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Postembryonic neurogenesis in the optic lobe and central brain of *Drosophila melanogaster*

A dissertation submitted in partial satisfaction of the requirements for the Degree of Doctor of Philosophy in Molecular, Cell and Developmental Biology

by

Kathy Ngo

2016
ABSTRACT OF DISSERTATION

Postembryonic neurogenesis in the optic lobe and central brain of *Drosophila* melanogaster

by

Kathy Ngo

Doctor of Philosophy in Molecular, Cell and Developmental Biology

University of California, Los Angeles, 2016

Professor Volker Hartenstein, Chair

Neurogenesis or the proper formation of the nervous system requires three distinct phases: (1) early neurulation involving progenitor proliferation and specification; (2) progenitor migration and extension of fibers; and (3) neural differentiation and connectivity establishment. How connectivity is established remains to be poor understood. We investigated neurogenesis of the central brain and visual processing center in *Drosophila*. In *Drosophila* central nervous system (or central brain), there are 100 lineages, each derived from a single neuroblast; where neurons of one lineage remain in close proximity to their mother neuroblast. As described in the Appendix, we used cell type specific markers combined with global neuronal markers to serve as local landmark and mapped out how individual neuroblast lineages progress during development where gross anatomical changes are described. The fly optic lobe, the visual system processing center, is also highly modular. We show that early neurogenesis in the optic lobe is remarkably similar to vertebrates, following a “conveyor belt neurogenesis” (Ch. 4) and
show that Jak/Stat and Notch negatively regulates epithelium-to-neuroblast conversion, a non-canonical mode of neurogenesis (Ch. 2). To further gain insights on how connectivity is established in the optic lobe, we reconstructed the global architecture and connectivity of the optic lobe at sequential stages of development. Our analysis reveals three major structural/developmental hallmarks by which the optic lobe, compared to other regions of the fly brain, stands out: large scale neuronal movements, correlated temporal gradients in neuron production and differentiation, highly ordered retinotopic projections in between visual neuropils, and the formation of multiple layers within these neuropils (Ch. 3). The works described in Ch. 2-5 and Appendix 1-3 serve an important platform for understanding how the nervous system is formed in Drosophila.
The dissertation of Kathy Ngo is approved.

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2016
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Chapter 3 is a manuscript that is currently in preparation to be submitted. All the experiments and experimental design were done by KTN. Manuscript was written by KTN and VH. Chapter 4 is manuscript version currently under revision to Current Biology: [Joly J.-S., Recher G., Brombin A., Ngo K., Hartenstein V. A conserved mode of neurogenesis supports a deep homology between insect and vertebrate visual systems]. I worked under the guidance of VH and contributed to describing the development of the Drosophila optic lobe.

Appendix 1 is a reprint of [Lovick J.K., Ngo K.T., Omoto J.J., Wong D.C., Nguyen J.D., Hartenstein V. (2013). Postembryonic lineages of the Drosophila brain: I. Development of the lineage-associated fiber tracts. Dev Biol 384(2): 228-57], with permission from Elsevier (Lic #3890380660794). I devised an optimized protocol for labeling tracts during metamorphosis which Fig. 5-13 were based on. Experiments for Fig. 5-13 were contributed equally by JL, KTN, and JO. This project was directed by VH.

Appendix 2 is a reprint of [Wong D.C., Lovick J.L., Ngo K.T., Borisuthirattana W., Omoto J.J., Hartenstein V. (2013). Postembryonic lineages of the Drosophila brain: II. Identification of lineage projection patterns based on MARCM clones. Developmental Biology 384, 258], with permission from Elsevier (Lic #3890380722435). The manuscript was contributed by DW, JL and KTN. KTN contributed experimentally to Fig. 4 and 6. The genetic construct to make single
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Appendix 3 is a reprint of [Hartenstein V., Younossi-Hartenstein A., Lovick J.K., Kong A., Omoto J.J., Ngo K.T., Viktorin G. (2015). Lineage-associated tracts defining the anatomy of the Drosophila first instar larval brain. Developmental Biology 406: 14-39], with permission from Elsevier (Lic # 3890380306945). KTN contributed to Fig. 1, 2, and 5. The figures of other experiments were carried out by JL, AK, JO, and VH. JO, AK, and VH contributed to the manuscript. The manuscript was prepared by VH. This project was directed by VH.

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**SELECTED POSTER PRESENTATIONS AND TALKS**


Chapter 1.

Step-by-step guide to neurogenesis
Chapter 1.

Step-by-step guide to neurogenesis

The visual system in both vertebrates and invertebrates requires a precise topographic map, with the correct neuronal subtype specified in a tightly-regulated spatiotemporal fashion. The formation of the retinotopic map requires the production of cells types in a highly regulated manner. Such processes in Drosophila still remains to be poorly understood. This chapter is two-fold, (1) giving a general survey of the developmental mechanisms regulating Drosophila neurogenesis followed by (2) a comparative review of the conveyor belt mechanism of neurogenesis shared by both vertebrates and invertebrates.

1. A survey of neurogenesis: how to make a central nervous system

A common feature of the central nervous system found in all bilaterian animals is its relatively high number of cells, and diversity in cell types. This is undoubtedly true for large vertebrate brains, which possess many billions of neurons and glial cells; but it also applies to microscopic invertebrates, such as nematodes or flatworms, where nerve cells, though much less numerous, still amount to 20-40% of the overall number of cells in the body (for C. elegans: Sulston et al., 1983; for Drosophila melanogaster: Campos-Ortega and Hartenstein, 1985). The second characteristic of the nervous system, compared to other organs of the body, is the diversity in cell types and their specific interconnections. To generate precise and reproducible patterns of electric activity that choreograph muscle contractions (and the workings of other effectors, such as ciliated cells or glands) into meaningful behaviors with adaptive value, nerve cells need to take on a large variety of shapes and form highly specific connections.

To produce many cells, specify different cell types, and generate invariant connections among neurons puts a heavy burden on the developmental process generating the central nervous system, called neurogenesis. Neurogenesis can be broken down into three major
morphogenetic steps which overlap in time: (1) birth and proliferation of neural progenitors; (2) migration of neural precursors and extension of nerve fibers; (3) specific branching and formation of connections of nerve fibers. I will briefly characterize all three phases of neurogenesis, and then provide a more detailed overview of the mechanisms of early neurogenesis, which I have studied in my thesis for the genetic model, *Drosophila melanogaster*.

1.1. Early neurogenesis

Progenitors of the nervous system arise within a specialized domain within the ectoderm, called the neurectoderm. These progenitors internalize into the interior of the animal, and embark on a prolonged period of proliferation. To account for the high cell number of neurons alluded to above, proliferation of neural progenitors lasts considerably longer than that of other cells; for example, in the *Drosophila* embryo, cells in general undergo 2-4 divisions after blastoderm formation, which lasts to about 25-30% of development, before becoming postmitotic and differentiating into various cell types, such as epidermis, muscle, or gut. By contrast, neural progenitors undergo 8-10 divisions during an extended period of proliferation that lasts until about 60% of embryogenesis (Hartenstein and Campos-Ortega, 1985; Hartenstein et al., 1987).

The mechanism by which neural progenitors split from the neurectoderm, the way they arrange within the embryo, and their pattern of proliferation differs among animal taxa. For most taxa, early neurogenesis has not been investigated in detail, since specific molecular markers were, until very recently, only available for a few “model species”, such as mouse, chicken, *Xenopus*, zebrafish, *Drosophila*, and *C. elegans*. In vertebrates, early neurogenesis comprises the invagination of the entire neurectoderm (“neurulation”) and subsequent formation of an epithelial tube, the neural tube. Neural progenitors within the neural tube divide symmetrically, leading to an enlargement of the neural tube. At later stages, the orientation of the mitotic spindles tilts, such that with each division, one daughter cell will be pushed out of the neuroepithelium. This
daughter cell will often become postmitotic and differentiate as a neuron (Paridaen and Huttner, 2014). In several regions of the neural tube that give rise to much larger structures, notably the forebrain vesicle, cells that have moved out of the neuroepithelium establish a secondary proliferatory center, called the subventricular zone, which is able to generate many more neurons than the neuroepithelium alone (Bayor 1980a, b; Gotz and Huttner, 2005; Huttner and Kosodo, 2005; Paridaen and Huttner, 2014).

In *Drosophila*, early neurulation takes a different shape than neurulation in vertebrates. Instead of invaginating as in vertebrates, the fly neurectoderm gives birth to individual progenitors, called neuroblasts (Campos-Ortega and Hartenstein, 1985). Neuroblasts form a relatively small population of cells, when compared to neural progenitors in vertebrates. Each neuroblast is uniquely specified by a mechanism that involves a class of bHLH transcription factors, called proneural genes (Campuzano and Modolell, 1992; Chan and Yan, 1999; Delidakis and Artavanis-Tsakonas, 1992; Guillemot, 1995; Hinz et al., 1994). Turned on by the intricate pattern of segmentation genes, proneural genes are expressed in small domains, called proneural clusters (Hinz et al., 1994). For example, at the onset of neurogenesis, the proneural gene l(sc) is expressed in four patches of 8-10 cells each within each hemisegment of the early embryo (Skeath and Carroll, 1992; Skeath and Doe, 1998). Each proneural cluster gives rise to a single neuroblast, which is achieved by a mechanism of lateral inhibition. Thus, the Notch ligand Delta (Dl), which is first upregulated in the entire proneural cluster in response to proneural gene expression, becomes increasingly concentrated in one cell, the presumptive neuroblast, which thereby activates the Notch pathway in all other cells of the proneural cluster (Cabrera 1990). Binding of Delta causes a conformational change followed by a cleavage of the Notch receptor. The released intracellular Notch fragment (NICD) moves into the nucleus and upregulates the expression of the bHLH repressor, Enhancer of split, E(spl) (consisting of the loci mδ, mγ, mβ, m3, m5, m7, m8 and groucho; Artavanis-Tsakonas and Simpson, 1991;
Notch activity in these cells negatively feeds back on the proneural genes, resulting in the abandonment of a neural fate in these cells. By this mechanism, a proneural cluster produces only one neuroblast, whereas all other cells become epidermal progenitors (Campos-Ortega and Knust, 1990; Artavanis-Tsakonas and Simpson, 1991; Posakony 1994; Lewis 1996; Chan and Jan, 1999). Not all parts of the *Drosophila* nervous system arise by individualized neuroblasts. Instead, parts of the neurectoderm, in particular those that give rise to the visual system, invaginate and undergo an initial phase of epithelial growth, similar to the vertebrate neural tube. I will highlight this (for flies) “non-canonical” mode of neurogenesis in a later section of this chapter.

1.2. Morphogenesis of the neural primordium: progenitor migration and extension of fibers

In vertebrates, postmitotic neurons undergo considerable migrations before sending out their first axons. Generated within, or adjacent to, the neuroepithelium (called the ventricular layer because it borders the inner lumen of the neural tube, which becomes the ventricle of the nervous system), postmitotic neural precursors move basally to form the mantle layer of the expanding neural tube. Migration is guided by the long apical-basal extensions of the neuroepithelial cells, which at these later stages of neurogenesis are called “radial glia”. Nerve fibers growing out of the neurons of the mantle layer are preferentially directed basally, and form the outermost layer of the neural tube, called the molecular layer. This layer becomes the white matter (due to the myelin ensheathing long nerve fibers in the molecular layer); the mantle layer, housing mostly cell bodies and shorter nerve processes, becomes the gray matter. In some regions, in particular the forebrain, many postmitotic neurons migrate through the molecular layer and establish a layered zone of gray matter at the surface, which becomes the cerebral cortex. Aside from these radial migrations, neurogenesis is also dominated by tangential migration, whereby neuronal precursors move parallel to the plane of the neural tube. Well
studied examples are the tangential migration of cerebellar granule cells, and of cortical interneurons born in the ventral part of the forebrain vesicle (Cooper et al., 2013; Kawaji et al., 2004).

Migration is not a prominent feature during *Drosophila* neurogenesis. Neuroblasts generally remain at the position where they were born and divide in an asymmetric, stem cell-like pattern (Homem and Knoblich, 2012; Zhong and Chia, 2008). Each division renews the neuroblast, and produces a smaller cell, the ganglion mother cell (GMC) that remains attached to its mother neuroblast. The GMC undergoes one more division, generating two postmitotic neurons. This process repeats itself 5-8 times in the embryo, and many more times in the larva. As a result, each neuroblast produces a primary lineage of 10-16 neurons in the embryo, and a much larger secondary lineage of about 150 cells in the larva (Bello et al., 2008). Primary lineages differentiate first and form the functional neurons of the larva; secondary neurons, after sending out a single fiber, hold off with differentiation until the pupal period (Hartenstein et al., 2008).

Lineages split into smaller units with cells adopting different fates. First, the two daughters of GMCs have different fates, called “A” and “B” fates. The differentiation between these two fates depends on Notch activity, with the A-fate relying on high Notch, and the B-fate on the absence of Notch (Truman et al., 2010). Neurons with the A- and B-fates form two so-called hemilineages. Secondly, Neuroblasts undergo temporal phases of differential gene expression, which equips GMCs/neurons born during these phases with different genetic “addresses”. This process has been best studied for primary lineages (Brody and Odenwald, 2005) and more recently rapidly proliferation Type II neuroblasts (Bayraktar and Doe, 2013). In the case of primary lineages, most neuroblasts express the transcription factor Krueppel (Kr) during their first round of division, followed by Huckebein (Hb), Nubbin (Pdm-1), Castor (Cas), and Grainy head (Gh) during subsequent divisions (Brody and Odenwald, 2005). These transcription factors
interact with each other, and are thought to provide neurons that “inherit” them due to the time of their birth with specific fates (Brody and Odenwald, 2005; Maurange 2012).

Neurons of one lineage remain close to their mother neuroblast, with earlier born neurons generally pushed away from the neuroblast by later-born siblings. Upon the onset of differentiation, neurons send out a single nerve fiber. Fibers of one hemilineage bundle (fasciculate) with each other, so that in the end, each hemilineage produces a discrete fiber bundle, called primary or secondary axon tract (Hartenstein et al., 2008). These axon tracts form a blue-print of connections in the center of the developing brain. Mapping lineages at the larval and adult stage provides one with a map of “macro-connectivity” of the fly brain. Thus, what amounts to defined fiber bundles connecting anatomically defined subdivision of the vertebrate brain, such as the nigro-stratal tract (substantia nigra to striatum) or geniculo-cortical tract (corpus geniculatum to visual cortex), is represented by lineage associated tracts in the fly brain. For example, four lineages send their axons in the antennal lobe tract, which connects the olfactory center (antennal lobe) to the mushroom body (Lai et al., 2008; Das et al., 2013). Other, defined lineages generate the longitudinal system of small field neurons of the central complex (Lee lab ref), or the visual input pathway to the central complex (Omoto et al., manuscript in prep).

Even during metamorphosis, there is no large-scale migration of neural progenitors. Clonal analysis has shown that cells of a given lineage remain close to each other in the adult brain (Wong et al., 2013). An exception is seen in the shifts in location of entire lineages or hemilineages that occur in response to non-isometric growth of the brain. These shifts in location, in our interpretation, do not correspond to canonical cell migrations (in the sense this term is applied to the movement of neural precursors in vertebrates), because only the cell bodies move after its fiber has already established connections in the neuropil. I will provide a
more detailed description of this cell movement in the developing *Drosophila* brain in subsequent chapters of my thesis.

1.3. *Neuronal differentiation and establishment of connectivity*

This phase is without doubt the most complex event shaping the nervous system. Each neuronal process branches according to its specific fate, and contacts specific target neurons. A wealth of studies has provided us with a list of signaling mechanisms that either act as gradients, or as specific “recognition molecules” during the formation of specific neuronal connections. Much progress has been made in understanding how the strictly homotopically ordered connections within the visual system of both vertebrates and invertebrates (“retinotopic map”) are established. In the vertebrate retinotectal map, repellant signals of the ephrin family and their receptors (reviewed in Xu and Henkenmeyer, 2012) account for the ordered connectivity, as will be discussed in more detail in Chapter 3 of my thesis. Ephrins are likely to play a role in *Drosophila* as well (Boyle et al., 2006). However, more detail is known about how another system of repellants, the semaphoring/plexin system, is involved in the ordered formation of the retinotopic map (Cafferty et al., 2006; Hsieh et al., 2014; Komiyama et al., 2007; Lattemann et al., 2007; Pecot et al., 2013; Sweeney et al., 2007, 2011; Yu et al., 2010).

In the *Drosophila* central brain and ventral nerve cord, the lineage mechanism of neurogenesis, introduced in the previous section, plays a pivotal role in controlling neuronal connectivity. Lineages, or part of lineages, form structural modules, whereby specific compartments, such as the antennal lobe or ellipsoid body, are scaffolded by individual lineages, or small groups of lineages. In the *Drosophila* larva, the olfactory center, called antennal lobe, receives input from 21 olfactory receptors (Vosshall and Stocker, 2007). The terminals of these receptors form large bulbous endings, called glomeruli. Glomeruli are structurally and functionally unique entities, with neurons expressing a defined set of olfactory receptors, always targeting the same glomerulus (Liang et al., 2013; Stocker et al., 1990; Su et al., 2009; Vosshall and Stocker, 2010;
Vosshall et al., 2007). Each glomerulus is connected to one postsynaptic projection neuron. All projection neurons form part of a single lineage, BAmv3 (Das et al., 2013), and there is, for most glomeruli, a strict 1:1 relationship between presynaptic receptor and postsynaptic projection neuron. One can speculate that the mechanism in place here is that, first, one (out of approximately 100) lineages get specified as a whole, possibly at the stage of the founding neuroblast, to recognize olfactory receptor terminals. Subsequently, individual neurons of this lineage, born sequentially and thereby equipped with dynamically changing genetic information (Boyle et al., 2006; Cafferty et al., 2006; Hsieh et al., 2014; Komiyama et al., 2007; Lattemann et al., 2007; Pecot et al., 2013; Sweeney et al., 2007, 2011; Yu et al., 2010), are instructed to specifically interact with certain receptors. A similar mechanism is likely to operate in many other compartments of the brain. In view of their important role as “organizers of connectivity” at the level of entire compartments (e.g., BAmv3-antennal lobe) as well as of individual neurons, the mapping of lineages throughout development represents an important project in Drosophila neurobiology. As reported in the appendices of my thesis, I was part of a team of students who carried out this project over the past several years.


The by far largest part of the Drosophila brain is occupied by the optic lobe, which represents a series of layered neuropils that receive and process input from the compound eye. Early neurogenesis in the optic lobe proceeds in a manner that differs from the above described fixed lineage mechanism encountered in the central brain. Rather than individual neuroblasts delaminating from proneural clusters, the neuroectodermal domain giving rise to the optic lobe invaginates and forms a neuroepithelium (“optic anlage”), not unlike the optic vesicle that forms the primordium of the vertebrate retina (Green et al., 1993; see Ch. 6 for more details). As will be described in a subsequent section of this chapter (Ngo et al., 2010; see Ch. 2), the optic anlage undergoes an initial phase of symmetric divisions, greatly enlarging the number of neural
progenitors. Subsequently, following a linear temporal gradient, the anlage converts into asymmetrically dividing, neuroblast-like progenitors which produce the neurons of the optic lobe.

The type of neurogenesis encountered in the optic lobe of Drosophila (and arthropods in general; Harzsch et al., 1999) bears much resemblance to the analogous process in vertebrates, as will be presented in Chapter 4 of my thesis. If one looks at teleosts, in particular, both the retina and its target, the tectum, form structures that continuously grow through the life of the animal (Johns 1977; Meyer 1978; Raymond and Easter, 1983; Raymond et al., 1983). This is made possible by a growth zone located around the margin of the retina and tectum. The growth zone consists of three adjacent domains that function like a conveyor belt. Most peripherally is a narrow band of epithelial stem cells which perpetually undergo self-renewing divisions. In next zone, facing inward, daughter cells produced by the stem cells enter a phase of rapid amplifying proliferation. Next comes the zone of postmitotic neural precursors, which, when differentiating, continuously add to the existing population of neurons of the retina and tectum. This “conveyor belt” mechanism of neurogenesis is found in the visual system (retina and tectum) of all vertebrates and may have evolved to account for the large number of orderly connected cells. Interestingly, the way in which neurons are generated in the Drosophila visual system bears much resemblance to the conveyor belt mechanism: as in vertebrates, one sees a neuroepithelial growth zone (optic anlage) flanking a zone of rapidly dividing neuroblasts, followed by gradually differentiating neural precursors.

In the remaining two sections of this introductory chapter I will provide more detail concerning the early development of the Drosophila optic lobe, which then sets the stage for the second Chapter 2, a published paper in which I investigated the role of the Notch and Jak/Stat signaling pathways during the epithelial-to-neuroblast transition of the larval optic lobe. Chapter 3 looks at the second phase of optic lobe development that takes place during metamorphosis. Chapter 4 compares the conveyor belt-like mechanism of neurogenesis in vertebrates and Drosophila.
final chapters (5-7) are published papers that document the anatomy of neural lineages in the *Drosophila* central brain.

3. Early development of the optic lobe

The fly visual system consists of photoreceptor cells (derived from the imaginal eye discs) and its target neurons located in the optic lobe. Photoreceptors project in a retinotopic order to the optic lobe, part of the brain that processes exclusively visual information, and that has been homologized with the nuclear layers (synaptic strata) of the vertebrate retina tectum (Sanes and Zipursky, 2010; Erclik et al., 2009; Strausfeld 1971; Cajal and Sanchez, 1915; Joly et al., in revision)

The larval visual system is derived from the posterior region of the embryonic head (procephalon), the optic lobe placode (OLP). At St. 13, the OLP invaginates from the embryonic head, forms a flattened vesicle and remains epithelial in character, expressing a number of molecular signatures including *sine oculis* (so), *tailless* (tll), and FasII (Cheyette et al., 1994; Daniel et al., 1999; Green et al., 1993). So, tll, and Fas2 is expressed in the developing OLP as early as St. 5 (Cheyette et al., 1994; Grenningloh et al., 1991) where tll becomes preferentially expressed in the anterior and posterior lip of the OLP in the mid embryo. Interestingly, overexpression of tll results in a cyclopic phenotype in which the optic lobes become fused at the dorsal midline (Daniel et al., 1999); however, the regulator(s) that activate or maintain tll expression in the OLP remains to be identified.

The placode, consisting of about 30-40 progenitor cells remains as one structure and begins separating into the inner and outer optic anlagen (IOA, OOA), respectively during the first twenty-four hours after the first molt (Ngo et al., 2010; Egger et al., 2007). Given the large number of optic lobe neurons (about 100,000, compared to less than 3,000 in the central brain), proliferation of the optic anlagen continues throughout a long period of development, from
embryo to early pupa, and is further boosted by a two-phase mechanism which is unique to the visual system. In a first phase (which does not occur in the central brain or ventral nerve cord), the optic anlagen grow by symmetric cell division to a size of several thousand epithelial progenitor cells (Ngo et al., 2010; Egger et al., 2007; Hofbauer and Campos-Ortega, 1990). The OOA of the early larva can be pictured as an expanding rectangular sheet of epithelial cells. They express a number of proteins known to be enriched in epithelial cells: PatJ, DE-Cadherin (DEcad), Crumbs (Crb), Scribble (Scrib) as well as the proneural gene, scute, suggestive of their neural potential (Egger et al., 2007). During the second larval instar, the OOA becomes subdivided into two domains, visibly subdivided by a furrow called lamina furrow. Cells lateral of this furrow (OOAl) give rise to the lamina; the much larger medial domain (OOAm) form the distal medulla.

During the second phase, which begins halfway through the larval period, the progenitors undergo an epithelial-mesenchymal transition (EMT), becoming neuroblasts that enter a phase of asymmetric cell division, each neuroblast producing a lineage comprising ~100 neurons. The molecular pathways controlling the EMT of optic lobe progenitors has been elucidated by recent studies (Apitz and Salecker, 2015; Morante et al., 2013; Orihara-Ono et al., 2011; Egger et al., 2010; Ngo et al., 2010; Reddy et al., 2010; Yasugi et al., 2008, 2010). At around the time when the lamina furrow divides the OOA into a lateral and medial domain, epithelial cells along the edges of these domains convert into asymmetrically dividing neuroblasts. These neuroblasts express classical asymmetric determinants (Ngo et al., 2010; Yasugi et al., 2010, 2008; Egger et al., 2007): Deadpan (Dpn), Asense (Ase), Inscutable (Insc; Roegiers and Jan, 2004; Knoblich 1997). Several other determinants are required for asymmetric division. The Par protein complex, consisting of aPKC and the conserved PDZ domain proteins Bazooka (Par-3) and Par-6, localizes apically and directs localization of the cell fate determinants such as Prospero, Numb, and the adapter proteins Miranda and Pon to the basal cell cortex (Betschinger et al.,
2003). The resulting polarity generates one identical cell and one developmentally restricted Prospero-positive ganglion mother cell (GMC), which will lead to two differentiating neurons after another round of division.

Neuroblasts “bud off” progeny in the direction perpendicular to the plane defining the OOA. Because of this directed proliferation, neurons born first come to lie at ever increasing distances from the neuroblast/OOA. At the same time as the neuroblasts divide, new rows of neuroblasts appear as, one by one, rows of epithelial cells along the medio-lateral-axis convert into neuroblasts.

As will be described in the subsequent chapter, the description of OOA development indicates that two spatio-temporal gradients are built up in the OOAm. One gradient, directed along the medio-lateral axis of the OOAm (“ml-gradient”), describes the sequence in which rows of neuroblasts are formed; the second gradient, directed from the surface inward, perpendicular to the plane of the OOA (“z-axis”), underlies the order in which each neuroblast produces neurons (“z-gradient”). The ml-gradient correlates with the anterior-posterior axis of the medulla (Fig. 1-1). Thus, axons that grow towards the first-born OOAl neurons, derived from the most medial row of neuroblasts, are the R7/8 axons originating from posterior retina, as well as L- neurons from the posterior lamina (Meinertzhagen and Hanson, 1993). The next set of axons, arriving later, captures neurons of the next (more lateral) row of OOAm neuroblasts. What this implies is that the ordered progression of NE-NB conversion may match the progression of ingrowing axons, and that this matching may be important for the formation of an ordered medulla neuropile. Such gradients can account for the sequential generation of different neuronal cell types (Hasegawa et al., 2011; Morante et al., 2011).

