineages, including the EBa1, SIPp1, and PBp1 lineages, they are all relatively small and probably become rather exclusive for the CX due to a premature loss of their non-CX neuronal offspring. These phenomena implicate that most, if not all, NBs underlying the development of higher brain centers produce diverse classes of neurons that innervate distinct sets of neuropils. Such complex organizations may reflect the extensive inter-neuropil networking in the deep insect brain region.

Despite the complexity, all the NB lineages apparently derive in stereotyped patterns. Hemilineage identity and neuronal birth order explicitly govern the derivation of diverse neurons from a common progenitor in the conventional lineages (Jacob et al., 2008; Kao and Lee, 2010; Lin and Lee, 2011). The composition of two distinct hemilineages is evident in those lineages where NB clones were paired with two distinct sister neurons as revealed by twin-spot MARCM (Fig. 6 – 9). Aided by the single-neuron trajectories, separate neurite tracks can often be followed from cell body regions into discrete sets of neuropils. The CX neurons can therefore be traced back to one of the sister hemilineages in a given NB clone. Great heterogeneity exists among the CX neurons of the same hemilineage. Beside the previous knowledge about the orderly production of six distinct LAL-to-CX neurons by the LALv1 lineage (Yu et al., 2009), the EBa1 and SMPad2 lineages make a specific CX neuron at the beginning of their larval round of neurogenesis (Fig. 7). Moreover, each neuron in the ultra-short PB lineage is probably unique and again predetermined by the birth order as the first sibling consistently acquires a characteristic elaboration pattern different from the remaining lineage (Fig. 7). Given the minimal cellular redundancy observed in each hemilineage, it is intriguing to detect the homology in the FB innervation between the two CREa lineages (Table 2). In fact, these two CREa lineages show overall gross similarity except the presence of additional Y-shape neurons in the CREa1 NB clone (Fig. 5). The distinction could result from selective loss of an entire hemilineage from the CREa2 lineage. This observation provides evidence for possible NB lineage duplication followed by divergent evolution of the duplicated NB lineages in the derivation of diverse NB lineages needed for building a complex brain.

The gross complexity is further striking in the PAN NB lineages. These vertebrate-type neuronal lineages yield many more neurons and exhibit comparable, if not higher, offspring heterogeneity than the conventional lineages. The progeny heterogeneity with minimal cellular redundancy is evident among sibling neurons derived from a single transient-amplifying precursor (TAP). For instance, the first larval-born DM1–6 and DL7 TAPs make a fixed number of diverse adult neurons, ranging from six to nine depending on the lineage origin (Fig. 2). These TAP clones show complex morphologies analogous to their parental NB clones (data not shown), despite drastic differences in the clone size. Some of them contain CX neurons but carry many additional non-CX neurons. Furthermore, despite the overall similarities, the first larval-born TAP clones have acquired stereotyped fine neurite elaboration patterns that differ from the collective neurite elaboration patterns generated by all later-derived TAPs of the same PAN lineage (data not shown). These phenomena clearly

demonstrate great cellular diversities within a single TAP sublineage and across sibling TAP sublineages. The production of a specific set of diverse neurons by a given TAP and the orderly derivation of distinct TAP sublineages from one common PAN NB further implicate that the vertebrate-type PAN NBs yield specific arrays of diverse neurons and glia through temporal fate patterning along the axes of NB division as well as TAP proliferation.

The enormous progeny complexities conceal individual neurite trajectories, making it not possible to discern the CX projectome from NB clonal morphologies. However, our characterization of 95 of the 106 NB clones (Yu, Awasaki et al., submitted) that constitute the entire central brain (excluding the subesophageal ganglion) should reveal nearly all the CX neurons, their developmental origins, and the possible CX wiring diagram. About the small-field CX neurons, we demonstrate their derivation as eight isomorphic sets that derive from four otherwise distinct PAN lineages in each brain hemisphere. The isomorphic neurons of diverse types are segregated into distinct clones based on neurite trajectories rather than neuron types. In other words, the DM1–4 progenitors are comparably programmed to make analogous types of small-field CX neurons but each produces a different isomorphic set to target different equivalent subdomains within the CX. The clonal-based patterned innervations provide an overall view over how the various CX substructures are interconnected into an intricate network (Fig. 3 and 7).