What adds to the structural complexity of the larval optic lobe is the fact that the OOA, so far pictured as a simple, rectangular sheet, bends along the dorso-ventral-axis. As a result, instead of forming straight rows along the dorso-ventral axis, cells are aligned in C-shaped curves. What
this spatial transformation means when looking at optic lobes sectioned along the “standard” frontal plane is that the OOA is sectioned twice, once dorsally, and once ventrally.

The inner optic anlage (IOA) also undergoes a NE-NB conversion, bending along the dv-axis, similar to what has been described above for the OOA. Briefly, in the late larva, the IOA consists of a C-shaped epithelial component. Further laterally, neuroblasts derived from the IOA (IOAnb) form a mass of cells that is also bent, and therefore seen twice in a frontal section. The IOA neuroblasts produce two populations of neurons. Neurons pushed anteriorly (or outward, taking into account the curvature of the IOA) become the proximal medulla (Mp); those pushed interiorly, or centrally, become the lobula and lobula plate. The precise timing of NE-to-NB are intricately regulated by multiple signaling pathways, their functions very complex as their roles are cell-type specific. The roles of these pathways will be reviewed below.

4. Major signaling pathways that regulate non-canonical epithelium-to-neuroblast transition

Classically, neuroblasts of the central brain and ventral nerve cord are specified in a two-step process (Chan and Jan, 1999; Campos-Ortega and Knust, 1990; Technau and Campos-Ortega, 1985; Hartenstein and Campos-Ortega, 1984). During the first step, discrete clusters of neuroectodermal cells (“proneural clusters”) express a combination of regulatory genes, the proneural genes, which makes the cells with neural potential “competent” to form neuroblasts. In the second step of neuroblast specification, called lateral inhibition, all cells express uniform levels of both activated Notch and Delta, having the potential to inhibit or be inhibited by its surrounding neighbors (Artavanis et al., 1995; Kooh et al., 1993; Fehon et al., 1991). Cells of each proneural cluster would then “compete” with each other to become a neuroblast.

By contrast, in the larval optic lobe, neuroblasts generation does not occur by a classical lateral inhibition mechanism. Rather, as briefly described above, neuroepithelial cells undergo an epithelial-to-mesenchymal transition, express the proneural gene and converts into an
asymmetrically dividing neuroblast. This process occurs in a tightly regulated spatiotemporal manner such only a few cells at any one time are converted to neuroblasts and key molecular players regulating temporal conversion of neuroblasts have been identified by a number of groups including: Notch, JAK/STAT, EGFR, and Fat-Hippo pathways (Egger et al., 2010; Ngo et al., 2010; Perez-Gomez et al., 2013; Reddy et al., 2010; Wang et al., 2011; Yasugi et al., 2008, 2010; see Fig. 1-2). Fat-Hippo regulates NE proliferation in the early larval stages together with Notch and will not be discussed here (Reddy et al., 2010). The roles of EGFR and Notch will be reviewed below. The roles of JAK/STAT in neuroepithelial-to-neuroblast transition will be discussed in greater details in Chapter 2.

Neuroblast conversion is induced by the proneural gene, lethal of scute (l’sc). L’sc is part of the proneural complex (AS-C), consisting of l’sc, scute (sc), achaete (ac), and ase that are clustered together (Martin-Bermudo et al., 1993; Alonso and Cabrera, 1988). The AS-C has a well-established role in NB delamination, adult sensory organ (SO) development, as well a number of regulatory roles in the central and periphery nervous system in Drosophila (Gomez-Skarmeta et al., 1995). L’sc has been shown to be sufficient for proneural function and activates neurogenic genes (Hinz et al., 1994). In the developing OOA, l’sc induces NB conversion (Yasugi et al., 2008).

4.1 EGFR promotes neuroepithelial-to-neuroblast transition

The EGFR signaling pathway has been very well characterized with multiple functions in many development contexts (Doroquez and Rebay, 2006). The Ras pathway triggered by the epidermal growth factor (EGF) pathway activates the ETS transcription factor Pnt that exists in two isoforms, PntP1 and PntP2 with the former locally induced in transitional neuroepithelial cells (Klambt 1993; Scholz et al., 1993; Shwartz et al., 2013). PntP1 and PntP2 is specific to the EGFR and Ras signaling, respectively (Klambt 1993). Signal binding of the secreted Spitz (s-Spi) ligand to the receptor transduces and translocating the downstream effector Ras-
activated protein kinase (MAPK) into the nucleus, phosphorylating the ETS-domain transcription factors PntP2. Induction of PntP1 activates the expression of lethal of scute (l’sc), in turn promoting neuroblasts (NB) transition (Yasugi et al., 2010).

Spitz is broadly expressed in the developing OOA while Rhomboid, which is required for Spi maturation, is expressed medial to l’sc. Loss of EGFR signaling in spi, rho, or pnt can cell-autonomously downregulate l’sc expression (Yasugi et al., 2010). Interestingly, Notch overactivation can induce the expression of l’sc, compensating for the loss of EGFR (Yasugi et al., 2010). Jak/Stat, Notch, and EGFR are mutually dependent on one another, although the exact molecular mechanism remains elusive.

4.2 Notch acts on multiple cell types in the outer optic anlage

The role of Notch in the larval outer optic anlage contrasts that of the lateral inhibition mechanism in the embryonic central nervous system (see Chapter 2 for more details). The Notch receptor is activated by two different ligands, Delta (Dl) and Serrate (Ser). Upon signal activation, Notch is proteolytic cleaved; the intracellular domain of Notch subsequently translocates into the nucleus where it associates with the transcriptional activator, Suppressor of Hairless [Su(H)] and activates downstream target genes. Among the most well-characterized Notch target genes are the Enhancer of Split Complex [E(spl)-C], comprising of seven genes: mδ, mγ, mβ, m3, m5, m7, m8 and groucho (Delidakis and Artavanis-Tsakonas, 1992; Jennings et al., 1994; Knust et al., 1992; Lecourtois and Schweisguth, 1995). In other developmental contexts such as the imaginal discs, Notch target genes have distinct expression patterns (Cooper et al., 2000; de Celis et al., 1996; Wech et al., 1999), suggestive of the possibility that Notch functions in the outer optic anlage is cell-type dependent.

In the early larva, expression of the Notch ligand, Delta and Notch activity is found ubiquitously at moderate levels in the outer optic anlage (OOA). At the stage when neuroblasts (NB)
conversion begins, the Delta/Notch pathway becomes dynamically centered around this
transition zone, following the ml-gradient of the OOA, with Delta expression showing a peak
level in transitioning neuroepithelial cells (tNE; Figure 1-2). These strongly Delta-positive cells
flank the already formed neuroblasts. The region of transitioning epithelial cells which shows
peak expression of DI have been previously shown to also express L'sc (Reddy et al., 2010).

Mutant analyses of DI, Notch, and Ser suggest each ligand activates a succinct set of Notch
target genes (Ngo et al., 2010; Perez-Gomez et al., 2013; Wang et al., 2011). Supporting this
notion, Perez-Gomez and colleagues observed that the Serrate (Ser) ligand is expressed in glial
cells (2013), where it was concentrated at the glial membranes in contact with the apical side of
NE cells. This suggests that Notch functions in the OOA are cell-type dependent. As expected,
different Notch target genes are expressed in different cell types. m8 and mγ is restricted to NB
and their progenies (Perez-Gomez et al., 2013; Weng et al., 2012); mβ and mδ is restricted to
surface glia; m7 being restricted to the neuroepithelium. Ser in the epithelial glia s required to
prevent premature neuroblast conversion as loss of ser results in ectopic activation of the l’sc
and PntP1 which are both sufficient to induce NB conversion (Yasugi et al., 2008, 2010). Delta
activates Notch in tNE and is required for the maintenance of the neuroepithelium (Weng et al.,
2012). The preferential expression of m7 in NE suggests that Delta-Notch activates E(spl)m7
target gene although this has yet to be tested. In glia cells, Ser-Notch interaction activates mβ
and mδ target genes to inhibit uncontrolled activation of the EGFR-Ras pathway and proneural
activation of l’sc (Perez-Gomez et al., 2013; Fig. 1-2).
Schematic representation of conveyor belt neurogenesis. Slowly dividing neuroepithelial stem cells (yellow) is flanked by rapidly dividing progenitor cells (asymmetrically dividing neuroblasts in *Drosophila*, green). Neuroblasts self-renewal by asymmetric division that gives rise to ganglion mother cells (GMCs). GMCs differentiate into neurons (gray).
Figure 1-2.

**Signaling model that regulates neuroepithelial-to-neuroblast transition.**

In the early larva, expression of the Notch ligand, Delta and Notch activity is found ubiquitously at moderate levels in the outer optic anlage (OOA). At the stage when neuroblasts (NB) conversion begins, Delta is expressed in transitioning neuroepithelial cells (tNE, dark yellow). Lethal of scute is expressed in the most medial Delta-expressing tNE (brown yellow). Serrate (Ser) ligand is expressed in glial cells (blue line), where it was concentrated at the glial membranes in contact with the apical side of NE cells. \(E(spl)m8\) and \(E(spl)m\gamma\) is restricted to NB and their progenies; \(E(spl)m\beta\) and \(E(spl)m\delta\) is restricted to surface glia; \(E(spl)m7\) being restricted to the neuroepithelium. Ser in the epithelial glia is required to prevent premature neuroblast conversion.
References


Chapter 2

Concomitant requirement for Notch and Jak/Stat during neuro-epithelial differentiation in the optic lobe
Concomitant requirement for Notch and Jak/Stat signaling during neuro-epithelial differentiation in the *Drosophila* optic lobe

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**Abstract**

The optic lobe forms a prominent compartment of the *Drosophila* adult brain that processes visual input from the compound eye. Neurons of the optic lobe are produced during the larval period from two neuroepithelial layers called the outer and inner optic anlage (OA, OA). In the early larva, the optic anlagen grow as epithelia by symmetric cell division. Subsequently, neuroepithelial cells (NE) convert into neuroblasts (NB) in a tightly regulated spatio-temporal progression that starts at the edges of the epithelia and gradually moves towards its center. Neuroblasts divide at a much faster pace in an asymmetric mode, producing lineages of neurons that populate the different parts of the optic lobe. In this paper we have reconstructed the complex morphogenesis of the optic lobe during the larval period, and established a role for the Notch and Jak/Stat signaling pathways during the NE-NB conversion. After an early phase of complete overlap in the OAs, signaling activities sort out such that jak/stat is active in the lateral OAs which give rise to the lamina, and Notch remains in the medial cells that form the medulla. During the third instar, a wave front of enhanced Notch activity progressing over the OAs from medial to lateral controls the gradual NE-NB conversion. Neuroepithelial cells at the medial edge of the OAs, shortly prior to becoming neuroblasts, express high levels of Delta, which activates the Notch pathway and thereby maintains the OAs in an epithelial state. Loss of Notch signaling, as well as Jak/Stat signaling, results in a premature NE-NB conversion of the OAs, which in turn has severe effects on optic lobe patterning. Our findings present the *Drosophila* optic lobe as a useful model to analyze the key signaling mechanisms controlling transitions of progenitor cells from symmetric (growth) to asymmetric (differentiative) divisions.

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**Introduction**

Neurons and most glial cells of the *Drosophila* brain are generated by a population of neural stem cells called neuroblasts ([Yu et al., 2006; Egger et al., 2008]). Neuroblasts divide asymmetrically, producing with each round of mitosis another neuroblast and a smaller daughter cell, the ganglion mother cell (GMC), which after one more round of division differentiates into neurons or glial cells. Neuroblasts of the central brain and ventral nerve cord (analog of the vertebrate spinal cord) are born in the early embryo; these neuroblasts are relatively few in number (less than 500 in all), and each one produces an invariant “lineage” of neurons/glia cells. By contrast, the optic lobe, in terms of cell number by far the largest part of the intact brain, is formed by neuroblasts that are born in the late larva from two neuroepithelial layers called the inner and outer optic anlagen (IOA, OOA; Meierzhagen and Hanson, 1993; Risbach and Hering, 2008). The optic anlagen arise in close proximity to the eye imaginal disc in the embryonic head ectoderm (Green et al., 1993). In the early larva, both IOA and OOA grow by symmetric cell division. With the beginning of the third instar (about half way through the larval period), neuroepithelial cells (NE) convert into neuroblasts (NB) in a tightly regulated spatio-temporal progression that starts at the edges of the epithelia and gradually moves towards its centers ([Hofbauer and Campos-Ortega, 1990; Meierzhagen and Hanson, 1993; Egger et al., 2007]). Neuroblasts divide at a much faster pace in an asymmetric mode, producing the lineages of neurons that populate the different parts of the optic lobe. The optic anlagen, as well as the neuroblasts and lineages derived from them, together form the complex optic lobe primordium that accounts for fully half of each late larval brain hemisphere.

Neuroblasts of the central brain and ventral nerve cord are specified in a two-step process. During the first step, discrete clusters of neuroectodermal cells ("proneural clusters") express a combination of regulatory genes, the proneural genes, which makes the cells with neural potential "competent" to form neuroblasts. Proneural genes encode DNA binding proteins that belong to the large family of
basic helix-loop-helix (bHLH) transcription factors, including the achaete-scute complex (AS-C) and their vertebrate homologs (Campuzano and Modelski, 1992; Guillemot, 1995; Kageyama et al., 1995). In the second step of neuroblast specification, called lateral inhibition, cells of each pronomeral cluster "compete" with each other to become a neuroblast. On the molecular level, this competition is mediated by the Notch signaling pathway, whose members are encoded by the so-called neurogenic genes (Campos-Ortega and Knust, 1990; Artavanis-Tsakonas and Simpson, 1991; Posakony 1994; Lewis 1996; Caan and Jan, 1999). Expression of the Notch ligand Delta is upregulated within the primordial clusters by AS-C gene. Binding of Delta causes a conformational change followed by a cleavage of the Notch receptor. The released intracellular Notch fragment (NCIS) moves into the nucleus and upregulates the expression of the bHLH repressor, Enhancer of split (E(spl)), (consisting of the lmd, m, mp, m3, n5, m7, m8 and groucho; Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992; Schorn et al., 1992). The expression of these genes down-regulates the transcription of the AS-C gene complex, causing the cell to abandon its neural fate and become epidermal. In neurogenic mutants (Lehmann et al., 1983), in which lateral inhibition is perturbed, expression of the proneural genes does not become restricted to individual cells, but persists in all cells of the proneural cluster (Calderon 1990; Martin-Bermudo et al., 1995; Ruiz-Gomez and Gaynes, 1993; Skeath and Carroll, 1992). As a result, all cells of the neurogenic region become neuroblasts, a phenotype called neural hyperplasia.

It stands to reason that the AS-C and neurogenic genes are also essential for the neuroepithelial-to-neuroblast (NE-NB) transition which occurs in the optic anlagen. Yasugi and colleagues (2008) have recently demonstrated that the proneural gene lethal of scute (lsc) is indeed expressed in a narrow band of cells that fall within the epithelium-to-neuroblast transition zone of the OOA. Use expression is negatively regulated by the Janus Kinase/signal transducer and activator of transcription (Jak/Stat) signaling cascade (Yasugi et al., 2008). Loss of Jak/Stat activity in Star92E clones does result in excess cells expressing lsc, and, subsequently, causing these cells to adopt a neuroblast fate. This finding matches similar results with Jak/Stat signaling in the vertebrate retina, where expression of the dominant-negative form of Stat3 promoted neurogenesis instead of astrogliogenesis (Gu et al., 2005). Furthermore, Reddy et al. (2010) show that the Hippo-Fat pathway signals a cell cycle arrest in the OOA epithelium, which in turns may be necessary for an upregulation of the Notch ligand Delta, and consequent activation of the Notch pathway.

In this paper we have reconstructed the NE-NB conversion in detail, and investigated the role of Notch signaling and its interaction with the Jak/Stat pathway in the Drosophila optic lobe. In the early larval optic lobe, prior to neuroblast conversion, both Notch and Stat activity is found ubiquitously in the optic anlagen. Subsequently, the expression domain of Jak/Stat and Notch signaling separate spatially, such that Notch activity remains high in the medial OOA, and Jak/Stat in the lateral OOA. During the third instar, a wave of enhanced Notch activity progressing over the OOA from medial to lateral controls the gradual NE-NB conversion. Epithelial cells at the medial edge of the OOA, shortly prior to becoming neuroblasts, express high levels of Delta, which activates the Notch pathway and thereby maintains the OOA in an epithelial state. Loss of Notch, as well as Jak/Stat, signaling results in a premature NE-NB conversion of the OOA, which in turn has severe effects on optic lobe patterning.

**Material and methods**

**Fly stocks**

Flies were grown at 25°C using standard fly media unless otherwise noted. The following mutant and transgenic strains were used in this study: N63 (Xu et al., 1992), Star92E (Baka et al., 2002), Star92EΔC, UAS-Su(H)DN, CyO, Actin-GFP (gift from Utpal Banerjee; Nagaraj and Banerjee, 2007), UAS-Star92ERWt (Kim et al., 2007), upd-4L4 (Halkier et al., 1995), dome-GAL4 (PG14, from Stephanie Roselli), esg-GAL4 (Goto and Hayashi, 1999), E(spl)m8-bacZ (Leconte and Schweisguth, 1995), F004032UGAP (Bech et al., 2007), nrn6-GAL4 (Paul Vallevera; Bloomington #7695), UAS-myr-RED (Henry Chang; Bloomington #7118) and tub-GLA86R (Ron Davis; Bloomington #7018). E(spl)m8-bacZ is a well-documented reporter for Notch transcriptional activity, containing Suppressor of Hairless binding sites. Star92EΔC is a strong hypomorphic allele of Star92E.

**Temperature shifts and lineage tracing experiments**

For temperature-shift experiments, embryos were collected and raised at 18°C. Hatching first-instar larvae of the Star92EΔC/Star92E; esg-GAL4, UAS-myr-RED/UAS-Su(H)DN, tub-GLA86R/+ and y/y (N1) genotype were shifted to the restrictive temperature (30°C) for various time intervals (e.g., 0-48 h, 0-56 h and 48-96 h). After shifting to restrictive temperatures for certain time intervals, larvae were grown at 18°C. Wandering third instar were dissected and fixed according to standard procedures (Ashburner, 1989).

**Lineage expression analyses**

were done with upd-gal4 using the Gal4 Technique for Real-time and Clonal Expression (G-TRACE), containing the following genotype: UAS-GFP, UAS-dsRedNerger, ubi-p35/FR-tqg-FRT-eGFP, allowing for marking of both cells in real-time and those derived from a particular lineage using fluorescent proteins (Evans et al., 2000).

**Immunohistochemistry and BrdU Labeling**

Samples were fixed in 4% formaldehyde in phosphate buffer saline (PBS, Fisher-Scientific, pH = 7.4; Cat. No. #BP399-4). Tissues were permeabilized in PBS (phosphate buffer saline with 0.1% TritonX-100, pH = 7.4) and immunohistochemistry was performed using standard procedures (Ashburner, 1989). The following antibodies were provided by the Developmental Studies Hybridoma Bank (Iowa City, IA): mouse anti-Dachshund (mAbD2a-2, 1:600), mouse anti-Delta (1:10), mouse anti-NeuTactin (BP166, 1:10), rat anti-DN-Cadherin (DN-DX #8, 1:20), rat anti-DN-Cadherin (DCAD2, 1:500), mouse anti-Fossinidulin (1D4, 1:500), mouse anti-Repo (BD2, 1:20). We also used mouse anti-β-galactosidase (β-Gal; Promega, Cat. No. #25580061, 1:100), rabbit anti-β-Gal (MP Biometicals, LLC, Cat. No. #0856032, 1:100), guinea pig anti-Delta (gift from Marc Muskhatchev, 1:1000), rabbit anti-Deadpan (Dpm; gift from Yuh Nung Jan, 1:500), rat anti-Crumbs (Crb; gift from Elisabeth Knust, 1:2000) and mouse anti-BrdU (BD Biosciences, Cat No. #347580). For BrdU staining, polyclonal antisera was pre-absorbed overnight prior to immunostaining. Secondary antibodies, IgG (Jackson Immunoresearch; Molecular Probes) were used at the following dilutions: Cy5-conjugated anti-guinea pig (1:250) and anti-rabbit (1:2000); Fluorescein (FITC)-conjugated anti-mouse (1:200), anti-guinea pig (1:250) and anti-rabbit (1:200); Cy5-conjugated anti-rabbit (1:100) and anti-mouse (1:100); AlexaFluor 488-conjugated anti-mouse (1:500), Alexa 568-conjugated anti-rabbit (1:300), and 546-conjugated anti-mouse (1:500). Phalloidin 546 and Phalloidin-Rhodamine (Molecular Probes; Cat No. #A22283, #B815, used to visualize actin filaments were diluted in PBTB (pH = 7.4, phosphate buffer saline, 0.1% Triton X-100, 2% BSA, 5% Normal Goat Serum, NCN), 1:100. TO-PRO-3 (Invitrogen, 1 μM in PBTB) was used as a nuclear stain. Tissues were mounted in Vectashield mounting medium (Vector, Burlingame, CA; #H1000).

For BrdU labeling, larvae were fed for the duration of the pulse with medium to which 1 mg/ml of BrdU was added. Subsequently larvae were transferred to normal food for the duration of the chase. For short pulses directly prior to fixation, dissected wandering third-
instar larvae were incubated in BrdU at room temperature (Sigma, 70 μg/ml) in PBS for 30 min. Samples were fixed in 4% formaldehyde in PBS and washed in 0.3% PBS. Denaturation in 2 N HCl for 30 min was followed by a second fixation with 4% formaldehyde in 0.1% PBS. Standard immunohistochemistry was performed as described (Ashburner, 1989).

Confocal microscopy

Staged *Drosophila* larval and adult brains labeled with suitable markers were viewed as whole-mounts by confocal microscopy (Biorad MRC 1024S microscope using Biorad Lasersharp version 3.2 software; lenses: 40× oil (numerical aperture 1.3; WD 0.17); and ISM 700 Image M2 using Zen 2009 (Carl Zeiss Inc.); lenses: 40× oil (numerical aperture 1.3)). Complete series of optical sections were taken at 2-μm intervals. Captured images were processed by ImageJ (National Institutes of Health, http://rsweb.nih.gov/ij/) and Adobe Photoshop.

Generation of three-dimensional models

Digitized images of confocal sections were imported into the Amira (http://www.amiravis.com). Since sections were taken from focal planes of one and the same preparation, there was no need for alignment of different sections. All models were generated using the Amira software package. Objects, including the different domains of the optic anlagen and the emerging neuropile compartments of the optic lobe, were manually segmented on the series of confocal images imported into Amira. These domains are visible based on cell size, cell shape, and texture in brain preparation labeled with anti-Neuromedin antibody, or with Phalloidin. Following segmentation, the program then generated surfaces which could be rendered in different colors and degrees of transparency.

Results

Structure and development of the larval optic lobe

The structure of the optic lobe primordium of the larva is highly dynamic and, towards the later stages, very complex. As a result, we currently have only a rudimentary understanding of how the different neuropiles and cell types of the adult optic ganglia map onto the larval optic lobe primordium. Moreover, the dynamic changes in stage that characterize the optic lobe at the different larval stages make it very difficult to interpret mutant phenotypes of genes controlling optic lobe development. We will in the following depict the normal development of the larval optic lobe, focusing on the outer optic anlage and its derivatives, the distal (outer) medulla and the lamina (Fig. 1).

The OOA of the early larva starts out as an expanding reticular sheet of epithelial cells, formed dorso-ventrally oriented columns of cells (Fig. 1A, B1). Starting at the late first instar and continuing throughout larval life, the OOA epithelium bends along the dorsoventral-axis (Fig. 1A–4). As a result, cells are aligned in C-shaped curves (Fig. 1A). What this spatial transformation means when looking at optic lobes sectioned along the “standard” frontal plane, as shown in Fig. 1A5 is that the OOA is sectioned twice, once dorsally, and once ventrally.

During the second larval instar, the OOA becomes subdivided into two domains, visibly separated by a furrow called lamina furrow (green line in Fig. 1A4, B1–8). Cells lateral of this furrow (OOA) give rise to the lamina; the much larger medial domain (OOAm) form the distal medulla. At around the time when the lamina furrow divides the OOA into a lateral and medial domain, epithelial cells along the edges of these domains convert into asymmetrically dividing medulla neuroblasts (Mnb; Meierzhagen and Hanson, 1993; Egger et al., 2007; Fig. 1B3). As shown in Fig. 1C–F, this transition can be followed effectively by labelling optic lobes with anti-Crumb (Crumb being expressed at the apical membrane of all ectodermally derived tissues; Tepass et al., 1990; Fig. 1C, D) and anti-Deadpan (a marker for neuroblasts; Ber et al., 1992; Fig. 1E, F). Once cells have converted to neuroblasts, they “budd” off progeny in the direction perpendicular to the plane defining the OOA. Because of this directed proliferation, neurons born first come to lie at ever increasing distances from the neuroblast/OOA (Fig. 1B4–8, shown only for OOAm). At the same time as the medulla neuroblasts divide, new rows of neuroblasts appear as, one by one, rows of epithelial cells along the medio-lateral-axis convert into neuroblasts (Fig. 1B5–8). In the late larva, medulla neuroblasts start to disappear. Thus, the lineages at the medial edge of the optic lobe, which had been the first to appear, are no longer capped by a neuroblast (arrowheads in Fig. 1A5 and B8). The fate of the medulla neuroblasts after they cease to divide has not yet been followed in detail. Similar to neuroblasts of the central brain, they are likely to undergo programmed cell death (Cenci and Gould, 2005).

The correlation between neuron position and birth date can be visualized by BrdU pulse-chase experiments, shown in Fig. 1G–I. Early pulses (24 h) result in fast labelling of medulla neurons located deep (arrows in Fig. 1G; red circles in Fig. 1I). In this experiment, BrdU is taken up by all cells of the epithelial OOA which, around 24 h, divide symmetrically. As the epithelium converts into neuroblasts, all neuroblasts “inherit” the (by then already diluted) label. When neuroblasts start their rapid asymmetric division, only the first born neurons receive enough BrdU to maintain detectable label; these are the neurons located deeply. Pulses administered at mid-larval stages (72 h; Fig. 1H) result in strong labelling of neurons located in the medial medulla at deep and intermediate levels (arrow in Fig. 1H; blue circles in Fig. 1J). In this experiment, the BrdU pulse reaches the OOA at a stage when the epithelial cells have all but ceased to divide, and the medial cells have converted into rapidly dividing neuroblasts. These are the cells that incorporate BrdU. Due to the rapid division/dilution of the label, only early born neurons, located deeply, receive enough label. Late pulses, followed by immediate fixation, result in labelling of most neuroblasts and superficially located neurons (arrows in Fig. 1E; green circles in Fig. 1J). At this stage, the most medial lineages no longer proliferate (arrowheads in Fig. 1J). The description of OOA development above indicates that two spatio-temporal gradients are in the OOA. One gradient, directed along the medio-lateral-axis of the OOA ("ml-gradient"), describes the sequence in which rows of neuroblasts are formed; the second gradient, directed from the surface inward, perpendicular to the plane of the OOA ("z-gradient"), underlies the order in which each neuroblast produces neurons ("z-gradient"; Fig. 1D3). The ml-gradient correlates with the anterior-posterior axis of the retina. Thus, axons that grow towards the first-born OOAm neurons, derived from the most medial row of neuroblasts, are the R7/8 axons originating from posterior retina, as well as I- neurons from the posterior lamina (Meierzhagen and Hanson, 1993; Fig. 1B5). The next set of axons, arriving later, captures neurons of the next (more lateral) row of OOAm neuroblasts, etc. What this implies is that the ordered progression of NE-N8 conversion may match the progression of ingrowing axons, and that this matching may be important for the formation of an ordered medulla neuromere. The significance of the z-gradient has not yet been investigated. It seems likely that, similar to what is known for lineages of the central brain and ventral nerve cord, it accounts for the sequential generation of different neuronal cell types (Grosskostenhauer et al., 2005).

Fig. 2 shows the different components of the larval optic lobe primordium in more detail. The epithelial part of the OOA (blue solid lines in Fig. 2A) is flanked medially and laterally by neuroblasts (blue circles) that produce the distal medulla (Md) and lamina (L), respectively. The medial neuroblasts generate lineages of the distal medulla that are directed centro-laterally (hatched lines with arrows in Fig. 2A). As explained in the previous section, the oldest lineage (11)
is the one situated furthest medially; the youngest, most recently born one (In) is the one furthest laterally. Within each lineage, central neurons (nc) are older than peripheral ones (np). Neurons form bundles of axons that gather at the base of the lineages. Along with ingrowing axons from lamina neurons (L) and retinal axons (not shown), this mass of fibers give rise to the neuropile of the distal medulla (MdnY; shaded blue).

The inner optic anlage (IOA) also undergoes a NE-NB conversion, bending along the dv-axis, similar to what has been described above for the OOA. The details of this transformation will be described elsewhere (Ngo et al., in prep). Briefly, in the late larva, the IOA consists of a C-shaped epithelial component (IOAep; red lines in Fig. 2A); note that, as for the OOA, the IOA sectioned along the frontal plane appears twice, once ventrally, and once dorsally. Further laterally, neuromasts derived from the IOA (IOAn; red circles) form a mass of cells that is also bent, and therefore seen twice in a frontal section. The IOA neuromasts produce two populations of neurons. Neurons pushed anteriorly (or outward, taking into account the curvature of the IOA) become the proximal medulla (Mp); those pushed inferiorly, or centrally, become the lobula and lobula plate (Lo; Fig. 2A). Fig. 2B–C illustrates the arrangement of the optic lobe neuromeres in the late larva, and their corresponding adult counterparts.

Activity of Notch and Jnk/Stat in the larval optic lobe primordium

A pivotal step in the development of the optic lobe is the formation of neuromasts from the OOA epithelium. As established for neuromast formation in the embryo, the number of neuromasts emerging
of the central brain and their early progeny. Stat activity is highest in glial cells. At the time when the OOA has expanded and is beginning to differentiate into a lateral and medial domain, Stat becomes increasingly restricted to the lateral domain (OOA; Fig. 3E). In the late larva, Stat is exclusively in the OOA and the lamina progenitors/ lamina neurons derived from it (Fig. 3I). The Jak/Stat ligand upd, after an initial phase of widespread expression in the OOA and IOA, also becomes restricted to the OOA and lamina (Fig. 3H). The restriction of expression can be confirmed experimentally by using the G-TRACE construct (GallTechnique for Real-time and Clonal Expression; Evans et al., 2009), which combines the Gal4/UAAsystem in concert with the FLP/FRT recombination system (Theodosis and Xu, 1998) to visualize both real-time and lineage-traced gene expression patterns. While real-time upd expression is restricted to the OOA and lamina during late larval development (marked by diRedStinger, in red; in Fig. 3H), a large proportion of the optic lobe is labelled by EGFP, demonstrating that these cells are derived from cells expressing upd at an earlier developmental stage. Consistent with the real-time expression of upd-gal4, done-gal4 real-time expression is also restricted to the OOA (Fig. 3I).

At the stage when the NE-NB conversion begins, the Delta/Notch pathway becomes dynamically centered around this transition zone, following the medial gradient of the OOA. Delta remains always restricted to the epithelial part of the OOA (Fig. 3E, F, I, J). Partially, it partially overlaps with the domain of Stat activity, i.e., it reaches into part of the OOA but does not extend all the way to its lateral rim. Medially, Delta expression shows a peak level in epithelial cells about to convert to neuroblasts (arrowheads in Fig. 3F, J). These strongly Delta-positive cells flank the already formed neuroblasts, which express the markers Deadpan (Dpn) and Asense (Ase; Egger et al., 2007; Wallace et al., 2000; Fig. 3G, k). The region of transitioning epithelial cells which shows peak expression of DI has been previously shown to also express Sce (Reddy et al., 2010). Additionally, Notch activity, monitored by expression of E(spl)m8, is high in the OOA, overlapping with the expression of Delta. In contrast to Delta, E(spl)m8 stays on in neuroblasts and their progenies (Fig. 3E, I).

Loss of Notch leads to a precocious release of neuroblasts from the OOA

The dynamic expression of Delta and E(spl)m8 in the optic anlage suggests that Notch signalling is involved in the ordered release of neuroblasts from the anlage. In order to investigate the function of Notch during larval neurogenesis, we used the temperature-sensitive N48 allele. Raising embryos at the permissive temperature resulted in wild-type early larvae; these were shifted to the restrictive temperature (30 °C) dissected at later larval stages, and stained with markers for neurons, neuroepithelium, and optic lobe epithelium. For the latter, we used an antibody against Cnamps which highly specifically labels the apical surfaces of the optic anlagen (Tepass et al., 1990; see Fig. 1C, D above). In wild-type, Cnaps expression labels the C-shaped belt that demarcates the outer optic anlage (Fig. 4A, C, B). In N48 mutant brains, the Cnaps-positive domain was strongly decreased in size and did not show its typical C-shape (Fig. 4B, D, S). This reduced size of the optic anlagen in late larval brains is caused by the premature conversion of epithelial cells into neuroblasts. Optic lobe neuroblasts and their progeny (CMCs and neurons) are positive for DII016 (Neurotactin, Nt) and Deadpan (Dpn), whereas the optic anlagen are distinctively BP106-negative and Dpn-negative (Fig. 4E, J). The misshapen optic lobes of N48 mutant larvae are covered by Dpn-positive neuroblasts (Fig. 4F, I) and their progeny. As in wild-type controls, DII-Cadherin (DIIcad in green, Fig. 4J, K) comes on in the deep layers of the medulla primordium, suggesting that differentiation of these cells has set in normally. At the same time, the evenly distributed columns of neurons and axons that represent the nascent medulla columns in the wild-type brain (Med in Fig. 4) are disrupted

Fig. 2. Layout of the late larval optic lobe. (A) Confocal cross-section of the late larval optic lobe of left brain hemisphere, labelled with anti-Neurotactin (white), epithelial inner and outer optic anlage (OOAep; magenta lines; OOAep, blue line) are strongly positive for anti–Neurotactin and appear dark. Neuroblasts arising from the outer optic anlage (Loa, cross circles; Mmp, blue circles) and inner optic anlage (IOAmp, magenta circles) are moderately strongly labelled; neurons and outgrowing axons are strongly labelled. Arrows on hatched lines indicate direction in which neurons are given off by their respective neuroblasts. Neurons of the distal medulla (Md) form discrete lines, each one emitting a bundle of axons towards centrally (arrow). Tips of these axons gather in a plexus that forms the axon core of the distal medulla neuropil (Mdnp, shaded blue). Lineages located medially belong to neuroblasts formed from the OOA (first; I); the further later a lineage is located (I), the later the corresponding neuroblast was formed. In each given lineage, neurons born early are located centrally, far from the surface (nc); late born neurons are peripherally (np). Neuroblasts of the I/OA give off neurons outward (purple arrow) and inward (red arrow). The former makes up the proximal medulla (MpI) and the latter makes up the holobal and holobal plate (Lo, see also digital models in panel E). Aspects of these neuron populations grow medially and form the proximal medulla neuropil (Mdp, shaded in purple), the holobal plate neuropil (Lmp, red) and the holobal neuropil (Lmp, green). (B, C) Confocal cross-sections of late larval brain (B) and adult brain (C), labelled with anti-Neurotactin to visualize optic neuropil (white), left hemisphere, lateral to the left, dorsal up. Optic lobe neuropiles of the larva and adult are rendered in corresponding colors; abbreviations and color code as in A. Bars: 40 μm.
in the mutant. Here, medulla neurons form more irregularly sized clusters with thicker axon bundles emanating from them (Fig. 4K).

In a significant fraction of N\textsuperscript{}\textsuperscript{ed} mutant brains, medulla neuroblasts derived from the OOA had all but disappeared, and a mass of tightly packed neurons covered the entire lateral surface of the brain (Fig. 4G; see also below for mutations in jak/Stat signalling). This suggests that the time interval during which medulla neuroblasts of N\textsuperscript{ed} mutant optic lobes are active (i.e., from their birth from the OOA to their disappearance) may be the same as in wild-type. Because they are born earlier in the Notch mutant, they also disappear earlier.

\textit{Notch related defects in optic lobe connectivity}

During the middle of the third instar larval stage, photoreceptor axons R1-R6 start growing into the brain and make connections with cells of the lamina (Selleck and Steller, 1991; Meinertzhagen and Hanson, 1995). R7/R8 axons continue past the lamina and terminate in the distal medulla; lamina neurons (L-neurons) also extend axons towards the distal medulla. The medulla neurons, in turn, connect to the lobula complex and the central brain. To explore the retinal axons connectivity defects within the optic lobe that is associated with N\textsuperscript{ed} mutants, we labelled the fiber tracts with Fasciclin II (FasII). These include the retinal axons, lamina axons, the posterior optic tract (medulla to central brain), and the axons from the lobula to the central brain (Nassif et al., 2003).

Not surprisingly, connectivity defects were profound in N\textsuperscript{ed} mutants, given that Notch is known to be required for patterning of retinal cell types (Cagan and Ready, 1989). Previous studies had shown that perturbation of Notch or Delta results in a neurogenic phenotype: most cells within the atonal (ato) intermediate group differentiate and express the fate of R8 cells (reviewed by Franklin and Mardon, 2002). As a result, later born R1-6 and R7 are either reduced or absent in neurogenic mutants (Cagan and Ready, 1989). In N\textsuperscript{ed} mutants, we observed that R8 neurons form axons which do not properly bundle into thin fascicles. Instead, these R8 neurons form thick and irregular bundles that completely bypass the lamina and terminate at a deep level within the medulla primordium (Fig. 4L–O).
Fig. 4. Loss of Notch signaling causes a premature NE->NB conversion and leads to structural defects in the optic lobe. Photographic panels (A–Q) are volume renderings (A–D, H–I) or frontal confocal sections (all others) of late third instar brain hemispheres labeled with different markers. Panels of left column (A, F, K, L, N, P) and panels C and H show wild-type control; panels of middle column (B, E, M, Q) and panels D and I are from N37 larvae raised at the restrictive temperature from first to third instar; (A–D) Abrogation of Notch signaling results in less of the OOA epithelium, labeled with anti-Crumb (Crb) antibody (yellow); BrM neurons are labeled with anti-Neurotactin (Nrt; blue) for reference. (E–K) Neuroblasts and neurons of the medulla, marked by anti-Deadpan (Dp; E–I) or anti-Nrt (L, K) cover the entire lateral surface of the optic lobe. This phenotype is most clearly visible in volume renderings shown in lateral view (H–I). Is wild-type (H), Dp-positive medulla neuroblasts (Mnb), shown in yellow color, occupy a C-shaped band medial to this view. "Peripheral" of the epithelial outer optic anlage (OOAp); Dp-positive neuroblasts derived from the inner optic anlage (IOAb) form a central cylinder. In N37 (I), the OOAp has converted prematurely to medulla neuroblasts (Mnb). Mdp, proximal medulla neurons; Mdc, central medulla neurons. (L–O) Double labeling with anti-Fasciclin II (Red, magenta) to mark fiber tracts and anti-DE-cadherin (Green, cyan) to mark the epithelium of the OOA. Panels L and M show a plane of section through the center of the optic lobe neuropile; N and O show the optic lobe neuropile at an anterior plane. Most retinal afferents (ra) terminate in the lamina neuropile in wild-type (Lap in L and O). In mutant, retinal afferents project in irregular angles of fibers to the deeper medulla neuropile (Mnp in M and O). Note strongly decreased optic lobe epithelium (OOAp) in mutant. (P, Q) Loss of lamina neurons in N37 mutants. Double-labeling with anti-DE-cadherin (green, neuropile marker) and anti-fushan (blue, magenta) labels neurons of lamina (L), as well as lobula (Lo) and mushroom body (MB); (R, S) Digital 3D models of late third instar larval brain hemispheres of wild-type (R) and N37 mutants (S). Models are shown in lateral view, anterior is to the left, dorsal is up. Models represent a view of the optic lobe surface, which is formed by the epithelial outer optic anlage (OOAp) and, more medially, the medulla neuroblasts (Mnb) derived from it. Lateral, and surrounded by the OOA, is the primordium of the lamina (L), proximal medulla (M) and lobula compex (Lo). Color code as in model shown in (Fig. 2. Other abbreviations: CA central brain; OOA, lateral domain of outer optic anlage; OOA, medial domain of outer optic anlage. Bar: 50 μm.

retina, the medulla primordium is also misshapen due to the fact that medulla neuroblasts/neurons are born in an abnormal temporal pattern. Thus, in wild-type, the epithelial OOA grows to a large size and forms the "dome" covering the entire lateral surface of the brain (see previous section). Subsequently, neuroblasts are released in a well-ordered succession from the margin of the OOA, resulting in the medulla primordium with a given (large) surface area and (small) depth. In the N37 mutant the OOA does not grow to a large size because the OOA epithelium prematurely converts into neuroblasts. As a direct result of such premature conversion, the medulla

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primordium as a whole and the medulla neuropile in particular, is larger in depth and smaller in surface area (Fig. 4R, S).

The effect of loss of Notch function on the lamina primordium is complex. Fast labeling of nascent lamina neurons is visible in the wild-type OOA. Axons of these neurons fasciculate with the afferent retinal axons (Fig. 4L, N; note that it is not possible to distinguish between retinal and lamina-derived axons in anti-FasII-labeled brains). In N4 mutants, Fast-positive neurons appear to be absent, where it cannot be detected in the immediate vicinity of the rudimentary OOA (Fig. 4M, O). This interpretation is further confirmed by the absence of the lamina neuronal marker, Dachshund (Dac). In wild-type, Dac expression appears at a low level in the OOA and is strongly upregulated in postmitotic lamina neurons (Fig. 4P). This lamina-specific expression of Dac is reduced or absent in N4 mutants (Fig. 4O).

Loss of Jak/Stat signaling mimics many aspects of reduced Notch signaling in the larval optic lobe

The use of molecular markers that switch expression as epithelial cells convert into neuroblasts had shown that the Jak/Stat pathway, similar to Notch signaling, may act as an inhibitor of neuroblast formation (Yasugi et al., 2008). To verify that the structural phenotype ensues from loss of Jak/Stat signaling follows this prediction, we used markers for optic lobe epithelium, neuroblasts, and neurons in the background of a temperature-sensitive mutation of Stat92E (Stat92EwM) with a strong Stat92E hypomorph (Stat92EwM/Stat92EwM). Late larvae that had developed at the restrictive temperature from hatching onward showed absence or significant reduction of the OOA epithelium as visualized by anti-Crb labeling (Fig. 5A–D). As observed in N4, the Dac-positive band of lateral cells representing the primordium of the lamina was greatly reduced (Fig. 5C, D).

Lineages of medulla neurons produced by prematurely converted neuroblasts occupied the entire surface area of the optic lobe primordium (Fig. 5E–I, L, I). Dpn-positive neuroblasts were typically strongly reduced or absent (Fig. 5F); due to their earlier time of birth, neuroblasts completed their proliferative activity earlier than in wild-type and consequently also disappeared earlier. Loss of stat signal resulted in premature differentiation, as shown by the increase in ELAV-positive cells as compared to wild-type (Fig. 5G–H). Moreover, Stat mutant brains dissected at an earlier stage (mid-third instar) had optic lobes with an increased number of neuroblasts (data not shown); similar to what is shown in the previous section for N4 mutants. The premature epithelial to neuroblast conversion of the OOA was confirmed by applying short 30-min BrdU pulses to Stat92E/Stat92EwM larvae. In wild-type controls, BrdU incorporation is mostly confined to the medial OOA where cells have converted into neuroblasts, which cycle much more rapidly than the epithelial cells (Reddy et al., 2010; Fig. 5K). In Stat92E/Stat92EwM mutants, BrdU incorporation is seen over the entire surface of the OOA, supporting the conclusion that these cells have prematurely converted into rapidly cycling neuroblasts (Fig. 5L).

Knock-down of Notch and Stat activity specifically in the optic lobe causes premature epithelium-neuroblast conversion

Both Notch and Jak/Stat activity are required not only in the optic lobe, but also in many cells of the central brain and other organs of the larva. To confirm that the optic lobe defects resulting from global inhibition of Notch or jak/Stat signaling are indeed the result of a local requirement for these signaling activities in the optic lobe itself, we...
used a Ga4 driver line, eg5-Gal4 (Goto and Hayashi, 1999), to direct the expression of mutant constructs to the optic lobe. eg5-Gal4 is expressed at high levels in both optic anlagen (IOA, OOA) and their derivatives from early stages onward (Fig. 6A, B). The line is not expressed significantly in the central brain; outside the CNS, eg5-Gal4 is seen in numerous adult-specific progenitor cells (e.g., those of the midgut; Ostheim and Spradling, 2006) that are not involved in the normal functioning of the larva. In the head, eg5 is expressed in a narrow belt of cells surrounding the eye field, but not in retinal photoreceptors (Lim and Tomlinson, 2006). To inhibit Notch signaling, we expressed a dominant-negative form of Su(H) under the control of eg5-Gal4 (Nagaraja and Banerjee, 2007); for the Jak/Stat pathway, we used a Stat52E RNAi construct placed under UAS control (Kim et al., 2007). The resulting phenotypes resembled the ones described above for the N^ and Stat5^ mutants, respectively. The epithelial outer optic anlage converted prematurely into neuroblasts that budded of neurons (Fig. 6D, E) compared to wild-type (Fig. 6C). Also, the loss of Dac-positive lamina neurons resembled the phenotype described for N^ and Stat5^ (Fig. 6E, F).

Notch and Stat activity are required during the late larval stage and are mutually interdependent.

Notch and Jak/Stat are both active throughout the development of the optic lobe. Specifically, both activities are seen in a fully overlapping pattern in the IOA and OOA of the early larva (see Fig. 3A-D). To further understand the temporal requirement for the two signaling pathways,

![Fig. 6. Optic lobe-directed expression of mutant constructs using the eg5-Gal4 driver line.](image)

(A, B) Frontal confocal sections of first instar (A) and early third instar (B) brain showing RFP expression, driven by eg5-Gal4, in optic lobe (magenta). Stat52E-GFP is labeled in green. (C) Frontal confocal sections of third instar wild-type brain labeled with anti-Dac (green), anti-Elav (blue), and anti-Duf (red). CB, central brain; IOA, inner optic anlage (IOA); neuroblasts from inner optic anlage; MnG, median ganglion; MhG, median hemiganglion; OOA, outer optic anlage. (D, E) Frontal confocal sections of late third instar brain in which a dominant-negative form (Su(H)^M) construct (Su(H)^M) or Stat52E-RNAi construct (E) under UAS control was driven by eg5-Gal4. Note loss of epithelial outer optic anlage and premature growth of medullary neurons. (F, G) Labeling with anti-Dac (red) and anti-Duf (green) illustrate strong reduction of lamina neurons in the medulla (M). (H) In frontal section (F) and volume rendering (G), MB, mushroom body (central brain). Bar: 40 μm.

![Fig. 7. Temporal requirement of Notch and Stat signaling in optic lobe development.](image)

(A–D) Frontal confocal sections of late third instar brain (equivalent of 96 h at 25°C) mutant for N^ (A, B) and Stat5^ (C, D). In panels of the left, larvae were raised at the restrictive temperature from hatching until 48 h later; in right panels, larvae grew at the restrictive temperature between 48 h and 96 h. (A) Timeline and schematic depiction of Notch/Stat function in optic lobe development. Despite their expression from early larval stages onward, Notch and Stat activity are only dispensable during the second half of the larval period, coinciding with the Ni-Ni conversion of the OOA. Thus, only late heat treatment caused noticeable abnormal phenotype, consisting of loss of epithelial OOA (arrowheads in D) and superficial position of medullary neurons (MnG in A, B). Peakexpression of Delta, seen in the anterior along fringe of IOA and OOA in wild-type or early heat-treated Stat5 mutant; (C, arrow) is only found in the IOA in late heat-treated Stat5 (D), suggesting a requirement of Stat activity for maintaining high OOA levels. Conversely, as shown in panels F and G, persistent Notch activity is required to maintain Stat in the OOA (F: wild-type expression of Stat5::GFP reporter; G: Stat5::GFP reporter in optic lobe where Notch activity was inhibited by an eg5-Gal4–Su(H)^M construct. Bar: 40 μm.)

we carried out temperature-shift experiments. Extended shifts (0–48 h) carried out in first and second instar larvae (before 48 h after hatching) had no overt effects on the structure of the optic lobe seen either in larvae dissected right after the temperature shift, or in larvae left to develop at the permissive temperature until 96 h (Fig. 7A, C). Only shifts of 24 h or more applied during the third larval instar (after 48 h) caused premature epithelial-neuroblast conversion as described in the previous sections (Fig. 7B, D). These results indicate that the early growth phase (by symmetric division) of the optic anlagen does not depend on Notch or Stat activity, even though both are expressed. Specifically, loss of Notch or Stat is unable to convert the early optic anlagen into neuroblasts prior to the mid-larval stage.

To test whether jak/Stat has the potential to inhibit Notch activation in the OOA, we visualized the expression of the Notch ligand, Delta in these mutants. Loss of Stat during the second half of the larval period resulted in downregulation of Delta in the OOA (Fig. 7D). The altered
patter of Delta expression was particularly conspicuous, since expression in the IOA was unchanged in the Stat92E*/Stat92E*G0 mutants, and contrasted sharply with Delta reduction in the OOA. By contrast, we found Delta expression to be unchanged in early larva mutant for Stat (data not shown). Consistent with this observation, loss of Stat prior to NE-NB conversion did not affect the expression of Delta (Fig. 7C).

As Delta expression is compromised in Stat- mutants, we also asked whether the converse is true; that is, is Stat signaling can be attenuated when Notch signaling is reduced. To address this, we chose Su(H)xH using eg-gal4 and placed Stat92E-GFP reporter in the background to assess how Jak/Stat signaling is modulated. As expected, the Su(H)xH in the OOA is reminiscent of what we have observed before, with the loss of DE-cadherin (marking the OOAep) in the mutant (Fig. 7G). Compared to wild-type, there is a severe reduction of Stat expression in the outer optic anlage (Fig. 7F, G). Stat signaling is completely abrogated in the OOA as well.

Together, these data suggest that there is a temporal requirement for both Jak/Stat and Notch signaling to ensure proper epithelial-to-neuroblast conversion in the OOA (Fig. 7E). Both signals are dispensable during early development, during the time of expansion in the epithelium, although they are continuously expressed during that time. Stat activity is needed to maintain the DI-induced wave of Notch signaling activity along the NE-NB conversion front. Conversely, Notch is also necessary to maintain Stat in the OOA. Both signals, thus work in cooperation with one another to allow for the formation of the highly ordered medulla structural characteristic for the optic lobe. Given the changing expression dynamics of Notch signaling and Jak/Stat signaling, nevertheless, the interaction between the two is most likely indirect.

Discussion

Neural progenitors give rise to the diversity of cell types seen in the central nervous system (CNS). Intrinsic factors expressed in progenitors, as well as extrinsic cues from neighboring cells specify cell fate. In both vertebrates and invertebrates, the specification of cell types follows a highly invariant spatio-temporal pattern. Typically, one can distinguish an early phase where the pool of progenitor (e.g., the neuroepithelium of the neural tube in vertebrates) expands by symmetric cell division. Subsequently, progenitors start leaving the pool of expanding cells and either directly differentiate into specific cell types, or undergo asymmetric divisions where one daughter keeps the properties of a progenitor, whereas the other differentiates (reviewed by Zhong and Chia, 2008).

Neurogenesis in the Drosophila optic lobe follows a similar pattern. Segregating from the embryonic neuroectoderm as a small epithelial placode, the optic lobe anlage undergo a phase of growth by symmetric cell division in the early larva, followed by a highly ordered transition into asymmetrically dividing neuroblasts. The mediolateral gradient that characterizes this transition in the OOA is correlated with the posterior—anterior gradient of eye development: photoreceptor axons of the earliest developing (posterior) row of ommatidia arise first and capture the first born neurons, formed (in case of the medulla) from the medial edge of the OOA. Later born axons occupy medulla neurons forming later, at increasingly lateral levels (Meinerzhagen and Hanson, 1995). It is to be assumed that this temporal match between target neuronal development and afferent axon development plays an important role for correctly wiring the optic lobe; this hypothesis, though, requires rigorous testing.

Notch activity controls the epithelium–neuroblast transition in the optic lobe

We show in this paper that the Notch pathway is critically involved in the ordered NE-NB conversion. The most significant effect resulting from decreasing Notch function in the larval brain was the reduction in size of the epithelial optic anlage, as shown by the loss of the epithelial mariner, Crib. It is therefore likely that the function of Notch in the optic anlage is to maintain its undifferentiated, neuro-epithelial state. The clonal analysis of Reddy et al. (2010) led to the same conclusion. This would match a similar function of Notch in the embryonic neuroectoderm, where Notch activity is also required for cells to stay epithelial (reviewed in Campos-Ortega and Knust, 1990). The only difference is the topology of the neuroblast (i.e., cell that moves out of the epithelium): in the embryonic neuroectoderm, neuroblasts are mostly scattered cells, surrounded on all sides by epithelial cells. In the optic anlagen, there is a continuous front where all epithelial cells convert to neuroblasts. However, this difference aside, the way in which Notch signaling acts and is controlled during the NE-NB conversion could be quite similar in the embryonic neuroectoderm and the late larval optic anlagen.