Regarding other CX neurons, we have recovered three PB neuron-containing lineages, three EB neuron-containing lineages, three NO neuron-containing lineages, and as many as ten FB neuron-containing lineages. Judging from neural and clonal architectures, most of them are probably substructure-characteristic intrinsic or input neurons. The PB neurons of DM5 and PBp1 lineages elaborate loosely through the entire PB and are likely PB-intrinsic neurons. By contrast, the DM6 lineage-derived PB neuron(s) might wire the lateral-most PB glomerulus with other CX substructures or neuropils outside the CX. About the EB neurons, the EBa1 descendants could relay information from BU/LAL/SMP/SIP/AOTU to the EB, the EB-targeting LALv1 offspring specifically collect inputs from LAL, and the DM6-derived EB neurons might wire additional neuropils with the EB. The LALv1 lineage also produces NO input neurons that extend from the LAL to specific NO subcompartments (Fig. 7; Yu et al., 2009). The other two NO-targeting lineages, including DM6 and SMPad2, show diffuse bilateral NO elaborations together with broad FB innervations. It is not clear about their projections outside the CX. Contrasting these two NO/FB-cotargeting lineages, the remaining eight FB lineages provide various inputs to specific layers of the FB. The FB-prominent DL1 and LALv1 lineages exhibit complementary layer-specific FB elaborations and convey messages from the SIP/SMP and LAL, respectively. Besides, multiple lineages, including EBa1, AOTUv4, CREa1, CREa2, and SIPp1, potentially wire SIP/SMP with various layers of the FB (Fig. 10). Furthermore, these SIP/SMP-related FB lineages, unlike the LALv1 lineage, preferentially innervate the nc82-dense layers of the FB (Table 2). All these observations implicate the SIP/SMP as the primary FB input center.

In sum, the development (as implicated through cell lineage analysis) of deep *Drosophila* brain regions, including the CX, is complex but remains stereotyped. Revealing the cellular composition of these lineages will uncover the complete repertoire of neurons within the brain, and lay the necessary foundation for studying brain development, evolution, and function.

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Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: TA, TL. Acquisition of data: JSY, H-HY, TA. Analysis and interpretation of data: JSY, TA. Writing of the manuscript: TA, TL. Technical and material support: YH, DP, J-CK. Study supervision: TL.

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Figure 1. General structures of the central complex (CX) and CX neurons

(A) Sagittal view of the central complex composed of four neuropils: the protocerebral bridge (PB), fan-shape body (FB), ellipsoid body (EB), and noduli (NO) (after Hanesch et al 1989).

(B) Schematic illustration of CX small-field and large-field neurons. Line drawing shows a dorsal view of the CX. Three types of small-field neurons, pb-fb-no, fb pontin, and pb-eb neurons, are shown in magenta, cyan, and green, respectively. Two types of large-field neurons, EB ring neuron and LAL (lateral accessory lobe)-fb neuron, are shown in orange and blue, respectively.

Figure 2. Gross morphologies of DM1–6 and DL1 NB clones

Composite confocal images of representative DM1 (A–C), DM2 (D–F), DM3 (G–I), DM4 (J–L), DM5 (M–O), DM6 (P–R) and DL1 (S–U) NB clones: flip-out MARCM clones shown in an nc82-counterstained standard *Drosophila* brain (left images), cell body regions of twin-spot MARCM clones with NB progenies shown in green and their paired TAP progenies in magenta (middle images), and the cell bodies of the co-existing TAP clones (right images). In all lineages, clusters of cell bodies are located in posterior brain (arrows in left images). The first sets of larval-derived neuronal progenies (arrowheads in middle and right images) exhibit different specific cell counts with N ranging from 6 to 9. Note presence of two clusters of cell bodies (outlined with dashed lines) in the DM4 and DL1 NB clones, and presence of glia-like progenies in the DL1 NB clone (bracket in S). Clones with cell bodies in right hemispheres were shown in all figures of this study. Scale bars equal 50 μ m.

Figure 3. DM1–4 NB clones innervate discrete sets of equivalent CX subdomains

Close-up views of CX elaborations in DM1 (A–C), DM2 (E–G), DM3 (I–K) and DM4 (M–O) NB clones (green), shown at different focal planes from the posterior PB region (left images), through the FB and noduli (middle images), to the anterior EB structure (right images). Their collective patterns of primary CX elaboration are further illustrated in the schematic CXs (D, H, L and P). White arrows: elaboration within the PB; brackets: elaboration within the FB; orange arrows: elaboration within the noduli; solid arrowheads: main elaboration within the EB; open arrowheads: minor elaboration within the EB. Scale bar equals 50 μ m.

Figure 4. DM5, DM6 and DL7 NB clones innervate different CX substructures

(A–I) Close-up views of CX elaborations in DM5 (A–C), DM6 (D–F) and DL7 (G–I) NB clones (green), shown at different focal planes from the posterior PB region (left images), through the FB and noduli (middle images), to the anterior EB (right images) structure. The PB elaboration of DM5 is intricately patterned with discrete glomerular concentrations (e.g. arrowheads and bracket in [A]). By contrast, DM6 targets only one PB glomerulus (open arrowhead in [D]), elaborates broadly in layer-specific densities within the FB (bracket in [E]), diffusely innervates the paired noduli (arrowheads in [E]), and occupies the EB with ring-like elaboration (arrow in [F]) plus sector-specific innervation (bracket in [F]). And DL1 selectively targets the top two as well as the bottom two layers of the FB (brackets in [H]), corresponding to the 7+6 and the 1+2 layers in [J]). (J) Confocal section of the FB showing layer-specific differential nc82 staining. Note that the alternative weak (w) and strong (S) immunostainings allow division of the FB into seven recognizable layers. (K–L) Confocal sections of the paired noduli at the posterior (K) versus anterior (L) focal plane, revealing three subcompartments: d- dorsal, m- middle, v- ventral. Scale bars equal 50 μ m.