Surprisingly, Notch activity, despite of its continued expression throughout development, appears to be dispensable during the earlier phase of optic lobe development during which the epithelial optic anlagen grow by symmetric mitosis. Neither early temperature shift experiments with N°, nor temporally restricted optic lobe expression of Su(H)DIN resulted in premature neuroblast formation. Also the active lifespan of the optic lobe neuroblasts appear to be independent of Notch activity. Optic lobes of late Ne larvae reared at the restrictive temperature were mostly devoid of (Vip—positive) neuroblasts, and lineages or overall volume of the medulla primordium were not noticeably enlarged.

Taken together, our findings in conjunction with other studies (Yasugi et al., 2008; Reddy et al., 2010) suggest the following model of Notch signaling in the larval optic lobe. Moderate levels of the Notch ligand DI, as well as Notch activity, are present in the entire optic lobe anlage of the early larva. Starting during the mid larval stage, the proneural gene lsc is expressed at the medial margin of the OOA (Yasugi et al., 2008). This expression sets in motion a cascade of events that result in the ordered NE-NB conversion. lsc locally upregulates Delta and other proneural genes (see; Wallace et al., 2000; this study) that promote first the conversion of OOA epithelium to neuroblasts, followed by rapid asymmetric division and neuronal differentiation. At the same time, once cells have converted to neuroblasts, Lsc and Delta are downregulated, even though N stays on in a dynamic manner in neuroblasts and neurons. Lsc remains high in a laterally moving band of cells at the medial OOA margin. A second mechanism that may act on the localized DI upregulation has been proposed in the recent study by Reddy et al. (2010), who propose that the cell cycle arrest in the OOA, caused by activation of the Fat/Hippo pathway, is prerequisite for the accumulation of DI Throughout the third larval instar, a continued, interdependent expression of Lsc and Delta in the OOA could be the mechanism that accounts for the slow, gradual release of neuroblasts from the OOA margin. Thus, Lsc is known to upregulate Delta in other neural precursors (e.g., Hino et al., 1994), and this could be the case also in the OOA. The Lsc induced peak in Delta levels at the medial OOA margin would then signal to its neighbors laterally, increasing Notch activity, and thereby preventing a premature advance of Lsc towards lateral.

How initiation and maintenance of Lsc expression is controlled is still unclear. Wingless (Wg), a known activator of proneural genes in other tissues (Gonzalez-Gaitan and Jackle, 1995; Ruifson et al., 1996) is expressed in a fairly restricted pattern in the apices of the OOA (Kaphegat and Kunes, 1994). It is possible that a long range effect of Wg could be responsible for Lsc activation along the OOA margin.

Notch and the maintenance of the undifferentiated state in the developing nervous system

A large number of studies in vertebrate and invertebrate systems alike suggest that the fundamental role of N in the developing nervous
system is to maintain cells in an undifferentiated (neuroepithelial) state at any given moment. Cells released from N activity enter a differentiative pathway (typically accompanied by structurally visible changes, such as a switch from epithelial cell to neuroblast, and/or a switch in mitotic behavior (symmetric vs. asymmetric). The temporally controlled release/birth of neurons from the neuroepithelium is often tied to different cell fates. This has been shown very convincingly in the retina of vertebrates and Drosophila. For example, in the vertebrate retina, the first wave of differentiation results in ganglion cells, the second wave of differentiation at a later point includes photoreceptors, followed by bipolar cells, and others (Young, 1985). If N activity is reduced at an early time point, the number of ganglion cells produced increases massively, at the expense of later born cell types (Austin et al., 1995; Dorsky et al., 1997; Jadhav et al., 2006; Silva et al., 2002; Cau and Blader, 2009). In Drosophila, the first retinal cell type to be born behind the morphogenetic furrow is the R8 photoreceptor. If at the time of R8 specification, N function is decreased, the number of R8 cells is increased, and other cell types born later are decreased (Cagan and Ready, 1989). With later pulses of N depletion, one gets different phenotypes; what they all have in common is that the cell types born at the time of the pulse are increased in number, the ones born later decreased.

In the Drosophila OOA investigated in this paper, it is the temporal progression of neuroblast formation that is controlled by N activity is linked to the coordinated growth between eye (and lamina) and medulla, derived from the OOA. However, it is well possible that the temporal progression is also tied into the control of different cell fates. The way in which the multitude of different medulla cell types map onto the larval optic lobe is not clear. It is most likely that (as in the lineages of the ventral nerve cord) most cell types are specified along the z-axis, which would imply that each part of the OOA (in the medio-lateral and dorso-ventral dimension) would produce the same cell types. However, it is well possible that some cell types which are actually not found in all medulla columns, such as wide field tangential neurons (Haase et al., 1984; Taghert et al., 2000), are produced by different parts of the OOA. Such cell types then might be affected by premature or delayed conversion of the OOA into neuroblasts; identifying specific markers that label cell types at early stages, and using such markers in the background of Notch loss or overactivity, will help clarify this question.

A role of the Notch pathway has been described for later stages in neural development, that is, the specification of neurons from ganglion mother cells (GMCs). Asymmetric neuroblast proliferation in the ventral nerve cord and brain produces a series of GMCs which each divides one more time into two, often different, neurons/flat cells (reviewed by Karaca et al. 2005). It has been shown that the fate choice between sitting pairs depends on Notch activity, both during embryonic and postembryonic stages (Spaanga and Doe, 1996; Sleath and Doe, 1998; Truman et al., 2010). Such may also be the case for the neuroblasts emerging from the OOA. The relatively high level of the Notch reporter, Eip[flp-lacZ] maintained in the OOA-derived lineages would speak for a continued role of Notch in these cells; however, detailed investigations of the neurogenesis of these lineages need to be carried out in order to address the potential later Notch function.

Interdependency of Notch and Jak/Stat Activity during the development of the nervous system and other organs

As previously reported (Yasugi et al., 2008; Reddy et al., 2010), we find that reduction in Stat activity causes a premature loss of the epithelial state of the OOA. This is accompanied by accelerated proliferation and gross abnormalities in the architecture of the optic lobe neuropile, as also seen in Notch mutant brains. Furthermore, continued activity of Stat in the epithelial OOA is dependent on Notch, and vice versa. The mutual interaction between both signaling pathways is most likely mediated by a number of intermediate steps. Thus, Delta levels are normal in optic lobes of StatT mutant brains up to 48 h after hatching. It is only during later stages, when the structurally visible premature change of OOA epithelium to neuroblasts occurs in the StatT mutant, that Delta expression is reduced. It remains to be seen what are the intermediate genetic events that interconnect the signaling activities of the Notch and Stat pathway.

The larval optic lobe represents but one of many scenarios in which interdependency between Notch and Stat have been reported. The types of genetic interactions between these pathways appear to be as diverse as the developmental events or cell fates which they control. For example, in the Drosophila ovary, mutual inhibition Notch and Stat set up the boundary between the stalk (Jak/Stat dependent) and the main-body follicle cells (Notch-dependent; Assa-Kunik et al., 2007). In the adult midgut, Notch is necessary for the differentiation of cells derived from the intestinal stem cells (ISCs) into enteroblasts and enteroctyes (Mitchell and Perrimon, 2006; Ohkaito and Spradling, 2006, 2007). Thus, high Notch activity in the enterocyte (EC) or enteroendocrine cell (EE), high Notch activity in the EB promotes the EC fate; low Notch activity allows for the formation of ECs; Jak/Stat signaling interacts with the Notch pathway at multiple steps: on the one hand side, it acts upstream in an activating manner. Thus, under stressful conditions (e.g. bacterial infection or JNK-induced stress), Stat functions to induce Notch to allow for self-renewal and proliferation (Boucher et al., 2008; Jiang et al., 2008). In addition, Jak/Stat is required during the differentiation of different ISC-derived cell types (Berbe et al., 2010; Lin et al., 2010; Liu et al., 2010). The same kind of dynamic and complex relationship between the two signaling pathways can be seen in the eye, where Stat function can both upstream and downstream of Notch (Chao et al., 2004; Gutierrez-Avino et al., 2009). A more recent report even suggests that Jak/Stat can function to inhibit Notch as well (Alahany et al., 2009). From all these studies, it is clear that many of the intermediates which link Notch and Jak/Stat signaling are still unknown. One can envision scenarios where subtle differences in the spatial distribution and timing of signals may contribute to how Stat and Notch interact during development. Drosophila optic lobe is likely to present a highly favorable system to address these complexities which impact the role of the two signaling pathways.
Chapter 3

Neurogenesis and layer formation in the *Drosophila* optic lobe occur in discrete steps during metamorphosis.
Abstract

Visual information processing is highly complex, involving columnar microcircuits that form a precise retinotopic map. The insect compound eye is organized into a hexagonal array of 800 ommatidia; each ommatidia contains eight photoreceptor cell types (R1-R8) that project to the optic lobe. The optic lobe consists of four ganglia: the lamina, medulla, lobula and lobula plate; where the medulla, lobula and lobula plate are stratified into ten, four and six distinct layers. The outer photoreceptor cells terminate in the lamina forming cartridges while the inner photoreceptor cells terminate in the distal medulla forming synaptic units called columns. Each column forms a single processing unit of the visual system and receives inputs from over 50+ classes of columnar and non-columnar neurons. Due to the complexity of cell types and the massive morphogenetic movement during metamorphosis in the optic lobe, the development of this structure remains elusive. In this work, we have three-dimensionally reconstructed each compartment using global markers to label the four ganglia and described the development of the three major fiber systems and the macroarchitectural changes that occurs as a result of such morphogenetic movements. Using cell-type specific drivers to label the major classes of retinal cell types in the optic lobe, we describe in great details, the development of these major cell types during development. Finally, we document the step-wise stratification of layers for medulla, lobula and lobula plate using a variety of synaptic markers and cell-type specific Gal4 drivers. These studies will lay the groundwork for future mutant analyses and the underlying molecular mechanisms regulating layer specificity in the optic lobe.
Introduction

Animals with complex, visually guided behaviors possess image forming eyes, whose neuronal projections to the brain form precise retinotopic maps. In vertebrates, image processing already takes place in the retina, which consists of three cellular layers separated by two synaptic strata. Photoreceptors (rods and cones) of the outer plexiform layer project their short axons towards the inner layer, formed by first order visual interneurons, the bipolar cells. Bipolar cells target the basally located layer of the second order visual interneurons, the ganglion cells. Several types of local interneurons (amacrine cells, horizontal cells) laterally connect bipolar cells and ganglion cells (Baier 2013; Masland 2001; Masland and Raviola, 2000). Ganglion cell axons leave the eye through the optic stalk and project in a retinotopically ordered manner to the contralateral optic tectum and dorsal thalamus. In insects, such as Drosophila, visual information is collected by the compound eye, which is formed by a large number of repetitive modules, called ommatidia. Each ommatidium possesses six outer photoreceptors (R1-R6) and two inner photoreceptors (R7-R8). Photoreceptors project in a retinotopic order to the optic lobe, part of the brain that processes exclusively visual information, and that has been homologized with the nuclear layers (synaptic strata) of the vertebrate retina tectum (Sanes and Zipursky, 2010; Erclik et al., 2009; Strausfeld 1971; Cajal and Sanchez, 1915; Joly et al., submitted)

The Drosophila optic lobe has become a propitious model system for analyzing the structure, development, and function of neural networks (Brand and Livesey, 2011; Sanes and Zipursky, 2010; Fischbach 2008). Parallel information processing relies on layer-specific synaptic connections, a feature common to both the insect and mammalian visual system (Cajal and Sanchez, 1915; Masland and Raviola, 2000). The optic lobe of the adult fly has four main compartments (“optic ganglia”): called lamina, medulla, lobula and lobula plate, each of which is further subdivided into multiple layers (Fig. 1). Photoreceptors involved in motion detection (R1-6) terminate in the lamina; R7 and R8, responsible for color vision, project to the outer medulla
This ordered projection subdivides the lamina and medulla into stereotyped, repetitive units, called cartridges in the lamina, and columns in the medulla (Hadjieconomou et al., 2011; Meinertzhagen and Sorra, 2001; Huang and Kunes, 1998; Huang et al., 1996; Meinertzhagen and O’Neil, 1991; Ready et al., 1976). Lamina interneurons (1st order visual interneurons, L1-L5), targeted by photoreceptors R1-R6 project to the medulla; medulla neurons (1st/2nd order visual interneurons), targeted by R7/8 and by L1-L5, either interconnect the distal and proximal layers of the medulla (medulla interneurons; Mi), or project to the lobula/lobula plate (transmedullary neurons; Tm/TmY; Takemura and Meinertzhagen, 2008; Bausenwein et al., 1992; Heisenberg and Buchner, 1977; Meinertzhagen 1976; Campos-Ortega and Strausfeld, 1972; Braitenberg 1967). Medulla interneurons and transmedullary neurons comprise a large number of subtly different types (Morante and Desplan, 2008; Fischbach and Dittrich, 1989). 3rd/4th order visual interneurons of the lobula plate (T2-T5; Oliva et al., 2014; Otsuna and Ito, 2006; Scott et al., 2002; Fishbach and Dittrich, 1989) form complex, retinotopically-organized connections between medulla, lobula, and lobula plate (Fig.1). Aside from these columnar neurons, the lobula/lobula plate, as well as the medulla, possess many different types of tangential interneurons (Morante and Desplan, 2008; Gao et al., 2008; Fischbach and Dittrich, 1989), whose widely branching neurites cover a large area of the visual field. These neurons transmit processed visual information to the central brain (Wernet et al., 2014). For example, motion of the visual field, resulting in adjustment of the direction of flight or walking, is processed by large tangential neurons of the lobula plate which directly project to premotor neurons in the dorsal slope area of the brain (Borst and Euler, 2011). Specific interneurons responding to color stimuli have been identified in the peripheral optic lobe and are required for phototactic behavior (Otsuna et al., 2014; Rister et al., 2007), though the central circuits mediating color-guided behavior have not been clearly identified. Recent work from
Aptekar et al. indicates that several classes of lobula neurons with axonal projection to discrete foci ("optic glomeruli") in the ventro-lateral protocerebrum encode basic visual features required for figure discrimination, which plays a crucial role in the context of pursuing prey, avoiding predators, and mating behavior (2015).

As a result of the complexity of cell types and connections in the optic lobe, the development of this structure is poorly understood. All neurons of the four optic ganglia are produced by a small epithelial placode ("optic placode) that invaginates from the neuromeroderm of the embryonic head (Meinertzhagen and Hanson, 1993; Green et al., 1993). In the late embryo, the optic placode has split into two layers, called the outer (OOA) and inner (IOA) optic anlage. The OOA gives rise to the neurons of the lamina (L1-L5) and medulla (Mi, Dm, Tm, TmY, Mt), while the IOA produces neurons of the lobula and lobula plate (T2-5; Apitz and Salecker, 2015; Apitz and Salecker, 2014; Pinero et al., 2014; Li et al., 2013a; 2013b; Suzuki et al., 2013; Hasegawa et al., 2011; Ngo et al., 2010; Egger et al., 2007; Green et al., 1993). Given the large number of optic lobe neurons (about 100,000, compared to less than 3,000 in the central brain), proliferation of the optic anlagen continues throughout a long period of development, from embryo to early pupa, and is further boosted by a two-phase mechanism which is unique to the visual system. In a first phase (which does not occur in the central brain or ventral nerve cord), the optic anlagen grow by symmetric cell division to a size of several thousand epithelial progenitor cells (Ngo et al., 2010; Egger et al., 2007; Hofbauer and Campos-Ortega, 1990). During the second phase, which begins halfway through the larval period, the progenitors undergo an epithelial-mesenchymal transition (EMT), becoming neuroblasts that enter a phase of asymmetric cell division, each neuroblast producing a lineage comprising ~100 neurons. The molecular pathways controlling the EMT of optic lobe progenitors has been elucidated by recent studies (Apitz and Salecker, 2015; Morante et al., 2013; Orihara-Ono et al., 2011; Egger et al., 2010; Ngo et al., 2010; Reddy et al., 2010; Yasugi et al., 2008, 2010). By contrast, little is known
about the specification of fate and connectivity of the multitude of neurons making up the optic lobe. For the medial domain within the OOA, which gives rise to the multitude of medulla neurons, cell fate appears to be mainly linked to the time of birth within the lineage produced by a progenitor (Bertet et al., 2014; Li et al., 2013a, 2013b; Hasegawa et al., 2011, 2013; Suzuki et al., 2013; Morante et al., 2011). On the other hand, the five different types of lamina neurons, derived from the lateral margin of the OOA, seem to be specified already at the level of the progenitor itself and are not time-dependent (Pinero et al., 2014; Selleck et al., 1992; Selleck and Steller, 1991). Nothing is known about the underlying mechanism of how the diverse cell types in the lobula and lobula plate are generated.

The analysis of optic lobe development is complicated by the fact that the optic anlagen and the neurons/nerve fibers they produce undergo complex morphogenetic movements before adopting their final position in the adult brain (Hasegawa et al., 2011; Morante et al., 2011). To address this problem, we used three-dimensional reconstruction models with global markers labeling the three different cortices (e.g. lamina, medulla, and lobula complex) and its corresponding neuropil, coupled with cortex-specific Gal4 drivers and R7/R8 markers. In this work, we describe in great detail, the macroarchitectural rearrangement of optic lobe cortices and neuropils, as well as the step-wise growth cone extensions of photoreceptor cells, lamina and medulla neurons. We also describe the step-by-step layer subdivision in the medulla, lobula, and lobula plate neuropils using the cell adhesion molecule, N-Cadherin coupled with a number of layer-specific markers. This work provides the fundamental basis for the macroarchitectural changes during optic lobe morphogenesis as well as the step-wise process of layer target selection for the optic lobe ganglia.
Materials and Methods

Fly Stocks

Flies were grown at 25°C using standard fly media unless otherwise noted. The following transgenic strains were used in this study (original source in parentheses): *esg-Gal4* (B. Edgar); *insc-Gal4; UAS-mCherry* (Bloomington Stock Center, Stock #8751); *Ln-Gal4* (L. Zipursky); *for^NP79-Gal4* (Kyoto Stock Center; Stock #103517); *NP3233-Gal4* (Kyoto Stock Center; Stock #113173); *Bsh-Gal4* (M. Sato; Hasegawa et al., 2011); *Drx^{GMR77F09}-Gal4* (Janelia Farms FlyLight Gal4 Collection; Bloomington Stock Center, Stock #46986); *acj6^{PG63}-Gal4* (L. Luo; Potter et al., 2010); *wg-gal4* (Giraldez et al., 2002); *Vsx-Gal4* (T. Erclik); *UAS-mCD8GFP on X, II, III* (Bloomington Stock Center; Stock #5130, #5136, #5137) was used to recombine the various Gal4 drivers. Genotypes used for labeling abbreviated cell types in the figures are listed below as a reference. Global markers are not listed. Refer to the main text for full name of cell types.
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<td>Nrv2-Gal4, UAS-mCD8::GFP</td>
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**Immunofluorescence**

Larvae and pupae were staged as previously described (Bainbridge and Bownes, 1981).

Fixation procedures for larval, pupal and adult optic lobes varied and are described below. For
larval brains, dissected tissues were fixed in 3.7% formaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 5.63 mM Na2HPO4, 6.37 mM KH2PO4; pH = 7.4) for 25 min. Staged pupal tissues were dissected and fixed in 4% methanol-free paraformaldehyde (PFA) in PBT (PBS with 0.1% Triton X-100) for 40 min at 4°C. Tissues were permeabilized in PBT (PBS with 0.3% Triton X-100) and immunofluorescence was performed using standard procedures with the exception of BP106 (Neurotactin, Nrt) and BP104 (Neuroglian, Nrg) labeling. For BP106/BP104 labeling in the pupae, dissected tissues were fixed on ice in PBS in PFA for 35-90 min (depending on the stage). Tissues were dehydrated and stored in ethanol at -20°C overnight. Tissues were rehydrated on ice and standard immunolabeling was performed. Adult tissues were fixed in 4% PFA in PBT (PBS with 0.3% Triton X-100). The following antibodies were provided by the Developmental Studies Hybridoma Bank (Iowa City, IA): mouse anti-Neurotactin (BP106, 1:10), mouse anti-Neuroglian (BP104, 1:30), mouse anti-Fas2 (1:4), mouse anti-Chaoptin (24B10, 1:10) and rat anti-DN-Cadherin (DN-EX #8, 1:20); mouse anti-Dlg (4F3, 1:10). Additional primary antibodies used in this study were: rabbit anti-Synaptotagmin (1/500; gift from H. Bellen); rabbit anti-V5 (1/100; #R960-25, Invitrogen). Secondary antibodies, IgG (Jackson ImmunoResearch; Molecular Probes) were used at the following dilutions: Dynalight 649-conjugated anti-rat (1:400), Cy5 anti-rat (1:700); AlexaFluor 488-conjugated anti-mouse (1:500) and 546-conjugated anti-mouse (1:500). Tissues were mounted in Vectashield mounting medium (Vector, Burlingame, CA, #H1000).

Lineage Tracing

Lineage tracing experiments were performed for for-Gal4 and Wg-Gal4 by crossing to transgenic flies with the genotype: UAS-Flp, Act5C-FRT(stop, y+)FRT-Gal4;;10xUAS-mCD8GFP/TM3, Kr-Gal4, UAS-GFP. Staged pupae were grown at 25°C, dissected, and brain tissues were harvested at desired time points for downstream immunofluorescence.
5-ethyl-2’-deoxyuridine (EdU) assays (Invitrogen) was used to label cells in S-phase. 2nd and early 3rd instar larvae with the genotype insc-Gal4; UAS-mCherry (to globally label axon tracts and neuroblasts) at 72h ALH were fed media containing 16 µg/ml bromophenol blue and 130 µM EdU for four hours. Larvae with EdU incorporated were dissected either immediately (Fig. 5M-N) or chased to wandering third instar larvae stage (Fig. 5O-P) and fixed in 3.7% formaldehyde in PBS (pH = 7.4) and permeabilized with 0.3% PBT. Samples were incubated in an optimized EdU reaction (provided by the manufacturer) containing: 425 µl Click-iT Reaction Buffer, 20 µl CuSO4, 1.2 µl AlexaFluor 647 Azide, 1 µl anti-GFP (Fig. 5O-P; polyclonal antibody conjugated to AlexaFluor 488; Molecular Probes, Cat No #A-21311), 50 µl 10x Click-iT Buffer Additive for 90 min and whole-mounted in Vectashield.

Confocal Microscopy

Staged Drosophila larval and pupal brains labeled with suitable markers were viewed as whole-mounts by confocal microscopy [LSM 700 Imager M2 using Zen 2009 (Carl Zeiss Inc.); lenses: 40x oil (numerical aperture 1.3)]. Complete series of optical sections were taken at 2-µm intervals. Captured images were processed by ImageJ (National Institutes of Health, http://rsbweb.nih.gov/ij/) and Adobe Photoshop.

Generation of three-dimensional models

Digitized images of confocal sections were imported using Trak-EM2 plug-in in FIJI software (Cardona et al., 2012; Schindelin et al., 2012). Since sections were taken from focal planes of one and the same preparation, there was no need for alignment of different sections. The optic lobe compartments (neuropils and cortices) were manually segmented using a global marker for
neural tracts (BP106 from P0 to P32; BP104 from P32 onwards) and the synaptic marker, DN-Cadherin (DN-Cad) within a series of confocal images. All surface rendered digital atlas models were generated using 3-dimensional viewer as part of the FIJI software package.
Results

Architecture of the optic lobe primordia in the late larva

The outer and inner optic anlagen of the early larva start out as small, crescent-shaped epithelial layers (OOAep, IOAep; Fig. 2A, B). The posterior, concave side of both optic anlagen is defined by the larval optic neuropil, which consists of the afferent larval photoreceptors (Bolwig’s nerve) and their target neurons (Sprecher et al., 2011; Fig. 2C, D). As larval development progresses, the OOA and IOA grow tangentially by symmetric cell division (described in Egger et al., 2007; Ngo et al., 2010; Yasugi et al., 2008). The curvature increases, such that the dorsal and ventral tips of the OOA and IOA come in close contact (Fig. 2F, G).

When referring to the topography of the optic anlagen, we distinguish a medio-lateral axis, which corresponds roughly to the medio-lateral body axis, and a dorso-ventral axis, which is curved, so that both the dorsal and ventral pole come to lie posteriorly (Fig. 2B, E). Around 48h ALH, the OOA and IOA start to convert into asymmetrically dividing neuroblasts (nb; Fig. 2H-J) which form distinct primordia of the optic ganglia (Fig. 2J). At this stage, the optic anlagen are more often termed outer (OPC) and inner (IPC) proliferation centers of the optic lobe in the recent literature (Fig. 2K-M; Apitz and Salecker, 2015; Bertet et al., 2014; Li et al., 2013b; Brand and Livesey, 2011). Thus, from this point onwards, we will adopt this terminology when referring to the late larva and pupa, and will reserve “optic anlagen” for the neuroepithelia of the early stages.

Molecular markers expressed in the optic proliferation centers and at later stages allow one to identify precursors of different cell types at the larval stage as well as to follow the fates of the different primordia throughout metamorphosis. To globally label neuronal cell bodies and nerve fibers, we used antibodies against Neurotactin (BP106) or Neuroglian (BP104) (Hortsch et al., 1990a, 1990b). Specific markers for subpopulations of optic lobe neurons expressed from early to late stages of development included Ln-Gal4 (L3/L4 neurons of the lamina; Zhu et al., 2009),
Dac (all lamina neurons, columnar neurons of the lobula and lobula plate T4/T5; Mardon et al., 1994), Bsh-Gal4 (medulla interneuron Mi1; Hasegawa et al., 2011), Vsx1-Gal4 (columnar neurons connecting distal medulla with proximal medulla, lobula and lobula plate, Tm, TmY; Erclik et al., 2008), Acj6-Gal4 and forNP0079-Gal4 (columnar neurons connecting the lobula, lobula plate and proximal medulla, Tlp, T2-T5; Potter et al., 2010b), and wg-Gal4 (tangential neurons of the medulla and lobula; Bertet et al., 2014; Fig. S1).

At the late larval stage, the optic proliferation centers have produced four main masses of immature neurons (“optic lobe primordia”) laid out in a way depicted in Fig. 2K-P. The OPC is divided by the lamina furrow into a small lateral domain (OPCl) and large medial domain (OPCm) which, by that stage, consists mostly of asymmetrically dividing neuroblasts (Fig. 2N). The OPCI generates the primordium of the lamina; the OPCm gives rise to the large primordium of the medulla (Fig. 2M, P), containing all of the different types of columnar medulla neurons (Mi, Tm, TmY, Dm; see Fig.1). At this point, the IPC has also split up into a large lateral component (IPC1), consisting of neuroblast-like (non-epithelial) progenitors, and a medial, epithelial component (IPCm; Fig. 2K-P). As shown in a recent study, there continue to be cells delaminating from the IPCm and migrating towards the IPC1 (Apitz and Salecker, 2015). The IPC1 gives off neuronal precursors in two directions, producing two separate primordia (Fig. 2L): (1) cells given off posteriorly into the concavity of the crescent-shaped IPC1, become the columnar elements of the lobula and lobula plate (Tlp, T4/T5; Fig. 1); (2) cells moving antero-laterally will become the “distal cluster” (Oliva et al., 2014), that contains the T-shaped neurons (T2/T3; Fig.1) which interconnect the lobula and proximal medulla. The IPCm, gradually, over the course of early metamorphosis, dissociates into numerous clusters of neuronal precursors which mainly give rise to the wide-field neurons interconnecting the lobula with the central brain. The development of these dispersed clusters of cells can only be followed with specific markers and will not be considered here.
The dorsal and ventral domains of both OPC and IPC differ in their developmental potential from the central domain. Defined by the expression of the morphogen Wg (Bertet et al., 2014; shown in green inset in Fig. 2O), they give rise to many types of glial cells, and therefore were called “glial proliferation zone” (GPZ; Edwards and Meinertzhagen, 2010; Chotard and Salecker, 2007). In addition, the dorsal and ventral tips of the OPC produce most, if not all, large-field tangential elements of the medulla.

**Morphogenesis of the optic lobe during early metamorphosis**

As a starting point for the following descriptions, Figure 3A-C schematically depicts the way in which neurons and their processes are arranged to compose the optic lobe. Neuronal cell bodies of columnar neurons (the large majority of cells of the optic lobe) form a layer (cortex or rind), several cell diameters deep (Fig. 3A). For the medulla and lamina, this layer is oriented in a parasagittal plane, and is defined by a horizontal (antero-posterior) axis, and a vertical (dorso-ventral) axis. The lobula and lobula plate are oriented perpendicularly to the medulla (Fig. 3A-C); their horizontal axis points medio-laterally (Fig. 3A). Neuronal processes assemble in a second layer, the neuropil, which coextends alongside the corresponding cortex (Fig. 3A). The large majority of neurons of the optic lobe are columnar neurons (blue neuron in Fig. 3A), which give off one main fiber directed perpendicularly to the plane of the cortex, thereby passing through the neuropil and (in case of projection neurons interconnecting different neuropils) exiting the neuropil at the side opposite to the cortex (Fig. 3A, blue neuron). The axis defined by the direction of these columnar axons will be referred to as “z-axis” (Fig. 3A). The main fiber of each columnar neuron forms branches at defined locations along the z-axis. The branch location of certain types of neurons were used to define discrete layers within the medulla and lobula/lobula plate (Fig. 3A, hatched lines; Fischbach and Dittrich, 1989).
Unlike most columnar neurons, tangential neurons (Fig. 3A, purple neuron) are confined to the edges of a compartment, and extend neurites oriented parallel to the plane of the corresponding neuropil (Fig. 3A, purple neuron). For example, the tangential neurons of the medulla (Mt) flank the anterior edge of this compartment and extend fibers (“Cucatti’s bundle) that penetrate the medulla neuropil anteriorly (Fig. 3B). Among columnar neurons, only few classes, notably the T2/T3 neurons (light blue neurons in Fig. 3B), are arranged in a way similar to tangential neurons. T2/T3 cell bodies fill a bar-shaped volume (“distal cluster”) located along the junction between posterior medulla cortex and lateral lobula plate cortex (Fig. 3B).

The above described architecture of the optic lobe compartments can be already recognized in the late larva (Fig. 3C), which can be best appreciated if looking at a horizontal section of the optic lobe (Fig. 3D, G, J and K). Neuronal precursors form cortices flanked by underlying neuropil primordia. However, the orientation of the compartments, with the exception of the lamina, differs significantly from the adult configuration. The horizontal axis of the medulla is directed medio-laterally in the larva (Fig. 3C), and then rotates to antero-posteriorly in the adult (Fig. 3B). For the lobula/lobula complex, the larval postero-anterior axis reflects the adult medio-lateral axis (compare Fig. 3C to Fig. 3B). As stated earlier (see Fig. 2A-E), the vertical, dorso-ventral axis of all compartments is bent in the larva, so that both dorsal and ventral tip point posteriorly. During metamorphosis, the primordia of the optic lobe cortex and neuropil undergo extensive proliferation, fiber growth, and morphogenetic movements. These events, graphically depicted in Fig. 3D-M and Figs. 4-6, will be discussed in the following, following the order: (1) reorientation of compartment axes; (2) directed growth; (3) formation of fiber systems (outer and inner optic chiasm); (4) rearrangement of neuronal cell bodies.
Changes in the axes of optic lobe compartments

The first change to occur in the orientation of optic lobe primordia is the straightening of the dorso-ventral axis, which happens during the first 36-40 hours of metamorphosis, and can be best appreciated when looking at parasagittal sections (Fig. 3F, I, M) of the brain or lateral views of the 3D digital models (Fig. 4D, H, L, P). By 12h after pupariation (P12), the shape of the primordia of the lamina and medulla has changed from its original C-configuration to that of slightly curved “banana” (compare purple and blue structures in Fig. 4A and 4E). This change equally affects the shape of the cell body layers (semi-transparent layers, Fig. 4E; Fig. 4G-H) and the underlying neuropils (solid layers in Fig. 4E; Fig. 4F). The case of the inner ganglia, lobula and lobula plate is slightly different. At the larval stage, the IPC (gray; inner C-shaped structure, Fig. 4B) follows the same curvature as the OPC (gray; outer C-shaped structure, Fig. 4B), as shown in Fig. 2L (green/yellow, IPC; blue, OPC) and Fig. 4B (gray C-shaped structures, Fig. 4B). As a result, the clusters of immature neurons budded off from the IPC towards the convex side and concave side also fill a curved volume (green and yellow, Fig. 4C-D) which then straightens out during early metamorphosis (green and yellow, Fig. 4G-H). However, the primordia of the neuropils of the lobula plate and lobula (green and yellow, respectively; Fig. 4A-B), formed by these neurons, exhibit a relatively straight dorso-ventral (vertical) axis from the larval stage when they first appear (Figs. 3F; 4A-B).

The second large-scale movement that takes place during the first half of metamorphosis is a global rotation of the optic lobe around the dorso-ventral axis. This movement can be best visualized in horizontal sections (dorsal view) of the optic lobe (Fig. 3D, G, J, K), but is also apparent when looking at dorso-lateral (columns 1 and 3, Fig. 4) or lateral views (columns 2 and 4, Fig. 4) of the corresponding digital 3D models. The medio-lateral axis of the medulla cortex and neuropil turns clockwise and, from about 48 APF onward, corresponds to the antero-posterior axis. Due to the curvature of the medulla (and the overlying
lamina and retina), the anterior edge of the medulla remains located more medially than the posterior edge (arrows “a” and “p” in Fig. 3B, K). The medulla cortex, located anteriorly of the medulla neuropil (if discounting for the curvature of the dorso-ventral axis) in the larva and early pupa (Fig. 3C-D, G), shifts to a position antero-lateral of the neuropil in the pupa and adult (Fig. 3J-K). The lobula (LO)/lobula plate (LP) performs a similar rotation as the medulla. In the larva, the plane of the LO/LP neuropils, has a posterior-to-anterior orientation (yellow and green, Fig. 3C; LO and LP in Fig. 3D). The IPCl-derived immature neurons (green neurons; Fig. 3C) forming the columnar elements of the LP are located laterally of their neuropils (compare location of IPCl to LP in Fig. 3D). Towards mid-pupal stages, the orientation of the LP rotates clockwise. As a result of this rotation, the cortex of cell bodies of the LP comes to lie posteriorly of the neuropils (compare purple LP neuropil to green LP cortex in Fig. 3K; Fig. 3B). Concomitantly, cell bodies of neurons produced by the IPCm (yellow in Fig. 3B-C) and initially located medially of the lobula neuropil (Fig. 3C), shift anteriorly to become the anterior cortex of the lobula (Lt, Fig. 3B).

The third movement shaping the optic lobe affects the lamina, which pivots counter-clockwise around the dorso-ventral axis. In the larva, immature lamina (LA) neurons are budded off posteriorly, away from the OPCI (Huang and Kunes, 1996; Fig. 3C-D). As a result, the lamina is “attached” to the lateral edge of the medulla, and the vertical plane (along antero-posterior axis) of the lamina (magenta, Fig. 3C) stands orthogonal to that of the medulla (horizontal or medio-lateral axis; blue, Fig. 3C). This relationship between lamina and medulla remains constant (compare ME and LA planes in Fig. 3D, G, F) until approximately 32h APF (Fig. 3J). Subsequently, between 32h and 48h APF), the lamina rotates counter-clockwise (compare LA neuropil positions in Fig. 3J-K) and “floats” anteriorly over the medulla cortex, so that the plane of the lamina now lies parallel to that of the medulla (Fig. 3B, K; see also 3D models in Fig. 4I, M where the lamina is positioned to the back or “anteriorly” in Fig. 4M compared to a more
posterior or frontal position in Fig. 4I). The formation of the outer optic chiasm is a direct consequence of this movement (Fig. 4O; see below).

**Directed growth of the optic lobe compartments**

During the first third of pupal development, all optic lobe compartments grow in size along the horizontal axis by a factor of approximately 4 (lamina), and 2-3 (medulla, lobula/lobula plate; Fig. 3). There is little or no growth along the vertical axis. To understand the growth of the optic lobe, discussed in more detail below, it is helpful to briefly recapitulate the development of the eye (Kumar et al., 2010; Ready et al., 1976), whose photoreceptors innervate the optic lobe, as well as the retinotopic order underlying this connection. The eye and optic lobe compartments are divided into modular units, the ommatidia (retina), cartridges (lamina), and columns (medulla, lobula/lobula plate). Units form vertical and horizontal rows. Photoreceptors R1-6 of the posterior-most vertical row of ommatidia project to the posterior-most vertical row of lamina cartridges; these cartridges, as well as R7/R8 of the posterior ommatidia, are connected to the most anterior vertical row of medulla columns (Hadjieconomou et al., 2011; Meinertzhagen and Sorra, 2001; Meinertzhagen and Hanson, 1993; Heisenberg and Buchner, 1977; Meinertzhagen 1976; Campos-Ortega and Strausfeld, 1972; Braitenberg 1967). In turn, the most anterior vertical row of medulla columns is connected to the most medial vertical row of columns in the lobula complex (Fig. 3B). These crossed connections create the outer and inner optic chiasm (Fig. 3B; see below). Along the dorso-ventral axis, connections are not crossed (Fig. 3E-F, H-I, L-M): dorsal ommatidia project to dorsal lamina cartridges and dorsal medulla columns, which in turn project to dorsal lobula columns; ventral units in the eye project to ventral units of the optic lobe.

The ordered connectivity between eye and optic lobe compartments is reflected during development by the temporal sequence in which the units of the visual system are born and
differentiate. In the eye imaginal disc, ommatidia located in a given vertical row differentiate at the same time. The vertical row formed by the first born ommatidia comes to lie at the posterior edge of the eye; more anterior rows are gradually added, at a pace of one row every 90 min (Meinertzhagen and Hanson, 1993; Selleck and Steller, 1991). In other words, the eye primordium grows and differentiates along the horizontal axis from posterior to anterior. The growth of optic lobe compartments along the horizontal axis also shows a temporal order, as depicted schematically in Fig. 5A-C. This was demonstrated in numerous studies for the lamina, where, during the larval stage, the first born (presumptive) posterior vertical row of cartridges differentiates concomitantly with the incoming axons of the posterior row of photoreceptor axons (Meinertzhagen and Hanson, 1993; Huang and Kunes, 1996; Selleck et al., 1992; Selleck and Steller, 1991). More and more anterior cartridges are added until the final size of the lamina is reached around 40h APF (Fig. 5D-F). A similar gradient in differentiation is detectable in the deeper compartments of the optic lobe when using markers for specific cell types. Labeling of a late larval or early pupal optic lobe with antibody against FasII or Ln-Gal4 reporter shows L3/4 neurons located in the posterior lamina, projecting towards the medial medulla (Fig. 5D-E). The size and staining intensity of axonal terminal is the medulla clearly follows a medio-lateral gradient, with the most medial ones (produced by the first born L3/4 neurons) being the largest and most intensely labeled (Fig. 5E). A similar gradient is visible when labeling medulla Tm/TmY neurons using Vsx1-Gal4 (Fig. 5H), where medially located neurons are strongly labeled and send long axons with terminal arbors towards the posterior part of the lobula. Vsx expression of neurons and axons becomes increasingly fainter when moving laterally in the medulla, or anteriorly in the lobula. Also neurons derived from the IPC, labeled by Dtx^{R77F09}-Gal4 (Fig. 5G) or acj6-Gal4 (Fig. 5I) exhibit this gradient. The expression of Acj6 in neurons generated by the IPC in the late larva already reveals a clear gradient (Fig. 5J-L). As described in a previous section, the IPCI is formed by a crescent-shaped array of neuroblasts which buds.
off immature neurons both posteriorly (i.e., into the cavity of the IPCl), as well as antero-laterally. The first expression of Acj6 occurs in the very center of the mass of immature neurons located in the IPCl cavity (Fig. 5J-K), furthest away from the IPCl neuroblasts. When adjusting for the curvature of the IPCl, this translates into the anterior-to-posterior gradient in neuronal differentiation described above (Fig. 5I, L).

The temporal gradient in differentiation exhibited by optic lobe neurons along the horizontal axis reflects a similar sequence in neuronal birth dates. This was shown in several previous studies for the OPC, which gives rise to the neurons of the lamina and medulla in a strict posterior-to-anterior and medial-to-lateral order, respectively (Piñeiro et al., 2014; Ngo et al., 2010; Yasugi et al., 2008; Egger et al., 2007; Hofbauer and Campos-Ortega, 1993; Meinertzhagen and Hanson, 1993). A similar order is observed in the IPCl (Fig. 5M-P). A pulse of EdU applied to a mid-third instar larva labels the crescent-shaped array of neuroblasts of the IPCl (Fig. 5M-N). When chasing the pulse to the late larval stage, labeling is seen in the center of the mass of immature neurons (Fig. 5O-P). This pattern indicates that each round of neurons born from the IPCl pushes the previously born neurons away from the IPCl, creating a peripheral-to-central (i.e., anterior-to-posterior) gradient in neuronal birth.

**Development of the fiber systems of the optic lobe**

The axons of optic lobe neurons form two major fiber systems, the outer and inner optic chiasm; which connect the lamina to the medulla, and medulla to the lobula/lobula plate, respectively. The outer chiasm is comprised of axons of L neurons and R7/8 photoreceptors, as well as a number of centripetal neurons projecting from the medulla to lamina (Fischbach and Dittrich, 1989). These fibers form thin bundles extending parallel to the horizontal plane, each bundle comprising the elements connecting one cartridge of the lamina to one column of the medulla (Sanes and Zipursky, 2010; Meinertzhagen and Sorra, 2001; Meinertzhagen and O’Neil, 1991;
Fiber bundles are surrounded by processes of glia and form a thin layer directly adjacent to the distal surface of the medulla neuropil (Fig. 8B', H).

The crossing of fibers of the lamina-to-medulla axon bundles in the horizontal plane can be seen as a direct result of the sequential timing of neuronal birth and differentiation, as described in the previous section, and shown in Fig. 5A-C (schematically) and Fig. 5D-F (labeling of L neurons). Axons of the first-born L neurons (and R7/8 receptors) extend all the way from their lateral origin in the lamina primordium (OPCl) to the medial edge the OPC, where the first-born medulla neurons are located (Fig. 5A, D). Axons of later born neurons terminate further laterally in the medulla, thereby crossing their older siblings (Fig. 5B, E, arrow). In the larva and early pupa, the shape of the chiasm (i.e., “cross”) is not yet visible, due to the fact that the lamina is oriented perpendicularly to the medulla. As the lamina rotates counterclockwise to the medulla, effectively aligning both neuropils (roughly) along the antero-posterior axis (Fig. 5C, F); axons are pulled into the cross-shaped configuration characteristic for the outer optic chiasm.

The spatial reorganization of lamina and medulla axon bundles during lamina rotation entails that axons are “dragged” through the mass of neurons forming the medulla cortex (Fig. 5E-F). Thus, prior to lamina rotation, the layer of bundles of L axons/photoreceptors enters the medulla from posteriorly (yellow arrow in Fig. 5E; see also Fig. 6B). After lamina rotation, this point has moved forward (yellow arrow in Fig. 5F), resulting in L fiber bundles radiating throughout the medulla cortex (Fig. 5F, 6C). This suggests that cell bodies of the medulla cortex do not tightly adhere to each other, but are able to let fibers glide past them over long distances.

The inner optic chiasm is more complex than the outer chiasm, since it is comprised of several different systems of fibers growing in different directions, as shown schematically in Fig. 6D. First there are the medullary systems, comprised of the axons of columnar Tm and TmY
neurons that interconnect columns of the medulla with columns of the lobula/lobula plate in a retinotopic manner (blue in Fig. 6D; labeled by Vsx1-Gal4 in green in Fig. 6E). These fibers are directed orthogonally to the plane of the medulla. After exiting the medulla neuropil, fibers converge and enter into the cleft between lobula and lobula plate, extending parallel to the surface of these neuropils (Fig. 6D-E). Secondly, one observes the lobula/lobula plate systems (purple in Fig. 6D), including the different classes of T neurons with cell bodies located posterior to the lobula plate, which form bundles of fibers with a trajectory that is orthogonal to the medullary Tm/TmY system. There are two main subsystems, T2/T3 neurons interconnecting the proximal medulla with the lobula, and T4 neurons connecting medulla and lobula plate (Fischbach and Dittrich, 1989; see Fig. 1). For clarity sake, only T2/T3 are shown in Fig. 6D and will be considered in the following. T2/T3 axons enter into the cleft between medulla and lobula/lobula plate from posteriorly and extend forward, running parallel to the inner surface of the medulla (purple in Fig. 6D; labeled by acj6-Gal4 in Fig. 6F). These fibers then make a sharp turn medially, entering the space between lobula and lobula plate.

Similar to what have been discussed for the outer chiasm above, the crossing of fibers of the Tm/TmY and T systems that generate the inner optic chiasm is also the result of the order in which these cells are born and differentiate (Fig. 5A-C). Early Tm/TmY neurons appear in the presumptive anterior medulla and project their axons to what will become the medial lobula complex (Fig. 5A). Likewise, early differentiating T neurons reach towards and innervate the presumptive anterior medulla, and medial lobula complex (Fig. 5A). Later neurons innervate more posterior positions of the medulla; on their way towards more lateral domains of the lobula complex, they have to cross the earlier formed fibers, thus forming the inner optic chiasm (Fig. 5B).

A remarkable feature of the three-dimensional organization of the inner optic chiasm is the alternating arrangement of the two fiber systems originating in the medulla (Tm/TmY) and lobula...
complex (T2/T3/T4), respectively (Fig. 6D). When viewed in frontal sections, the axons of the T2/T3 system leave the posterior cell cortex as a series of approximately 30 thick, regularly spaced bundles (“horizontal bundles”; “hbₘ” in Fig. 6) arranged in a vertical row (Fig. 6G-I; section “P” indicated in Fig. 6D). Each bundle is surrounded by a layer of neuropil glia, called “outer chiasm giant glial cells” (Edwards and Meinertzhagen, 2010; Tix et al., 1994; “gl” in Fig. 6J). At more anterior levels (section “A” indicated in Fig. 6D), approaching the cleft between lobula and lobula plate, each bundle splays out into a fan-shaped array of fibers. The T2/3 bundles alternate with stacks of axons of medullary Tm/TmY neurons (“hbₘ” in Fig. 6D, I, M). Numerically, stacks of T2/3 axons and Tm/TmY axons correspond to the number of horizontal rows of medulla columns (Fig. 6K-M). This 1:1 relationship between medulla columns and fiber bundles of the inner chiasm can be directly seen in sections aligned with the cleft between lobula and lobula plate (section “A” in Fig. 6D; Fig. 6M, M’). It appears that each stack of Tm/TmY fibers is generated by the axons emanating from all medulla columns of one horizontal row. Similarly, a T2/3 stack contains all of the axons connecting one horizontal row of medulla columns with the corresponding row of lobula columns (data not shown).

**Movement of neuronal cell bodies in the optic lobe cortices**

The original position of immature neurons in the larval optic lobe primordium is determined by the position of its progenitor, as well as its date of birth (Piñeiro et al., 2014; Li et al., 2013a; Li et al., 2013b; Suzuki et al., 2013; Hasegawa et al., 2011). This relationship has been investigated in detail for the OPC, but, as shown above (see Fig. 5K, P), also applies for the neurons produced by the IPC. However, cell bodies of several classes of neurons change their position during the course of metamorphosis, as shown for two classes of medulla neurons: Mi1 (labeled by the expression of Bsh-Gal4; Fig. 7A-C), and a subset of Tm/TmY neurons expressing Vsx1-Gal4 (Fig. 7C-F). Mi1 neurons are among the first born medullary neurons, and consecutively occupy a deep position in the primordium of the medulla cortex, furthest away
from their neuroblasts of origin (Hasegawa et al., 2011; Fig. 7A). By 24h APF, this position has changed: Mi1 cell bodies have moved along the z-axis and are now located relatively superficially in the developing medulla cortex; they will maintain this position throughout later stages into the adult (white arrowhead in Fig. 7B; Hasegawa et al., 2011). In the case of neurons expressing Vsx, the movement occurs along the vertical plane. In the larva and early pupa, Vsx expression is confined to neurons located in a central domain of the medulla primordium (Erclik et al., 2008; Fig. 7D, E). By 48h APF, Vsx-positive neuronal cell bodies have spread out along the vertical plane to occupy all domains of the medulla cortex (Fig. 7F).

It is important to note that the above described movements only affect the position of neuronal cell bodies, not the (immature) terminal arborization formed in the medulla neuropil. Shortly after the birth of a neuron, it extends a process that enters the emerging medulla neuropil at a defined position (“c” in Fig. 7C). For most neurons, this position corresponds to the position of the cell body within the cortex. By contrast, the Vsx-positive neurons, “delivered” only in the central part of the medulla, emit axons that fan out along the vertical axis (yellow arrows, Fig. 7E). Axons of neurons located dorsally in the Vsx-expressing cluster project dorsally and enter the medulla neuropil at its dorsal edge; neurons located ventrally project in that direction and enter the ventral edge of the neuropil (yellow arrows, Fig. 7E). During the movement of the cell bodies that occurs between 24h and 48h APF, the position where axons enter the neuropil and form connections does not change; instead, the position of a cell body within the cortex approaches the position of its axon in the neuropil. This type of movement is different from the “migration” of immature neurons in the canonical sense (for example, movement of cerebellar granule cells in vertebrates; Cooper 2013; Komuro and Yacubova, 2003), where the entire cell, prior to formation of fibers, moves, and connections are established after the cell body has reached its destination.
The development of layers within the optic lobe neuropils: medulla

The fact that branching of different populations of neurons occurs at specific locations along the z-axis of the neuropil was used to define layers in the medulla, lobula and lobula plate (Fischbach and Dittrich, 1989). For example, the upper strata of the medulla (layers m1-m5) are defined by the endings of lamina neuron classes L1-L5. Photoreceptors R7 have their terminal bulbs right underneath L5, thereby defining layer m6. Medulla intrinsic neurons (Mi), as well as Tm and TmY neurons, define the layers of the proximal medulla (m7-m10). In an analogous manner, 6 layers were defined for the lobula, and four for the lobula plate (Fischbach and Dittrich, 1989).

Layers can also be recognized by globally labeling the neuropil with markers for proteins enriched in synapses, like Synaptotagmin (Syt), Bruchpilot (Brp, nc82) or DN-cadherin (DNcad), the markers used in this study (Fig. S2). Some layers are characterized by higher levels of DNcad labeling than others, suggesting a higher synaptic density in the former, and a lower density in the latter. Using DNcad in conjunction with specific layer markers, we reconstructed the development of neuropil layering in the optic lobe. The results, depicted in Figs. 8-10, will be presented in the following, starting with the development of the medulla, and ending with the lobula/lobula plate.

The superficial layers of the medulla, m1 and m2, show high DNcad signal, with m1 (blue in Fig. 8C; defined by c202-Gal4, which marks L1 neurons; Rister et al., 2007) exhibiting slightly less label than layer m2 (Fig. 8C, C’). Layers m3, m5 and m6 show the least amount of DNcad signal. M3, defined by the endings of the majority of R8 photoreceptors (Fig. 8A, C), is divided into a middle stratum (3b) with slightly higher DNcad intensity, flanked by two thin dark bands (3a, 3c; Fig. 8C’). The lower band receives terminals of L3, marked by the expression of Ln-Gal4 (Fig. 8E, E’). Layers m5 and m6, defined by the terminals of L5 and R7, respectively,
are both very low in DNcad staining (Fig. 8C, C'). By contrast, the narrow m4 layer, receiving the terminals of L4 (marker: Ln-Gal4; FasII) shows a high DNcad signal (Fig. 8E, E'). M7 and m8 represent the domain where the lowest stratum of the distal medulla (m7) borders the upper stratum of the proximal medulla (m8). These two layers receive dense innervation by the large medulla tangential (Mt) neurons, which are derived from the GPZ of the OPC, and can be labeled by lineage tracing cell populations derived from wg-Gal4 (see Materials and Methods; Bertet et al., 2014; Fig. 8F). Both layers exhibit moderate levels of DNcad, and are separated by a thin band of low DNcad (arrowhead in Fig. 8C, C', F, F'). The deep layers of the proximal medulla, m9 and m10, receive the terminal arborizations of Mi1 neurons, marked by Bsh (Fig. 8D). They are indistinguishable in terms of DNcad intensity (Fig. 8D, D').