Figure 5. Seven additional NB clones with CX elaboration, as revealed by flip-out MARCM
 Composite confocal images of representative EBa1 (A–C), LALv1 (D–F), AOTUv4 (G–I), CREa1 (J–L), CREa2 (M–O), SIPp1 (P–R) and PBp1 (S–U) NB clones: flip-out MARCM clones shown in an nc82-counterstained standard *Drosophila* brain (left images). They innervate various CX substructures, as shown in the confocal sections of EB or PB (middle images) and FB & NO (right images) in the original Z stacks. Various regions of elaboration are pointed with diverse marks. For example, the open arrowhead in [F] indicates the innervation of the contralateral noduli by the LALv1 clone. Scale bars equal 50 μm . $n > 3$ for all lineages.

Figure 6. Overall projection of seven NB clones with CX elaboration, as revealed by twin-spot MARCM

Composite confocal images of twin-spot MARCM EBa1 (A), LALv1 (B), AOTUv4 (C), CREa1 (D), CREa2 (E), PBp1 (F) and SMPad2 (G) NB clones with NB progenies shown in magenta and their paired GMC progenies in green. The entire clones within the nc82-counterstained brains (blue) are shown. The associated GMC clones contain one or two neurons (yellow arrows) depending on the lineage identity. Scale bar equals 50 μ m.

Figure 7. Innervation in CX in the seven additional NB clones with CX elaboration

Innervate various CX substructures, as shown in the confocal sections of EB (left images), FB/NO (middle images) and PB (right images) in the original Z stacks (Figure 6). Innervations of multiple layers of FB are shown with brackets. Note the differential elaborations of the NB clones (white marks) versus their paired GMC clones (yellow marks). Scale bar equals 50 μm .

Figure 8. Neurite trajectories of various NB clones that show CX elaboration (1)
 (A–D) Composite confocal images of twin-spot MARCM clones, EBa1 (A–C and M), LALv1 (D–F and N), AOTUv4 (G–I) and CREa1 (J–L), with NB progenies shown in magenta and their paired GMC progenies in green. The entire clones within the nc82-counterstained brains (blue) are shown on the leftmost images. The cell bodies (dashed outlines), primary tracks (directed in arrows with dashed lines that represent out-of-focal-plane projections), and neuropil innervations (indicated with abbreviated neuropil names) are followed from anterior to posterior brain regions as shown from left to right images. Composite confocal images of the 1st larval-born GMC clones (green) of the EBa1 (M) and LALv1 (N) lineages are shown. Note the innervation of multiple neuropils by various single neurons. Scale bar equals 50 μ m.

Figure 9. Neurite trajectories of various NB clones that show CX elaboration (2)
 (A–C) Composite confocal images of twin-spot MARCM clones, CREa2 (A–C), SMPad2 (D–F) and SIPp1 (G–I), with NB progenies shown in magenta and their paired GMC progenies in green. Composite confocal images of the 1st larval-born GMC clones (green) of the CREa2 (J) and SMPad2 (K) lineages are shown. Scale bar equals 50 μm.

Figure 10. Schematic diagram of possible CX neural projections

Possible wiring patterns within the central complex (small-field neurons, left) and between the CX and other brain neuropils (large-field neurons, right). Lineage origins are shown; dashed lines indicate potential projections.

Table 1

Primary Antibody Used in This Study

Antibody	Dilution	Source	Immunogen
anti-GFP	1:1000	Rabbit polyclonal (IgG Fraction) Invitrogen Cat. No. A-11122	GFP from <i>Aequorea victoria</i>
anti-mCD8	1:150	Rat monoclonal (clone 5H10) Invitrogen Cat. No. MCD0800	Mouse CD8 alpha chain
anti-DsRed	1:500	Rabbit polyclonal Clontech Cat. No. 632496	<i>Discosoma</i> red fluorescent protein (DsRed)
anti-Bruchpilot	1:30	Mouse monoclonal (nc82) DSHB	<i>Drosophila</i> head homogenate

Table 2

Relative Innervations of Clonal Neurons in FB Layers

	FB layers						
NB clones	1	2 strong	3	4 strong	5	6 strong	7
EBa1			+		+(1st)		
LALv1	++	+	+++	+	++	+	++
AOTUv4				+++	++	++	++
CREa1		+++					
CREa2		+++					
SMPad2			+(1st)	+(1st)			
SIPp1					+	++	++
DM1							
DM2							
DM3							
DM4						+	+
DM5							
DM6	++	+	++	+	++	+	++
DL1	++	+++				+++	++

Table 3

Relative innervations of clonal neurons in NO subcompartments

	NO subcompartments					
NB clones	ventral-c	middle-c	dorsal-c	ventral-i	middle-i	dorsal-i
EBa1						
LALv1	+	+		+++	+++	+(1st)
AOTUv4						
CREa1						
CREa2						
SMPad2		+(1st)			+(1st)	
SIPp1						
DM1	+++	++	++			
DM2	+++	++	++			
DM3	+++	++	++			
DM4	+++	++	++			
DM5						
DM6	+	+	+	+	+	+
DL1						