The layered organization of the medulla neuropil emerges during the second half of pupal development. At 72h APF, the pattern of DNcad labeling closely resembles the adult (not shown). Going backwards in time to 48h APF, the number of layers exhibiting different levels of DNcad intensity gets reduced, and the overall thickness of the medulla neuropil decreases, as schematically shown in Fig. 9B. At 48h APF, intermediate and deep layers (m7-m10) are thinner, but show a similar DNcad labeling as in adult, with m9/10, containing Bsh-positive terminals of Mi1 (Fig. 8J, J'), at high intensity, and m7 and m8 at moderate density; whereas a band of very low DNcad signal marks the m7/m8 boundary (arrowhead in Fig. 8I, I', L, L'). As in the adult, this band contains Wg-positive tangential axons (Fig. 8L). By contrast, layers m1 to m6 of the distal medulla show a stratification of DNcad intensity that is simpler than the adult pattern (compare Fig. 8C' to 8I'). Most notably, the wide layer that was lowest in DNcad signal in the adult, including m5 and m6, does not exist at 48h APF. The distal medulla consists of two layers of high DNcad signal, separated by a narrow band of low density. Based on the labeling of the superficial of the two DNcad-positive bands with Bsh-Gal4 (Fig. 8J) and Ln-Gal4 (Fig. 8K), which are expressed in L1 (terminals in m1), L2 (terminals in m2), and L5 (terminals in m1
and m2); the more superficial DNcad\textsuperscript{high} bands correspond to m1 and m2. The deep band harbors the terminals of L4 (labeled by \textit{Ln-Gal4}), L5 (\textit{Bsh-Gal4}), and R7 (24B10), implying that this band represents the primordium, or “protolayer” (pm) for m4-6. Interestingly, the narrow stratum 3c, containing terminal arborizations of L3 (labeled by \textit{Ln-Gal4}; arrow in Fig. 8K) is included in protolayer pm4-6. In other words, this protolayer encompasses the deep part of m3, and all of m4-m6 (schematically shown in Fig. 9B).

Two events are temporally correlated with the transition in medulla layering that occurs between 48h APF and eclosion: the differentiation of neuropil glia, and the extension of photoreceptor R8 axon terminals, analyzed previously (Edwards et al., 2012; Ting et al., 2005). During early pupal stages, R8 terminals are held back at the surface of the medulla neuropil, where they form a “transient R8” layer superficial to m1 (Fig. 8I). Between 48h and 60h APF, terminals extend basally, to occupy their final position within m3. At around the same stage, astrocyte-like medulla neuropil glia (ALG), labeled by \textit{NP3233-Gal4} (Edwards and Meinertzhagen, 2010; Omoto et al., 2015), extend their processes into the neuropil. Processes become strongly concentrated around photoreceptor terminals in layers m3 and m6, as well as tangential axons at the m7/m8 boundary (Fig. 8B, H). It is possible that this layer-specific growth of glial processes, which do not form synapses, is causally related to the appearance of DNcad-negative bands in m3 and m5/6. However, the higher concentration of glia is not likely the only cause for decreased DNcad density, since the DNcad\textsuperscript{low} stratum demarcating layer m3 is present at P48, even though glial processes just start to form around that stage (Fig. 8H, arrowhead).

Going further backward in development to 32h APF (P32; Fig. 9A-A’), 24h APF (P24; Fig. 9C-G’) and earlier (P12; Fig. 9H-M’) towards late larval stages (Fig. 9N-R), the thinning of the medulla neuropil and simplification of the DNcad banding pattern continues. During these early stages, the medulla neuropil has a characteristic wedge-like shape, the pointed end, which
represents the earliest stage of development, located laterally, where the still active OPC keeps adding neurons (Fig. 9C). Near the further developed medial edge of the medulla neuropil one can distinguish three layers: a superficial and a deep layer with strong DNcad signal, separated by a band of moderate signal (Fig. 9D, D’). Based on the expression of specific markers, the superficial DNcad\textsuperscript{high} layer represents the protolayer for m1-m6 (pm1-6). From about P24 onward, a thin DNcad\textsuperscript{low} band demarcates the nascent m3 (Fig. 9B, D, D’). The deep DNcad\textsuperscript{high} layer corresponds to m9 and m10, and the intermediate DNcad\textsuperscript{moderate} layer contains m7 and m8 (Fig. 9B, D, D’). Detailed analysis of specific markers expressed at these early stages clearly demonstrates that, despite of the homogenous DNcad signal density, the protolayers are polarized: different cell types form nascent arborizations at different depth. For example, L3 and L4 (Ln-Gal4, FasII) terminate in the center of protolayer pm1-6, where m3/4 will form later (Fig. 9F, I, L, Q); whereas terminals of L5 (Bsh-Gal4) or R7 (24B10) terminate deeper (Fig. 9E, I, K, N, P). On the other hand, it is also evident that different cell populations react to signals dictating their ultimate level of termination at different time points. Markers expressed in L4 (Ln-Gal4; FasII), later forming separate spatially superficial branches in m1 and m4 (Fig. 8E, K), have endings that span almost the entire thickness of protolayer m1-6 (Fig. 9F, I, L, Q), suggesting that the filopodia of these neurons do not yet respond to cues that later lead to their separation into superficial (m1) and deeper (m4) branches.

Even at its earliest stage of development, when the medulla neuropil represents a single protolayer which includes all of m1-m10, a polarization is clearly noticeable. Axon tips later destined to occupy the distal layers of the medulla (e.g., L4) are concentrated in the upper strata of pm1-10 (Fig. 9Q); those neurons which will innervate the proximal medulla (e.g., Vsx; T4) cluster at the lower boundary of pm1-10 (not shown). Arborizations of neurons that will branch in both distal and proximal medulla (e.g. Mi1 neurons labeled by Bsh-Gal4) initially fill the entire thickness of pm1-10 (Fig. 9P, right). As the transition from the single layer (pm1-10) to triple
layer (pm1-6/pm7-8/pm9-10; right panel in Fig. 9B) occurs, Bsh-positive terminals become segregated by the nascent pm7-8 into a deep layer in pm9-10, and a superficial layer in pm1-6 (left in Fig. 9P). The appearance of pm7-8 is correlated with, and could be causally linked to the arrival of axons of tangential neurons (labeled by expression of wg-Gal4; Fig. 9G, G', M, M') which penetrate into the medulla protolayer from its lateral edge.

In the larva and first day of metamorphosis, the primordium of the medulla neuropil is capped distally by a layer of elevated DNcad intensity (arrow in Fig. 9N, N'). This transient layer, which decreases in thickness from laterally (earlier stages of medulla development) to medially (later stages), is formed by a plexus of immature fibers of medullary neurons which assemble in the deep layer of the cortex (transient medullary plexus, TMP; arrow Fig. 9N'; white arrowhead in Fig. 6A-B). In other words, it does not become part of the medulla neuropil. Afferents from the lamina, which define the outer surface of the medulla neuropil (arrows in Fig. 6A-A') penetrate in between the medulla neuropil and the TMP (arrowhead in Fig. 6A-A', B).

The development of layers within the optic lobe neuropils: lobula complex

DNcad signal is distributed homogenously over the lobula plate (Fig. 10A'-D'), showing no correlation to the four layers defined on the basis of differential terminal arborization of T and Tlp neurons (Fischbach and Dittrich, 1989). Likewise, processes of neuropil glia are evenly distributed throughout the LP neuropil (Fig. 10A-B). In the lobula, DNcad labeling reveals three layers: a narrow distal layer of high DNcad signal, a wide proximal zone of moderate signal, and an intermediate layer of low signal (Fig. 10A', C'). Glial density is correlated with this layering: processes of the lobula ALG (also called “chandelier glia” in the literature) are most concentrated in the intermediate stratum of the lobula, and least in the distal lobula, with the exception of a very thin surface layer (Fig. 10A-B).
Labeling of columnar neurons innervating the lobula complex indicates that the DNcad-rich distal domain corresponds to layers 1-3, which are innervated by the T neurons labeled by acj6-Gal4 and for-Gal4LT (Fig. 10C; Fig. S2). Acj6-positive and For-positive arborizations are distributed at a low level diffusely over the lobula plate (Fig. 10C; Fig. S2). Labeling with Vsx1-Gal4, expressed in a subset of Tm and TmY neurons (Li et al., 2013b; Erclik et al., 2008), also shows a diffuse innervation of the lobula plate, as well as a concentration of processes in the proximal and intermediate lobula (layers 4-6; Fig. 10D). This matches the previous description of Tm/TmY neurons (Fischbach and Dittrich, 1989), according to which most classes (among them evidently the ones expressing Vsx) have terminals in the proximal lobula layers (Fig. S2). The proximal lobula is also strongly innervated by the classes of wide-field Lt neurons (Fischbach and Dittrich, 1989); a representative class of Lt neurons with arborizations in the proximal lobula, concentrated in layer 4, is marked by the expression of for-Gal4 (Fig. 10C).

Developmentally, a distinct lobula and lobula plate neuropil can be recognized from late larval stages onward (Fig. 11A-A’). The lobula exhibits a subdivision into a distal, DNcad-rich domain and a proximal DNcad-poor domain already at the onset of metamorphosis (Fig. 11A’, B’). Immature, Acj6- and For-expressing T-neurons reach into the superficial layer (Fig. 11A; Fig. S2), indicating that it constitutes the protolayer LO1-3. At P12, this protolayer is transiently subdivided into a more superficial stratum with moderate DNcad signal (white arrow, Fig. 11C-C’), and a deeper part with very high signal (Fig. 11C-C’). By P24, the final pattern of DNcad expression is established, with evenly high signal in protolayer LO1-3, moderate signal in the deeper part of LO4-6, and low signal in the domain bordering LO1-3 (Fig. 11E-H’).

The projection of T neurons of the lobula complex is largely restricted to its proper neuropil protolayer (i.e., LO1-3; Fig. 11A, C, E, G) from early stages onward. This also applies to some other neuron populations, such as the Lt neurons labeled by for-Gal4, which from early pupal stages onward are concentrated in protolayer 4 (Fig. 11E, G). However, there are some groups
of neurons that innervate the lobula at protolayers which do not ultimately correspond to the adult pattern. The Vsx-positive Tm/TmY neurons, whose final destination are the proximal strata of the lobula (Fig. 10D), initially terminate in the distal protolayer LO1-3 (Fig. 11D-D', 11F-F', H-H'). Between P24 and P48, Vsx-positive projections extend towards deeper layers to reach their final pattern at P72 (Fig. 10D).

Discussion

By reconstructing the global architecture and connectivity of the optic lobe at sequential stages of development we provide a dynamic map that will help in future studies to understand the formation of specific neuronal circuits, and to interpret experimental findings. Our analysis reveals three major structural/developmental hallmarks by which the optic lobe, compared to other regions of the fly brain, stands out: large scale neuronal movements, correlated temporal gradients in neuron production and differentiation, highly ordered retinotopic projections in between visual neuropils, and the formation of multiple layers within these neuropils.

Morphogenetic movements during optic lobe development

The position of cells in the optic lobe undergoes profound change between the late larval stages, when most cells are born, and the mid-pupal stage when the adult architecture and connectivity of the optic lobe is established. On the one hand, cell masses forming the cortices of the different optic lobe compartments move in toto; for example, the cortex of the medulla and lamina changes from its initial hemicylindrical shape to a rectangular shape; the lamina, initially perpendicular to the medulla, shifts forward and becomes oriented parallel to the medulla. On the other hand, neurons within a given cortex change position relative to each other; for example, medulla Tm neurons starting close to the center of the cortex shift in position all the way to the edges.
Long-range cell migration occurs in numerous types of neural precursors in the developing vertebrate nervous system. It entails the protrusion and adhesion of the leading edge, followed by detachment of cell bodies and cytoskeletal contractions (Cooper, 2013). Cell migration has been extensively described in the cerebellum, neocortex, chick optic tectum and the mouse superior colliculus (SC; Watanabe and Yaginuma, 2015; Omi et al., 2014; Sugiyama and Nakamura, 2003; Tan et al., 2002). In these systems, neural precursors migrate at an early stage, prior to sending out axons and forming connections with other neurons. It stands to reason that the migration event plays an important in establishing proper connectivity: neural precursors prevented from moving would not reach the domain where they are able to contact their proper synaptic targets.

The movements of neurons observed in the Drosophila optic lobe do not appear to follow the canonical mechanism of neuronal migration described in vertebrates. Thus, neuronal precursors emit axons that establish contact with the nascent neuropil already before their movement. This contact remains stable throughout development; only the cell bodies change their position, either along the z axis (e.g., from deep to superficial, as in case of the medulla Mi1 neurons), or along the vertical axis (as described here for the Vsx-positive Tm neurons. A similar type of movement was also described for several clusters of neurons in the central brain, where two hemilineages, produced by a common neuroblast, are located right next to each other in the larval brain, but move apart during the course of metamorphosis (Lovick et al., 2013). Likewise, several lineages located at the lateral surface of the central brain are displaced to dorsal or ventral positions by the growth of the optic lobe that occurs between early and late larval stages (Lovick et al., 2015). The fact that the displacement of neuronal cell bodies of some cell populations in the optic lobe and central brain occurs after the outgrowing fibers have reached and entered the neuropil also suggests that the movement is not essential for the connectivity of these neurons.
The mechanism by which cell bodies move in the *Drosophila* central brain or optic lobe has not been elucidated. The characteristic elongation and production of lamellipodia at the leading edge, described for “canonical” migration of neuronal precursors in vertebrates, is not apparent. This suggests that the movement is not active, but maybe caused by mechanical forces acting upon the cell bodies from the outside. In the central brain, such a passive movement seems to occur. Thus, if growth of the optic lobe was prevented by ablating optic lobe progenitors at an early stage (Lovick et al., 2015), central brain lineages did not move, but retained their position in the lateral brain cortex. At the same time, their axonal projection in the neuropile appeared unchanged, indicating that cell body movement is not essential for establishing connectivity.

**Temporal gradients in neuronal birth and differentiation**

A second hallmark of *Drosophila* optic lobe development is the presence of correlated temporal gradients underlying the birth and differentiation of neurons (reviewed in Campos-Ortega and Hartenstein, 1984; Meinertzhagen and Hanson, 1993). The first-born photoreceptors form a vertical row at the (presumptive) posterior edge of the retina. Their outgrowing axons encounter the row of first born lamina and medulla neurons, to which they establish contacts. Successively later born photoreceptors connect to later targets. We observed similar gradients in the deeper compartments of the optic lobe along either the mediolateral or anteroposterior axis: L3/4 and Tm/TmY neurons in the medulla; and IPCI derived proximal medulla and lobula plate neurons. The gradients in neuronal differentiation are reflective of the neuronal birthdates in both the OPC and IPC (Fig. 5M-P; Pineiro et al., 2014; Ngo et al., 2010; Yasugi et al., 2008; Egger et al., 2007; Hofbauer and Campos-Ortega, 1993; Meinertzhagen and Hanson, 1993). Additional studies are needed to address the question how precise the correlation between birthdate and connectivity in the deeper layers of the optic lobe really is; more importantly, the relationship between cell type, birth order, and connectivity needs to be established. Thus, incoming axons...
contact multiple target cell types, including different types of next-order projection neurons and local interneurons (Takemura et al., 2013). Given that, at least in the medulla, different types of neurons are born at different time points (Morante et al., 2013), it would be important to find out which of these is the “primary target” of the incoming axon, and whether this target (among all the cells that eventually are contacted by the axon) is always the first to differentiate.

Correlated temporal gradients underlying the formation of neurons and their targets have also been observed in other arthropod systems, notably crustaceans (Elofsson and Dahl, 1970; Harzsch et al., 1999). Here, three major growth zones, called P1, P2 and P3, were described for several species. P1 and P2 are neighboring each other in the head ectoderm, and produce, following the same posterior-to-anterior gradient as in insects, the retina (P1), lamina (P2), and medulla (P2). P3, located further medially in the ectoderm, is associated with the formation of the deep layers of the optic lobe. The minute visual system of the anostracan Daphnia magna, which possesses only 12 ommatidia projecting in a retinotopic manner on their target neuropil (lamina), has served as a classical model system to investigate the role of the temporal gradient in neuron production in controlling connectivity (Macagno, 1978; see below).

The temporal dynamics of neuronal birth and retinal axon extension has been documented in great detail in vertebrates. In Xenopus development, the retina grows by concentric accretion in which cells are added to the periphery where cell division is maintained at the dorsal and ventral ciliary margin (Hollyfield 1971; Straznicky and Gaze, 1971). This growth gradient results in a position-dependent birth order of retinal cells, whereby the oldest cells are located in the central retina and younger cells are located peripherally. Similar central-to-peripheral birth order of retinal cell types have also been reported in chicks, goldfish, other frog species, cat, and rat (Mednik and Spring, 1988; Drager 1985; Reh and Constantine-Paton, 1983; Rager 1980; Hollyfield 1968, 1971, 1972; Fujita and Horii, 1963; Sidman 1961). Unlike the retina, which shows a concentric mode of growth, the tectum grows along the anterior-posterior
axis in many vertebrates including chick, fish and frogs (Crossland 1979; Crossland et al., 1975; Gaze et al., 1974; Meyer 1978; Straznicky and Gaze, 1972; LaVail, 1971a, b; LaVail and Cowan, 1971). Anterior neurons are the first to differentiate, and neurons are continuously added by a growth zone extending in a crescent-shaped domain along the posterior margin of the tectum.

**The chronotopic organization of axon pathways in the visual system**

Axonal connections within the visual system follow a strict retino-topic order, which makes it possible that an image of the visual field is generated in the brain. In flies, retinal photoreceptors project onto the lamina, whereby both dorso-ventral axis is maintained. At the next two stages, the projection of the lamina onto the medulla, and the medulla onto lobula/lobula plate, the dorso-ventral axis is maintained, but the antero-posterior axis is reversed in the two optic chiasms. The location of axons within the chiasms faithfully reflects the location of the cell body in the compartment of origin; for example, axons of neighboring neurons in the lamina are also adjacent to each other in the outer optic chiasm. This implies that, given the posterior-to-anterior gradient of neuronal birth in eye and lamina, the position of axons in the chiasms also reflects birthdate of the neuron of origin (chronotopy).

The relationship between birthdates of neurons and their targets and neuronal connectivity is more complex. Projections between the photoreceptors and their targets in the retina (bipolar cells, retinal ganglion cells) are short, and maintain the axes of the visual field. The long projection of the retinal ganglion cells to the contralateral tectum, or thalamus, reverts both axes, with medial (=anterior) retinal ganglion cells connecting to the posterior tectum, and dorsal cells to the lateral (=ventral) tectum (Goodhill and Xu, 2005). This implies that birthorder of retinal ganglion cells (central to peripheral) and their targets in the tectum (anterior to posterior) does not match. Even more, the divergent birth orders necessitate a constant
reorganization (“shifting connections) of retinotectal projections (Reh and Constatine-Paton, 1984). Thus, as new neurons are constantly added at the posterior margin of the tectum, the early-born RGCs have to break their initially formed connections in order to maintain the retinotopic projection. This amazing shift in connectivity (which goes on while the visual system performs its function!) has been demonstrated in frogs and fish, as well as chick (McLoon 1985; Reh and Constatine-Raton, 1984; Easter and Struemer, 1984; Gaze et al., 1979; Schmidt 1977).

Whereas the birth order of retinal ganglion cells and their tectal targets is not correlated, a chronotopic order does exist in the axonal projection between retina and tectum, the optic nerve (retina to chiasm) and optic tract (chiasm to tectum. In goldfish and chick, newly grown fibers from the peripheral retina are added at the ventral surface of the nerve head (Finlay and Sengalaub, 1989; Easter et al., 1984; Easter et al., 1981; Rager 1980). In *Xenopus*, one also observes a chronotopic order of fibers which changes between optic nerve and tract (Taylor, 1987). In the nerve, axons are ordered concentrically, with younger axons originating in the retinal periphery, located at nerve perimeter, and older axons located at the nerve center. As the optic nerve passes through the chiasm, a reorganization of fibers occurs, such that young axons occupy a ventral position, and older fibers a dorsal position (Taylor, 1987). In mammalian vertebrates such as rodents, although there is a central-to-peripheral gradient of RGC formation (Drager 1985; Sidman 1961), there is no clear chronotopic ordering of the retino-collicular projection (Simon and O’Leary 1990, 1992). This is due to the fact that in mammals. RGCs initially project to many incorrect targets. Secondary axonal pruning plays a crucial role in establishing the mammalian vertebrate retinotopic map.

It stands to reason that the correlated temporal gradients underlying the formation of visual neurons and their targets, and or the chronotopic ordering of the tracts by which visual neurons project on their targets, play a role in the process that controls the formation of the
retinotopic projections. This has been directly confirmed in classical ablation experiments in the small crustacean Daphnia (Macagno 1978, 1979, 1981). It was found that retinal axons form connections with target neurons in the lamina in the order in which they arrive; if retinal axons 1, 2, 3 normally contact targets A, B, C, and axon 2 is ablated, then the remaining axon 3 will contact target B. Thus, in the experimental situation, axon 3 is next in line after axon 1; it arrives in the lamina primordium adjacent to 1, and occupies the next available target, which is B. No specific “labeling” of retinal axon and its target is needed.

The mechanism controlling connectivity has to be different in the vertebrate visual system. First, as explained above, birth orders of RGCs and their tectal targets are not correlated. Accordingly, experiments where either retina or tectum was rotated, still allowed for an orderly formation of a retinotopic map (Sharma and Hollyfield, 1980). An abundance of studies has made it clear that the graded distribution of the repulsive signals of the ephrin family and their receptors receptor in the RGCs and tectum, respectively, play a crucial role in the patterning of the retinotectal projection (Lemke and Reber, 2005). This does not exclude additional roles of local cell-cell interactions between the chronotopically ordered retinal axons. Work in zebrafish has shown that altering the chronotopy results in defects in retinotectal map formation (Pittman et al., 2008). The authors used an anti-sense morpholino of ath5 to remove neurons born before 42 hpf (hours post-fertilization) to show that axons of later-born, peripheral RGCs failed to exit from the eye to the tectum. Peripheral RGC axons remain in the RGC layer without entering the intraretinal region of the nerve heard. In addition, adding misrouted axons to the retinal projection caused significant pathfinding defects among the adjacent, normal fibers, indicating that the proper projection depends on interactions between axons.

The case of Drosophila, despite its wide use as a favorable model system in developmental neurobiology, is unclear. Local interactions between retinal axons, mediated by signals such as Robo 2, are important for the projection from eye to lamina and medulla (Pappu
et al., 2011). Global secreted signals, such as the ephrins, are present in the eye and optic lobe and could play a role similar to that described for vertebrates (Dearborn et al., 2012). Earlier experiments of Kunes et al. (1993) had also indicated that, along the dorso-ventral axis, chemical labels are important for the connections between retina and lamina. However, the importance of the correlated temporal gradients in neuronal birth that exist along the anterior-posterior axis has not been tested thus far.

**Development of layering in the visual neuropils**

The prominent layering of the insect medulla and deeper optic lobe compartments presents an interesting phenomenon that primarily relies on the subdivision of the cell membrane of individual neurons into small subcompartments that are able to interact only with specific synaptic partners. Thus, even though the fiber of medulla interneurons Mi1, or Mi15, which receive input from photoreceptor, extend throughout the entire thickness of the medulla neuropil, contact with R8 is made only at a specific depth which defines layer m3 (Takemura et al., 2013). This principle applies for all of the connections made between neurons in the medulla neuropil, as well as in the lobula and lobula plate. It should be noted that the layers as discussed here for the fly optic lobe neuropils are very different from the entities called “layers” in many regions of the vertebrate brain, such as the cerebral or cerebellar cortex, which are defined by entire cells, including cell bodies, and are generated by the migration of neuronal precursors. The layers of the optic tectum of vertebrates, on the other hand, bears much similarity to the insect optic neuropils: cell bodies are all located apically, near the ventricular layer, and send long dendritic fibers towards the basal (outer) surface; layers are generated by specific inputs that contact tectal dendrites only at specific depths (Meek, 1983).

There are different ways in which the layering of the medulla (and other visual neuropils) could evolve (Fig.12), depending on the time course in which input to the medulla arrives, and
medulla interneurons emit branches at different positions. Layers could be established sequentially (Fig.12A), with one type of input and its corresponding postsynaptic branches appearing first, followed gradually by others. The opposite extreme scenario (Fig.12B) would be if all layers develop simultaneously at an early stage and merely grow in thickness by increasing branch number and synapse formation. Both scenarios can be clearly dismissed by the observations reported here and in the previous literature. Thus, the definitive layers of the medulla, visible from approximately 60h APF onward, are preceded by protolayers, in which terminal branches of different afferents and their targets, which are later separated in different layers, overlap (Fig.12C).

The underlying molecular basis for restricting contacts to specific subdomains of the neuronal membrane could include highly localized expression of specific “recognition molecules” along the length axis of the columnar neuronal fibers, as well as diffusible attractive or repulsive signals acting on neurons and restricting their sites of contact to specific positions. In Drosophila, recent studies have provided some insight into the mechanisms guiding lamina neurons (L1-5) and photoreceptors (R7/8) to their proper layer in the medulla neuropil. R7 and R8 establish their final projections in the medullary m6 and m3 layers in a two-step process: R7/R8 first extend their growth cones to the respective temporary layers; by the mid pupal stage, they reach their final target destination (this work; Ting et al., 2005). The molecular network controlling the proper layer targeting of R7 includes Drosophila N cadherin, along with the phospho-tyrosine phosphatases PTP69D and Lar; while R8 growth cone extension requires Capricious (Caps), Flamingo (fmi, a transmembrane Cadherin) and the putative receptor, Golden Goal (ggo), as well as Robo-3 and Slit (Hofmeyer and Treisman, 2009; Nern et al., 2008; Tomasi et al., 2008; Chen and Clandinin, 2008; Ting et al., 2005; Lee et al., 2001). Ggo and Fmi are both expressed in the photoreceptor axons, with the highest protein expression in the youngest axons. By contrast, in the optic lobe, Fmi is expressed in the target and
photoreceptor axons while Ggo is preferentially expressed in the axons (Tomasi et al., 2008). R8 photoreceptors lacking fmi and ggo remain at the distal medulla and fail to extend their growth cones. Similar phenotypes have been observed for R7 photoreceptors lacking NCAD. Nettins and its receptor, Frazzled (Fra) have been recently identified as additional players in controlling R8 layer specificity (Timofeev et al., 2012). R8 axons lacking Fra stop extending their growth cones at the more distal layer.

Transcription factors that regulates the expression of these cell adhesion molecules have also been identified through forward genetic screens (Mencarelli and Pichaud, 2015; Morey et al., 2008; Petrovic and Hummel, 2008). Sequoia (S) regulates the expression of NCAD while R8-specific Caps expression relies on NF-YC, which represses Senseless, ensuring that R7 axons do not express R8-specific Rhodopsins and cell surface proteins (Morey et al., 2008; Petrovic and Hummel, 2008). Caps expression in R8-cells have been shown to be controlled by the homeodomain transcription factor, Orthodenticle (Otd; Mencarelli and Pichaud, 2015), suggestive of a redundant regulative mechanism of Caps by Otd and NF-YC. Such feedback loops and redundancy may be crucial for ensuring tightly-regulated layer-specificity in a cell-type specific manner.

Similar mechanisms have been observed for L1-L5 neurons, regulated by many cell surface proteins including N-Cadherin (NCAD), NetrinA/B, Semaphorin-1a, and PlexinA (Pecot et al., 2013; Timofeev et al., 2012; Hadjieconomou et al., 2011; Nern et al., 2008). In the late larva, the medulla neuropile represents as a single protolayer. As development progresses, the neuropile transitions to three protolayers (pm1-6/pm7-8/pm9-10). During this early transition, L3-5 terminals span the entire pm1-6 (Fig. 9F, 9L, 9Q). By P48, the arborization patterns have become refined to pm1-2 and pm4-6 (compare Fig. 9F to 8L), which confirms the description of Ln-Gal4 at the pupal stage in Pecot et al. (2013). In the early to mid-pupal stages, L4/L5
reaches to pm6 (labeled by 24B10 endings in Fig. 8L) and retracts to m4/5 due to the repulsive actions of Sema-1a -PlexA interactions in the serpentine layer (Fig. 8E; Pecot et al., 2013).
Neuronal connectivity in the *Drosophila* visual system.

Photoreceptors (red) are located in the retina and project in a precise retinotopic manner to the visual processing center of the brain, the optic lobe. The optic lobe has four synaptic compartments (from distal to proximal): lamina, medulla, lobula, and lobula plate. The medulla synaptic layer is subdivided to the distal and proximal region, separated by the serpentine layer. R1-R6 photoreceptors terminate in the lamina while R7-R8 terminate in the medulla. Lamina interneurons (L1-5; purple), targeted by R1-R6 project to the distal medulla; medulla local interneurons (Mi) interconnect the distal and proximal medulla; projection neurons (Tm, Tmy) connect to the lobula and lobula plate. Columnar neurons also connect lobula and lobula plate (Tlp), and lobula/lobula plate to the medulla (T2-4). Large tangential neurons of the medulla (Mt), lobula (Lt) and lobula plate (Lpt) transmit processed visual information to the central nervous system. Several types of local neurons interconnecting neighboring medulla columns (Dm, Pm), as well as projection neurons (T1, Y) are omitted.
Figure 3-2.

Architecture of the larval optic lobe primordium.

3D digital models and confocal sections of the developing larval optic lobe at the 1st instar (A-D), 2nd instar (F-I), and late 3rd instar (J-P) larval stages. Models present two different views for all three stages: posterior view (A, F, K), and lateral views (B, G, L). Insets at lower right corner of panels delineate orientation (a anterior; d dorsal; l lateral; p posterior). The outer optic anlage (OOA; for late stages called outer proliferation center, OPC) is shown in blue; inner optic anlage (IOA; later stages: IPC) is shown in yellow (epithelial medial inner proliferation center; IPCm) and green (mesenchymal lateral inner proliferation center; IPCl). Panel (M) shows a cutaway model of the optic lobe of a late larva. In this model, the optic lobe is digitally cut along a horizontal plane [indicated by arrowheads in adjacent panel (L)], and a dorsal view of the cut surface and bottom bottom part is presented. Areas shaded in gray correspond to the primordia of the optic ganglia (LA lamina; LO lobula; LP lobula plate; Med distal medulla; MEp proximal medulla) generated by the optic proliferation centers. Arrows in (K-M) indicate the direction in which optic proliferation centers convey postmitotic neurons. Drawing in panel (E) presents a schematic lateral view of larval brain (CB central brain; VNC ventral nerve cord) which illustrates the shape and location of the epithelial outer optic anlage. Note the curvature of the dorso-ventral axis which places the dorsal and ventral edge of the OOA close to each other.

(C-D, H-J, N-P): z-projections of confocal sections (approximately 15-20 µm thickness) of the optic lobe at the 1st instar (C-D), 2nd instar (H), and late 3rd instar (I, J, N-P). Panels (C, F, H, I, N) are cross sections along the vertical plane, (D, J, O) are parasagittal sections, and (P) is a horizontal section. The epithelium of the optic anlagen is either marked by DE-Cadherin (in red; C-D, N-P) or mCD8-GFP driven by esg-Gal4 (in red; H-J). Neuroblasts are marked by Deadpan (in green, H-J). Larval optic neuropil (LON) is marked by foraging (forNP0079-Gal4>UAS-
mCD8-GFP; green in C-D), and the central brain (CB) neuropil is marked by Discs Large (in blue, C-D). In panels (N-P) Dachshund (blue) labels neural precursors of the lamina (LA) and lobula/lobula plate (LO/LP). forNP0079-Gal4 >UAS-mCD8-GFP; green) exclusively labels the lateral domain of the inner proliferation center (IPCl). Inset in (O) shows expression of a wingless reporter (wg-Gal4>UAS-mCD8-GFP) in the dorsal and ventral tips of the outer proliferation center (OPC).

The developing OOA and IOA start out as epithelial, C-shaped layers (A, B, F, G) curved along the dorso-ventral axis. Beginning at the 2nd instar, the lamina furrow (lf) separates the medial and lateral outer optic anlage (OOAm/OOAl; OPCm/OPCl). Starting around that same stage, epithelial (ep) cells of the OOA are converted into neuroblasts (nb; H). The inner optic anlage also transforms into neuroblasts, starting around 48h AH (H). At this stage, one discerns a medial domain, still fully epithelial (H; IOAm) and a lateral domain (H; IOAl) which is mesenchymal; lateral cells in the IOAl start expressing Dpn (arrow). By the late larval stage (I, J), most cells of the OOA and IOAl are Dpn-positive.

Abbreviations used in this and all following figures: cb central brain; cl medulla column; cor cortex; ep epithelium; IPCl lateral domain of inner proliferation center; IPCm medial domain of inner proliferation center; LA lamina; lf lamina furrow; LO lobula; LP lobula plate; mc medulla cortex; ME medulla; MEd distal medulla; MEP proximal medulla; nb neuroblast; np neuropil; OOC outer optic chiasm; OPCl lateral domain of outer proliferation center; OPCm medial domain of outer proliferation center; TMP transient medulla plexus; VLP ventrolateral protocerebrum
Figure 3-3.

Morphogenesis of the optic lobe during metamorphosis.

(A) Schematic cutaway model of the adult optic lobe, delineating orientation of medulla (ME), lobula (LO) and lobula plate (LO) relative to the body axis. For each of the optic ganglia, the cellular cortex and underlying neuropil is shown. Neuropils are subdivided into layers along the z-axis, as shown for the medulla (hatched lines). The large majority of neurons in all optic ganglia are columnar neurons (represented by the medulla neuron shown in blue) whose main fiber penetrates the neuropil along the z axis. Tangential neurons (purple) project parallel to horizontal plane of the neuropil. (B-C) are schematic horizontal sections of the optic lobe of the adult (B) and late larva (C). The location of optic lobe neuropils and orientation of major fiber systems is indicated by shaded areas and lines; the color code introduced here is consistently employed throughout the remainder of the figures (magenta: lamina; blue: distal medulla; cyan: proximal medulla; green: lobula plate; yellow: lobula). In (C), location of the optic anlagen (gray) is indicated. Major systems of columnar neurons include lamina projection neurons (L), distal medulla projection neurons (Tm), proximal medulla projection neurons (T2/T3), lobula plate projection neurons (Tlp). Also shown are tangential neurons of medulla (Mt) and lobula (Lt). Note that primordia of optic ganglia and fiber systems are already formed in late larva, but undergo a rotation to reach their adult configuration. “a” and “p” in panel (B) indicate anterior and posterior edge of medulla, respectively. For further detail, see text.

(D-M) Z-projections of confocal stacks (15-20 µm thickness) of optic lobe labeled with anti-BP106 (green, neuronal cell bodies in cortex and axonal tracts) and anti-DNcad (magenta, neuropil). Panels of left column (D, G, J, K) show horizontal sections; middle column (E, H, L) represents frontal sections; right column (F, I, M) parasagittal sections.
Insets at lower right corner of panels of row (D, E, F) delineate orientation of panels (a  anterior; d  dorsal; l  lateral; p  posterior). Panels of upper row (D-F) depict white prepupa (P0); second row (G-I) 12h after puparium formation (P12); third row (K-M) 48h after puparium formation (P48). A horizontal section for P32 is shown in panel (J). For abbreviations, see legend of Figure 2.
Figure 3-4.

Morphogenesis of the optic lobe during metamorphosis.

All panels show digital 3D models at the pupariation (P0; first row, A-D), 12h after puparium formation (P12; second row, E-H), 40h after puparium formation (P40; third row, I-L), and 72h after puparium formation (P72; bottom row, M-P). Panels of first column (A, E, I, M) and third column (B, F, J, N) present view from dorso-lateral posterior; second column (B, F, J, N) and fourth column (D, H, L, P) show lateral view. Insets at lower right corner of panels of first row (A-D) delineate orientation of panels (a anterior; d dorsal; l lateral; p posterior). In panels of first and second column, developing neuropils are shown in saturated colors, using the color code indicated at bottom of figure. In these two columns, the epithelial part of the outer and inner proliferation centers (OPC, IPC) is in gray. In panels of first column, cortices of the lobula plate/proximal medulla are omitted to permit view of the neuropils. The cortex of the distal medulla (MED) and lamina (LA) is rendered in semi-transparent, unsaturated colors. In second column, all cortices are omitted. In third and fourth column, cortices are shown in saturated colours.
Figure 3-5.

Directed growth and differentiation of the optic lobe.

(A-C) Schematic horizontal sections of optic lobe of late larva (A), 24h pupa (B), and 48h pupa (C). Neuropils are shaded in gray. Direction of growth in these and following panels is indicated by arrows (e early born; l late born). Insets at lower right corner of panels delineate orientation of panels (a anterior; d dorsal; l lateral; p posterior). Early born neurons are represented in saturated colors (lamina L neuron: red; medulla Tmy neuron: blue; medulla-lobula neuron (T2/T3): green); late born neurons of the same classes are shown in light colors in panels (B, C). (D-F): Directed differentiation of the lamina and distal medulla. Z-projections of horizontal confocal sections of the optic lobe at L3 (D), P24 (E), and P48 (F). Optic lobe neuropils in these and all following panels are labeled by anti-DNcad (magenta). Lamina projection neurons (L3/L4) are labeled by anti-FasII (green in D) or In-Gal4>UAS-mCD8-GFP (green in E, F). Note that early born, more mature L3/4 axon terminals are thicker and show stronger GFP signal than later born, immature terminals. Hatched lines in (E) and (F) outline boundaries of medulla cortex. White arrows in these panels point at early born L3/4 axons, yellow arrows at late born ones. Asterisk marks position where these axons cross. Note that position of crossing point moves as the lamina shifts forward and becomes oriented parallel to the medulla between P24 and P48 (movement indicated by gray arrow in E), resulting in formation of the outer optic chiasm (indicated by asterisk in F). (G-I) Directed differentiation of the proximal medulla and lobula/lobula plate. Panels show horizontal sections of optic lobes of 24h or 12h pupae. Discrete neuron populations are labeled by Drx-Gal4>UAS-mCD8-GFP (T2-T5 neurons, in G), vsx-Gal4>UAS-mCD8-GFP (Tm neurons, in H), and acj6-Gal4> UAS-mCD8-GFP (T4/5 neurons in I). For all of these labeled neuron populations, terminal fibers are more pronounced in, or restricted to, the earlier born, more mature parts of the proximal medulla, lobula and lobula plate. Later formed, immature parts of these neuropils (asterisks in (G, H) are devoid of fibers at P24. (I and
show gradient in neuronal maturation in lobula plate cortex. T4/5 neurons, forming a major subpopulation of cells located in this cortex, show strong expression of acj6 marker in posterior, more mature part of the cortex; expression decreases towards anteriorly. This gradient appears in the late larva, and reflects birth order of neurons, as shown in (J-L). The three panels represent frontal (J), sagittal (K) and horizontal (L) section of optic lobe. Anti-DNcad (magenta) labels neuropil (strong label) and cell bodies/fibers (faint label). The C-shaped inner proliferation center (IPCl; unlabeled) buds off progeny towards the center. Earlier born cells are pushed centrally (away from the IPCl) by later born ones. Earlier born, more mature cells start expressing acj6; later born, less mature cells, closer to the IPCl (l) are acj6-negative. (M-P) demonstrate the directed growth in the IPCl using BrdU incorporation. (M, N) represent frontal section (M) and sagittal section (N) of 76h larva pulsed with EdU over 4h prior to fixation. EdU label (green) is incorporated into the IPCl and the OPC. (O, P) present a frontal section (O) and sagittal section (P) of wandering third instar larva pulsed between 72 and 76h, and chased to fixation at 100h. EdU-positive cells have vanished from the IPCl and are found in neural precursors, last divided between 72 and 76h, and now located in center of cell mass that becomes lobula plate cortex (LP). General label (magenta) of neurons (cortex, neuropils) by insc-Gal4; UAS-mCherry (M-N) or Jupiter::GFP (O-P). For other abbreviations, see legend of Fig.2.
Figure 3-6.

Development of fiber systems forming inner and outer chiasms.

(A-C) Confocal sections of the optic lobe at third instar larva (L3, A-A'), 24h after puparium formation (P24; B) and 48h after puparium formation (P48; C). (A) represents a horizontal section; (A', B, C) are frontal sections. Insets at lower right corner of these and following panels delineate orientation of panels (a anterior; d dorsal; l lateral; p posterior). Neuronal cell bodies and fibers (green) are labeled by anti-BP106 (A-B) or anti-BP104 (C); anti-DNcad (magenta) labels neuropil and some fiber systems, including axons of lamina neurons. At the larval stage up to P24, the planar neuropils of the medulla (MEd) and lamina (LA) are oriented perpendicularly to each other (※ in A). Axons of lamina neurons, as well as axons of R7/8 photoreceptors (white arrows in A and A') penetrate the lamina neuropil and then pass tangentially over the surface of distal medulla. Terminals of retina/lamina fibers form regularly spaced, DN-cad-positive clusters that foreshadow the medulla columns (cl in A'). Neurons forming the medulla cortex (mc in A) are located medially of the lamina. Axons of medulla neurons (blue arrow in A) intersect the plate of lamina axons at a right angle before entering the medulla neuropil. Outgrowing processes of several subpopulations of immature medulla neurons form a transient fibrous plexus located distal (outside) of the lamina axons (arrowhead in A, B). By P48, the lamina has shifted anteriorly, such that the planes of the lamina neuropil and medulla neuropil are parallel to each other (see Fig.5E, F). The masses of axons connecting lamina and neuropil (arrow in C) are dragged into the medulla cortex (mc). The crossing of these axons, representing the outer optic chiasm (OOC), falls into the center of the medulla cortex. (D) Schematic illustrating the main fiber systems of the inner optic chiasm (IOC). Masses of axons formed by the transmedullary neurons (shown in blue) are split into horizontal slices (hbM), whereby each slice contains axons from one horizontal row of medulla columns. Similarly, axons of T neurons connecting medulla columns with lobula and lobula plate
(only T2/3 shown, in magenta) form equal number of slices (hbL). Slices of transmedullary axons and T axons alternate, giving the inner optic chiasm, as seen in frontal sections (see panels G-M below) its characteristic striated appearance. (E-F) Horizontal sections of optic lobe 48h after puparium formation, showing medulla Tm neurons (E; green, labeled by Vsx1-Gal4) and lobula plate T neurons (F; green, labeled by Drx-Gal4) fiber systems. Neuropil is labeled by anti-DNcad (magenta). (G-J) Frontal confocal sections of optic lobe of larva and early pupa, illustrating spatial arrangement of fiber systems of inner optic chiasm during early metamorphosis. In (G, H), plane of section is slightly posterior of the inner optic chiasm, as indicated by “P” in panel (D). Axon bundles of T2/3 neurons (hbL; green, labeled by anti-BP106) are seen as they enter the chiasm from posteriorly. (I, J) Confocal sections representing plane “A” as shown in panel (D). General neuronal labeling by anti-BP104 (magenta). In (I), bundles of Tm neurons (hbM), labeled by Vsx1-Gal4 (green) alternate bundles of T neurons (hbL). In (J), glia of inner optic chiasm (labeled by Nrv2-Gal4; green) is seen to surround individual hbL bundles. (K-M') One-to-one numerical relationship between the fiber bundles formed by transmedullary neurons and T neurons. General neuronal labeling by anti-BP104 (white in (K-M)). (K) shows tangential section of medulla at level indicated by “H1” in panel (D). Note regular hexagonal arrangement of medulla columns (cl). One vertical row of columns is indicated by magenta circles. (L) presents tangential section at a deeper level, proximal to the medulla neuropil, indicated by “H2” in panel (D). Faint magenta circles indicate where the vertical row of columns visible in (K) project onto the plane shown in (L). Green circles are placed on all hbL bundles. Note identical number and spacing of columns and hbL bundles. (M, M') represent a section at the plane indicated by “A” in panel (D). The medulla neuropil is cross-sectioned, and medulla columns (cl) are clearly visible. Vsx-Gal4 labels hbL bundles formed by Tm neurons. Note equal number and spacing of these bundles and columns, as indicated by blue circles in (M'). For abbreviations see legend of Figure 2.
**Figure 3-7.**

**Cell body movements in the medulla cortex.**

(A, B) Horizontal confocal sections of optic lobe of late larva (L3, A) and early pupa (P24, B). Medulla interneurons (Mi1) are labeled by Bsh-Gal4; general labeling of neurons by anti-BP104 (red) and neuropil by anti-DNcad (blue). The vertical axis of the medulla cortex is shown by gray rectangle. Shortly after their birth, Mi1 cell bodies are close to medulla neuropil [arrow in (A)]; after 24h, they have shifted to distally towards the outer surface of the medulla cortex [arrowhead in (B)]. Top part of panel of (C), showing a schematic cross section of medulla cortex and neuropil, schematically depict the cell body movement. (D-F) Confocal sections of optic lobe of late third instar (L3, D), early pupa (P24, E) and mid-stage pupa (P48, F). Labeling of Tm neurons by Vsx1-Gal4. General labeling of neurons by anti-BP104 (red) and neuropil by anti-DNcad (blue). Vsx1-positive neurons originate from a central domain of the outer proliferation center [OPCm; in between white arrows in (D)], whereas the dorsal and ventral wings of the OPCm (yellow arrows) are devoid of this cell type. The same spatial restriction is still visible at P24 (E), where Vsx1-positive cell bodies are located in the central part of the cortex. Note that axons of the neurons at the dorsal rim of the Vsx1-positive cluster (arrowhead) project dorsally, traverse the cortex, and enter the neuropil at its very dorsal edge (double arrowhead); likewise, neurons at the ventral rim project ventrally. The mass of these fibers, which fill the deeper part of the medulla cortex, form the transient medullary fiber plexus (TMP; see Fig.6B and 9R). By P48 (F), cell bodies have spread out to fill the entire cortex (white arrows and yellow arrows coincide). All TM axons extend parallel, and the TMP has disappeared. For abbreviations see legend of Fig.3. The movement of cell bodies is depicted schematically in bottom part of panel (C). The entrypoint of a representative Vsx1-positive axon, indicated by “e”, remains the same before and after the movement of cell bodies.
Layering of the medulla neuropil in the adult and mid-stage pupa.

(A-L') Frontal sections of medulla neuropile, labeled by DNcad (magenta in A-L; gray in A', B'', C'-L'), and layer-specific markers: anti-chaoptin (24B10; labeling of photoreceptors R7/8; green in A, C, I; blue in E, K); c202-Gal4 (labeling of lamina L1 neurons; blue in C); Bsh-Gal4 (labels medulla Mi6 neurons; green in D, J); Ln-Gal4 (labels lamina L3/4 neurons; green in E, K); wg-Gal4 (labels precursors of medulla tangential neurons; green in F, L). The top two rows of panels (A-F') show adult stage; bottom rows (G-L') pupa at 48h after puparium formation (P48). For a given pair of panels (e.g., C, C'), the upper one shows anti-DNcad label (magenta) in conjunction with marker; the lower one anti-DNcad only (in gray). Panels of left column (A, A', G, G') presents overview of medulla neuropil at low magnification; hatched lines outline domain of neuropil shown at high magnification in the four columns to the right. Panels (B-B'') and (H) show labeling of neuropil glia expressing the marker NP3233-Gal4 (green). In the adult medulla neuropil, anti-DNcad labels distinct strata differing in signal intensity. Double labeling with cell type-specific markers, which define layers m1-m10, permits one to assign DNcad pattern to these layers, as described in detail in the text. White arrowhead in (C, C', F, F', I, I', L, L') indicates serpentine layer (boundary region between m7 and m8). White arrowhead in (H) marks the DNcadlow stratum (nascent layer m3) prior to the formation of significant glial processes. Note differences in anti-DNcad pattern between late pupal and adult stage illustrated in panels (C') and (I'), respectively. Layer m3, in the adult, is represented by a thin band with moderate DNcad signal ("3b") flanked by bands of low signal ("m3a", "m3c"). In the 48h pupa, m3 is narrower and typically represented by a single band with low or moderate DNcad level. Most conspicuously, the DNcad-low band representing layers m5/6 in the adult (C, C') is absent in the pupa (I, I'); here, terminals of m5/6 specific neurons (L1, R8), that coincide with a DNcad-low
stratum in the adult (C), are found in the lower part of a DNcad-high band (I). This band represents the “protolayer” for m4-m6.
Figure 3-9.

Layering of the medulla neuropil in the late larva and early pupa.

The design of this figure corresponds to that of the preceding Fig.8, presenting frontal sections of medulla neuropil labeled with anti-DNcad and layer-specific markers. Top row (A, A’)
represents 32h pupa; (B) schematically shows the developmental changes of the medulla neuropil layering as demonstrated with anti-DNcad from L3 to adult. Panels of the second and third row (C-G’) represent 24h pupa; rows four and five (H-M’)12h pupa; rows six and seven (N-R) late third instar larva. (R) illustrates the transient medullary plexus (TMP), formed by subsets of medulla neurons, which does not form part of the medulla neuropil. The distal boundary of this neuropil is defined by the incoming lamina/retina axons, visible as a DNcad-negative band (arrow). For details, see text.
Layering of the late pupal lobula neuropil.

(A-D’). Frontal sections of P72 optic lobe labeled with DNcad (magenta in A-D; gray in A’-D’), and colabeled with NP3233-Gal4 [labels glia; green in (A,B)]; for-Gal4 [lineage tracing; labels T neurons; green in (C)]; and Vsx1-Gal4 [labels Tm neurons; green in (D)]. (B, B’) represent a plane of section anterior to that in (A, A’), showing only the lobula (LO) and not the lobula plate (LP). The DNcad expression in the lobula neuropil reveals three layers, including a central layer with moderate signal intensity flanked by a proximal and distal layer with higher intensity. Processes of neuropil glia occur at a slightly higher density in the intermediate layer than the flanking proximal and distal layers (B). The distal layer contains terminals of T neurons (C), identifying this wide band as combined lobula layer 1-3 (Fischbach and Dittrich, 1989). Vsx1-positive Tm neurons are confined to the proximal and intermediate layers, indicating that they comprise lobula layers 1-4 which receive the large majority of Tm/Tmy neurons (Dittrich and Fischbach, 1989). LO (numerically labeled), discernible by various markers and intensity of DNcad: distal LO layers (1-3) is preferentially labeled by for-Gal4 (C); while proximal LO layers (5-6) are labeled by Vsx1-Gal4 (D). Both the distal and proximal LO layers show high DNcad. Layer 4 of LO is a thin layer that separates the distal and proximal LO with very low DNcad labeling (A’, C’). The LP is uniformly labeled by DNcad and there appears to be no clear layer separation with the present markers. The lamina is not shown. Orientation: lateral, right; medial left; ventral, down; dorsal, up. Other abbreviations: MEp, proximal medulla; MEd, distal medulla; IOC, inner optic chiasm. All sections are taken at approximately 15-20 µm thickness.
Figure 3-11.

Layering of the lobula neuropil during metamorphosis.

Frontal sections of the optic lobe labeled by for-Gal4 lineage tracing (green; A-A’, C-C’, E-E’, G-G’) and Vsx1-Gal4 (green; B-B’, D-D’, F-F’, H-H’) and counterstained with DNcad (magenta in A-H; gray in A’-H’) at late larval stage (first row; A-B’), 12h pupa (second row; C-D’), 24h pupa (third row; E-F’), and 48h pupa (fourth and fifth row; G-H’). The tripartite subdivision of the lobula neuropil is visible from P24 onward (E, E’). Prior to this stage, a thin, DNcad-rich band demarcates a protolayer for 1-3, in which for-Gal4-positive axon terminals are concentrated [arrowhead in (C)]; further proximally, the nascent lobula neuropil, which is in broad contact with the central brain neuropil (cb), has a moderate DNcad signal [protolayer 4-6 in (A-C’)]. Note that Vsx1-positive Tm axons, concentrated in proximal layers 4-6 in the adult lobula (see Fig.10D), are in the intermediate layer 4 at P48, and in distal layers 1-3 prior to that [arrow in (F)]. White arrows in (C, C’) point at superficial band of moderate DNcad signal that is continuous with the striated pattern of fiber bundles forming the inner optic chiasm (IOC). This band could correspond to layer of glial cells associated with the IOC. For abbreviations, see legend of figure 2.
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Chapter 4.

Conveyor-belt neurogenesis: A conserved mode of generating a complex, homotopically patterned visual system in insects and vertebrates
Abstract

The visual systems of vertebrates and many other bilaterian clades consist of complex neural structures guiding a wide spectrum of behaviors. Homologies at the level of cell types and even discrete neural circuits have been proposed, but many questions of how the architecture of visual neuropils evolved among different phyla remain open. In this review we argue that the profound conservation of genetic and developmental steps generating the eye and its target neuropils in fish and fruit flies supports a deep homology of bilaterian visual circuitries. Fish retina and tectum, and fly optic lobe, develop from a partitioned, unidirectionally proliferating neurectodermal domain that combines slowly dividing neuroepithelial stem cells and rapidly amplifying progenitors with shared genetic signatures to generate large numbers and different types of neurons in a temporally ordered way. This peculiar “conveyor belt neurogenesis” could play an essential role in generating the topographically ordered circuitry of the visual system.
Introduction

Vertebrates and flies possess an image forming eye whose photoreceptors project onto a multilayered visual neuropil. Already in the early 20th century, Santiago Ramon y Cajal noted the striking similarities between the neuronal organization of the visual systems of vertebrates and flies (1). More recently, a wealth of molecular studies demonstrated that conserved transcription factors such as Pax6/Eyeless and Six/Sine oculis form a central part of the gene network that controls the development of the visual system of vertebrates and flies (2, 3). This conservation led to the proposal that invertebrate and vertebrate eyes share homologous cytological and neuroanatomical features already present in their common bilaterian ancestor (4). Focusing on the first steps of visual processing, performed by retina and optic tectum in vertebrates, or by lamina, medulla and lobula in insects, it has been argued that vertebrate and insect neuronal networks use similar design principles which could be explained by a common ancestry (5-7).

In this review we survey the commonalities between visual system development in bilaterian animals, with a special focus on Drosophila and zebrafish. We discuss the genetic and cellular aspects of visual system development, and explore the extent to which basic morphogenetic mechanisms, rather than the complex organs per se, are shared among vertebrates and insects. We propose that the existence of conserved developmental genetic and cytological properties indicates a “deep homology” (8, 9) of bilaterian visual circuitries.

1. Synopsis of the vertebrate and fly visual systems

The vertebrate eye is composed of multiple tissues derived from the neural ectoderm (neural retina, optic stalk, pigment epithelium), the epidermal ectoderm (lens), and the ocular mesenchyme or neural crest (corneal epithelium; iris). The neural retina is formed by a large array of ciliated photoreceptors (rods and cones) that project their short axons towards a central layer (inner plexiform layer) formed by first order visual interneurons, the bipolar cells (Fig.1).
Bipolar cells target the basally located layer of 2nd order visual interneurons, the ganglion cells. Several types of local interneurons, including amacrine cells and horizontal cells, laterally connect bipolar cells and ganglion cells. Ganglion cell axons leave the eye through the optic stalk and project in a homotopic (i.e., retinotopic) order to one of the superficial layers of the contralateral optic tectum and dorsal thalamus. Different afferents are segregated in separate layers (10). Several classes of local interneurons form connections vertically and laterally within the tectum.

The fly eye, which develops from the epidermal ectoderm, is composed of an outer layer of cuticle and lens tissue. The retina underneath contains stereotyped clusters (ommatidia) of photoreceptor neurons which project in a somatotopic order to the optic lobe, the part of the brain that processes exclusively visual information (11). The optic lobe has four main neuropil compartments, called lamina, medulla, lobula and lobula plate, each of which is further subdivided into multiple layers (Fig.1). Photoreceptors involved in motion detection (R1-6) terminate in the lamina; R7 and R8, responsible for color vision, project to the distal layers of the medulla. Columnar visual interneurons targeted by photoreceptors vertically interconnect the layers of the lamina and medulla, and project to the lobula and lobula plate. From here, processed visual information is relayed by higher order visual interneurons to the central brain. Tangential neurons, similar to vertebrate amacrines and horizontal cells, laterally interconnect the vertical columns of the medulla and lobula/lobula plate; Fig.1)

A number of different hypotheses have been advanced in regard to the comparison between different layers of the visual system in flies and vertebrates. These include the idea that the peripheral layer of photoreceptors of an ancestral invertebrate diversified into the multiple neural layers of the vertebrate retina (12); on the other hand, it has been proposed that the photoreceptor layer of the insect eye, in addition to the first neuropils of the optic lobe (part of the central nervous system), can be homologized to the multilayered retina (5, 6). Continuing
along this line of thought, the vertebrate tectum, the target neuropil of retinal ganglion cells, might correspond to the deeper layers of the insect optic lobe (i.e., lobula and lobula plate). Unfortunately, evidence (in the form of anatomical/functional neural properties, or the expression of molecular markers) is ambiguous, and no generally accepted model has been worked out yet. In this review we will follow the proposal of Sanes and Zipursky (6), that the neuropil layers of the fly optic lobe correspond to the intermediate and deep layer of the vertebrate retina, in addition to the tectum.

In conclusion, fly and fish visual systems share certain fundamental design principles that form the structural basis for feature detection and coverage of the visual field. Among these principles, aside from a number of conserved cell types, are: (1) a layered and modular architecture (the fly retina has been compared to a “neuro-crystal”); (2) the inclusion of many vertically and horizontally projecting neuronal cell types within each module and layer; (3) a tightly controlled connectivity of neurons within and in between modules; (4) strictly homotopically ordered connections between layers.

There exists a longstanding, unresolved debate about to what extent these conserved design principles point at a homology versus analogy between discrete elements of the visual system. It seems likely that at the cell type level, photoreceptor cells and certain classes of central target neurons receiving direct photoreceptor input were present in the bilateran ancestor and are thereby homologous (19). In the following we review evidence that a distinct conserved neurogenetic mechanism, optimized for the generation of an extended, modular neural network interconnected by homotopic projections such as those encountered in the visual system, exists in vertebrates and arthropods alike, and we propose that this mechanism could have been present at the root of bilaterian evolution.
2. Conserved embryonic origin and genetic specification of the fish and Drosophila visual systems

The neurectoderm of the early embryo is subdivided along the anterior-posterior and medio-lateral axis by a group of conserved transcriptional regulators. Genes expressed along the AP axis include the Hox genes, which define the posterior brain and spinal cord (vertebrates) or ventral cord (insects), and a set of genes that are expressed in domains of the anterior brain (Otx, Emx, Tlx and their insect homologs; (20-24). Medio-laterally the neurectoderm is comprised of three columnar domains defined by the expression of Vnd/Nkx (medial), Ind/Gsx (intermediate), and Msh/Msx (lateral; 25, 26).

In vertebrates and insects alike, the visual system develops from the anterior domain of the neurectoderm, characterized by the (partially) overlapping expression of Orthodenticle (Otx/Otd) and Tailless (Tlx/Tll), and the absence of genes of the Hox complex (Fig.2A, B). This domain, in vertebrates, includes both the anlagen of the neural retina and, posteriorly adjacent, the tectum (Fig.2A). Pax 6 and other transcriptional regulators, including Six1/3/6, and Rx, are expressed in domains that are nested within the Otx/Tlx domain. In particular, Pax 6 and its *Drosophila* homolog, twin of eyeless (toy), define the eye field that gives rise to the neural retina in vertebrates, and the eye plus optic lobe in *Drosophila* (27, 28; Fig.2A, B). The second Pax6 homolog, ey, appears in the *Drosophila* eye imaginal disc, but does not coincide with the eye field in the early embryo (27). The same applies to the Six family of transcriptional regulators, in which Six3/6 defines the boundaries of the eye field in the vertebrate embryo, whereas the *Drosophila* Six3/6 homolog, optix (opt), is expressed at later stages in the eye disc (N29). Interestingly, sine oculis (so), the *Drosophila* homolog of Six 1, whose expression outlines the placoidal ectoderm in vertebrates, fully overlaps with the eye field of the *Drosophila* embryo (27, 28).
In vertebrates, the eye field of either side initially occupies a dorso-lateral position in the alar plate of the forebrain, in between the anlagen of the ventral forebrain (septum, hypothalamus and optic stalk) and the dorsal forebrain (pallium; Fig.2A). The optic tectum maps posteriorly adjacent to the eye field, in the alar part of the midbrain vesicle. The alar plate coincides with the intermediate column, as defined by molecular markers (N30). Indeed, tectal lateral progenitors (but not retinal progenitors) express the intermediate column determinant, Gsx (29).

The eye field in *Drosophila*, similar to that of the vertebrate embryo, is also surrounded anteriorly and laterally by the anlage of the protocerebrum (the part of the fly brain likened to the forebrain; 22, 27, 28; Fig.2B). The expression domain of the *Drosophila* Gsx homolog, ind, overlaps with the most posterior part of the eye field that gives rise to the lobula complex (KN and VH, unpublished), indicating that both vertebrate optic tectum and fly lobula complex arise from the intermediate column within the neurectoderm.

During neurulation, when the neurectoderm is folded into the interior of the embryo, the eye field of vertebrates becomes fully incorporated into the forebrain vesicle of the neural tube, from which it then evaginates as the optic cup, composed of the multilayered neural retina and the pigment epithelium (Fig.3A-F). The tectum forms in the dorso-lateral domain (alar plate) of the midbrain vesicle (30; Fig.3A, G). In *Drosophila*, the anlagen of the optic lobe and the eye also internalize as two invaginating neuroepithelia, a mechanism that sets these regions apart from the remainder of the fly brain. The canonical mechanism by which the fly brain develops involves stem-cell like neuroblasts that delaminate from the neurectoderm (31). By contrast, the *Drosophila* optic lobe anlage invaginates and forms an epithelial vesicle, which subsequently breaks up into two sheets, the inner and outer optic anlage (Fig.1D). These give rise to the lobula complex and medulla/lamina, respectively (32-34; Fig.1E). The *Drosophila* eye also develops from an invaginating neuroepithelium, the eye imaginal disc, located anteriorly adjacent to the optic lobe anlage (28, 35; Fig.1D). Following a phase of growth and
differentiation that takes place in this invaginated state, the eye disc everts during metamorphosis to become the compound eye (Fig.1E). Development of the eye from an invaginated disc is a derived feature seen in dipterans; in other insects, as well as arthropods with compound eyes in general, the growing epithelium giving rise to the eye forms part of the externally located head epidermis.

In conclusion, progenitors of the photoreceptors and their layers of target neurons derive from a genetically and topologically conserved domain, the anterior intermediate column of the neurectoderm. From within this domain, the primordia of the eye and its target structure (optic tectum/optic lobe) develop as invaginating neuroepithelia. As will be discussed in the following section, proliferation of these primordia follows a peculiar, highly structured pattern, termed conveyor belt neurogenesis. We will first introduce this mechanism for the optic tectum and retina in zebrafish, before attempting a side-by-side comparison with its *Drosophila* counterpart.

3. The conveyor belt neurogenesis in the fish optic tectum and retina

At early stages of the development of vertebrates, proliferating progenitors of retinal neurons are found throughout the alar plate of the presumptive forebrain (36, 37; Fig.3A, D). Subsequently, neural retina progenitors become located in a lens-facing domain all around the optic vesicle. From there, they flow to their destinations in the ciliary marginal zone (38; Fig.3B, E). Hence, progenitors lie at a hinge (ciliary marginal zone; CMZ) between two cortical structures, the retinal pigment epithelium and the neural retina (39; Fig.3C, F). At later stages, most of the neuroepithelium differentiates into an ependymal layer and becomes semi-quiescent. However, active proliferation or proliferation potentials persist in a peripheral zone of the retina, resulting in a continuous growth of these structures (40-42). Similarly, dividing tectal progenitors become restricted to a peripheral hinge (tectal marginal zone; TMZ) between the optic tectum and torus semicircularis (Fig.3G-I). This hinge is formed when the torus
semicircularis invaginates into the brain and comes to lie below the tectum, which occupies the dorsal and lateral parts of the brain (Fig.3H, I; 43).

The CMZ and TMZ exhibit a peculiar mode of proliferation, which has been termed “conveyor belt neurogenesis” (44). Conveyor belt neurogenesis includes the following features:

(1) The external edge of the CMZ and TMZ (CMZe, TMZe) is formed by a ribbon of slowly dividing neuroepithelial stem cells (43, 45, 46; Fig.4A, B) which accumulate transcripts for nucleotide and ribosome synthesis enzymes (43). These genes form a synexpression group that provides a useful signature to compare neuroepithelial stem cells of the retina and tectum, as well as other stem cell types.

(2) The progeny of the peripheral stem cells forms an intermediate layer (CMZi/TMZi) of rapidly dividing progenitor cells (“amplifying progenitors”; Fig.4A, B). These cells accumulate proliferation-associated genes (43) that represent another large synexpression group between the CMZ and TMZ, as already noted by Ramialison et al. (47). Proliferation of cells within the CMZi/TMZi is limited to a few rounds (43) of divisions before cells exit the mitotic cycle.

(3) Finally, postmitotic neural precursor cells gather at the central edge of the CMZ/TMZ. In this region cell cycle exit proteins (Kip, Insm1) are expressed as in many other proliferative domains (18, 48).

As already suggested by Cerveny et al. (49), the synexpression groups discussed in the preceding section might reflect the tight functional link between eye and optic tectum, which need spatially and temporally coordinated developmental processes to establish a precise connectivity map. We propose that the convergence of the molecular signatures with the morphogenetic features (conveyor belt neurogenesis) found in the primordia of eye and optic tectum supports a serial homology between these two primordia. Both emanate from neighboring domains within the alar plate of the forebrain/midbrain, forming an anterior
morphological unit located in front of the midbrain hindbrain boundary. The chordate ancestor probably possessed a simple, tube shaped brain (50). Photoreceptors and their direct target neurons developed in the wall of this tube, forming a “proto-retina”. Neurons posteriorly adjacent to the proto-retina formed a “proto-tectum”. Directed proliferation of progenitors of photoreceptors and target neurons from a peripheral growth zone (conveyor belt neurogenesis) could have already been present in the ancestral state. Subsequently, the increase in the size of the eyes in vertebrates led to the evagination of the optic cup from the telencephalon. Evolutionary scenarios propose that to colonize deeper waters, where the light levels are lower, the animal’s photosensitivity was increased by expansion of the photosensitive region. For craniates, there may have been distinct advantage if such expansion occurred by lateral ballooning, so that the light-sensitive region was not shadowed by the protective cranium (51). Along with the increase in retinal size, the tectum also expanded to accommodate the growing number of retinal afferents.

4. Patterns of growth of the fly eye and optic lobe

The development of the visual system in insects and other arthropods () is also characterized by a mechanism of directed proliferation within a neuroepithelial growth zone. In hemimetabolous insects and crustaceans the eye originates from a narrow, vertically oriented growth zone in the anterior head epidermis from where rows of differentiating ommatidia are “budded off” towards posteriorly (N54; Fig.5A). In Drosophila, the eye is derived from an invaginated epithelium, the eye imaginal disc. Following a period of symmetric cell divisions during the early larval stages (Fig.5B, D), a dorso-ventrally oriented growth zone becomes apparent within the disc (Fig.5C, E); progenitors within this zone maintain their mitotic activity throughout larval development. Whether within this growth zone one can differentiate between a spatially ordered subpopulation of more slowly dividing “stem cells” and faster dividing “progenitors”, as in the case of the vertebrate retina/tectum or the optic lobe (see below), is not clear. Posterior of the growth zone,
differentiation sets in. The first cells that become postmitotic differentiate into the R8 photoreceptors, forming a highly regular pattern, with one cell defining the center of each protoommatidium (52-53; Fig.5C). Other cells surrounding the R8s undergo one final mitosis before exiting the mitotic cycle. To this first vertical row of nascent ommatidia are then added, one after another, more anterior rows of ommatidia. Decapentaplegic (Dpp) signaling, Hedgehog (Hh) signaling and Notch (N) signaling are responsible for the orderly progression and spacing of R8 production. Subsequently, the nascent R8 cells serve as “organizers” for the ommatidia, recruiting surrounding cells to adopt specific cellular fate. Both N and EGF signaling play dominant roles in this process (N57).

Proliferation of the optic lobe, whose neurons are the targets of retinal photoreceptors, is also characterized by spatio-temporally directed growth. During early larval stages, the inner (IOA) and outer (OOA) optic anlagen that derive from the invaginated embryonic head neurectoderm form expanding sheets of neuroepithelial cells that grow by symmetric cell division (11, 32, 34, 54, 55; Fig.5B, D). By the beginning of the third larval instar, the OOA is subdivided into two domains, visibly separated by the lamina furrow (Fig.5C, E). Cells lateral to the furrow (OOAl) form the lamina, while cells medial to the furrow form the distal medulla (OOAm). Note that the polarity of the neuroepithelium giving rise to the visual system is inverted in *Drosophila* compared to vertebrates. Thus, the apical membrane of the fly neuroepithelia faces outwards, while it is directed internally, towards the ventricle, in zebrafish (Fig.5E, inset; compare with Fig.3E, H). This inversion can be viewed as a result of the invagination of the neurectoderm in vertebrates: the primordia of the eye and optic tectum form part of the neural tube, whose apical membrane faces towards the ventricular lumen. Progenitor cells and neurons are given off at the basal side of the neuroepithelium, which faces the outer surface of the neural tube. In flies, the apical membrane of the eye and optic lobe neuroepithelia face outward (dotted lines in
Fig. 5E, inset); neural progeny is pushed basally, as in vertebrates, but this direction is oriented inward.

By the mid third larval instar, cytological and proliferative characteristics of the OOAm change towards a mode that closely resembles the conveyor belt morphogenesis outlined above for the zebrafish optic tectum. Slowly cycling neuroepithelial cells of the OOAm, comparable to the TMZe of the zebrafish tectum, convert into rapidly dividing progenitors, called neuroblasts in insects, following a tightly regulated temporal sequence (“proneural wave”) that begins at the medial edge and moves slowly laterally towards the lamina furrow (Yasugi et al., 2008; 53, 55; Fig. 5E, inset). As a result, neuroblasts born first occupy a position medially within the OOAm, whereas the lastborn neuroblasts are located laterally (Fig. 5E, inset; arrow “a” indicates gradient).

Neuroblasts divide asymmetrically, thereby creating a second gradient (Fig. 5E, inset, arrow “b”) that reflects both time of birth and differentiative fate of neurons. Each mitosis produces a large, peripherally located daughter cell that continues to cycle as a neuroblast, and a small internal cell, called ganglion mother cell, that divides once before differentiation (GMC; 55-57). Thus neurons born early are located deep within the layer of cell bodies (cortex), whereas neurons born late are superficial, close to the neuroblast.

Neurons derived from the OOAI (lamina) and the IOA (proximal medulla, lobula, lobula complex)) are also born in a temporally graded manner (55, 58; not shown). However, compared to the OOAm, little is known about detailed aspects of proliferation of the OOAI and IOA, such as the orientation of mitotic spindles, or the relationship between birthdate and cell fate.
5. A similar growth mode in the Drosophila and fish visual systems

In both vertebrate and Drosophila visual systems, photoreceptors and their target neuropils originate from neuroepithelial stem cell layers. Neuroepithelial cells are polarized, express apical markers and establish contacts with collagen-rich basal membranes. These cells undergo interkinetic movements and perform their symmetric mitoses at the most apical part of the neuroepithelium (43, 56). Epithelial stem cells convert into asymmetrically dividing progenitors that give off their fast-amplifying progeny. In the conveyor belt mode neurogenesis, resulting global cell movements are tangential, because early born cells are pushed away from the progenitor population by later born cells (Fig.4A-C).

Molecular signatures of the different zones within the fish eye and tectal marginal zone and the fly optic lobe also show similarities. As described in Recher et al. (43), retina and tectum neuroepithelium in zebrafish are characterized by the expression of factors involved in nucleotide and ribosome turnover. Importantly, Mycn appears as a central player in the neuroepithelium network of insects (59) and vertebrates (60). Following a siRNA screen in Drosophila, Neumüller et al. (61) demonstrated that a network of 39 genes involved in ribosome biogenesis are crucial for the survival of Drosophila neural stem cells. Analogies between neurogenesis in fly and mouse adult forebrains have previously been reported in several reviews (62, 63, -64), mainly on the basis of the succession of slow activated stem cells, generating fast transitory amplifying progenitors. In addition, Strausfeld and Hirth (65) proposed deep homology between insect central complex and mammal basal ganglia. Here, based on a previously proposed theoretical ground (9, 66), we propose that the observation of so numerous similarities between the conveyor belt neuroepithelial-based morphogenesis in Drosophila and fish, both in term of molecular signatures, ontogeny and cytology constitute strong arguments for deep homologies of the Drosophila eye/optic lobes and the vertebrate eye and tectum.
Conclusion

Comparative developmental and genetic studies support the hypothesis that the anterior brain (the domain specified by Otx/Otd) and the central nervous system of the trunk (expressing Hox genes) have separate evolutionary origins that date farther back than the appearance of bilaterian animals. The anterior nervous system (“apical nervous system”; 67) included sensory receptors and neuroendocrine elements that might have functioned in orienting the organism relative to light, gravity, and chemical composition of the substrate. The nervous system of the trunk (“blastoporal nervous system”; 67) consisted of reflexive sensori-motor neural networks controlling the locomotor apparatus. Both apical and blastoporal components had likely merged in the bilaterian ancestor, setting the stage for a course of evolution where enlarging, modular arrays of sensory receptors could exert a much more elaborate control over locomotion.

According to the currently prevailing view, the bilaterian ancestor most likely did not have a large, image forming visual system. However, it may have possessed a visual system formed by a considerable number of ciliary photoreceptors developing in the Six/Rx positive domain, as described for the annelid Platynereis (67). These photoreceptors (ancestral eye) projected their axons on neighboring target interneurons which, as a result, adopted the role to process visual input. Extant chordate species such as the amphioxus (68) also obviously suggest that early chordates already possessed axonal projections resembling the basic photosensory-motor circuits of the vertebrate forebrain. Moreover, early vertebrate fossils from the early Cambrian (69) already seemed to exhibit foraging behaviors.

We speculate that the generation of large neuron numbers and (more importantly) the formation of highly ordered, topologically specific neural connections was made possible by the advent of improved neurogenetic mechanisms, such as the conveyor belt neurogenesis discussed in this review (Fig. 6). Conveyor belt neurogenesis took advantage of generally available “elementary” components that formed part of the urbilaterian neurogenetic “tool kit”, such as the invagination
of neuroepithelia, and the delamination and oriented division of neural progenitors (31), and combined them in a manner that allowed for the temporally protracted generation of photoreceptors and their target neuropils. A specialized domain with conveyor belt characteristics may also exist in other parts of the neu ectoderm, such as the cortical hem of the vertebrate forebrain (70, 71). The conveyor belt combines a neuroepithelial, slowly dividing part with an adjacent fast dividing part; it can generate large numbers of neurons of different types in a temporally coordinated way, which is likely to be important for the formation of precise, homotopically ordered (e.g., retinotopic) projections. We propose that conveyor belt neurogenesis was a plesiomorphic trait in bilaterians and became increasingly more complex as imaging vision took more importance in the different bilaterian groups.

A hypothesis of homology between visual systems has important implications for more applied fields, such as biomedical research. By providing genetically tractable models for studying visual system biology, developmental studies of the insect optic lobe may enhance our understanding of congenital malformations at the cellular and molecular level. Genetic pathways affected in microcephaly and microphthalmia are indeed active in the mammalian embryonic brain and retina neuroepithelial progenitors (72). In mammals, these progenitors proliferate during embryonic development, become dormant in adults, but are importantly remobilized in regenerative processes (73). Studies of visual system evolution can be used as a powerful tool to identify key conserved pathways that are at the basis of pathologies in mammalian systems.
Figure 4-1.

Schematic representation of the visual neuropil layers and their connectivity in zebrafish and *Drosophila*.

A strict retinotopic organization of the visual neuropils is maintained throughout all layers of the retina to the optic tectum in vertebrates, and throughout all neuropils of the optic lobe in *Drosophila*.
Figure 4-2.

Embryonic origin and morphogenesis of the visual system in zebrafish (left) and *Drosophila* (right).

(A) Schematic of zebrafish late gastrula /early neurula embryo, dorsal view (redrawn from 74). The domains giving rise to the optic tectum and neural retina fall within the Otx-positive anterior neural plate. The anlage of the retina (eye field) is characterized by the expression of Pax6, Six1/3/6 and Rx. Grey transverse stripe posterior to the tectum indicates the midbrain-hindbrain boundary, which expresses Pax2/5/8 and Engrailed (En). (B) Fatemap of the *Drosophila* visual system at the gastrula stage. Otd defines a large domain within the dorso-anterior neurectoderm that gives rise to the protocerebrum and visual system. The Six1 homolog Sine oculis (So) and the Pax6 homolog Twin of eyeless (Toy) are expressed in the anlage of the visual system, that includes the eye and optic lobe. Expression of the Pax2/5/8 homologs Poxn and Dpax2 are observed in a narrow stripe of neurectoderm likened to the vertebrate midbrain-hindbrain boundary (21). Similar medio-lateral systems (medial: Vnd/Nkx; intermediate: Ind/Gsx (stippled); lateral: Msh/Msx) subdivide the neurectoderm in fish and flies. *Drosophila* Ind expression overlaps with the anterior lip of the optic lobe anlage, that gives rise to the lobula complex, while the zebrafish Ind homolog, Gsx, is expressed in the optic tectum. (C) Zebrafish brain and visual system at larval stage, lateral view (anterior to the left). (D, E) Lateral view of late *Drosophila* embryo (D) and 24h pupa (E), depicting the protocerebrum and associated visual system. Consistent color code used throughout (A-E) illustrates the relationship between early embryonic anlagen and their derivatives.
Figure 4-3.

(A-I) The tectal marginal zone (TMZ) and the ciliary marginal zone (CMZ) are serially homologous structures.

Schematic lateral views (A, B, C) and cross sections (D-I) of zebrafish embryos; yellow shading marks expression of proliferation genes (e.g., impdh2; 43). These genes first in appear in the entire alar part of the forebrain/midbrain, and then retreat to the stem cell zones of the tectal marginal zone (TMZ) and the ciliary marginal zone (CMZ). (A, D, G) At the 3-somite stage, expression of proliferation genes is in the dorsal part of the anterior neural tube. (B, E, H) At the 15-somite stage, the primordia of the tectum and retina become separated. The retina evaginates, forming the eye cup. Expression of proliferation genes becomes confined to the dorsal eye cup. Complex morphogenetic movements change spatial relationships within the midbrain. Here, proliferation genes retreat towards the mid-dorsal and the ventral part of the alar plate, which invaginates to form the torus semicircularis. (C, F, I). At the 25-somite stage, expression of proliferation genes become restricted to the TMZ and CMZ. The CMZ forms a transitional domain between the neural retina and pigmented epithelium, encircling the lens. Similarly, the TMZ forms a narrow, hinge-like region encircling the lateral and posterior tectum.
Figure 4-4.

Conveyor belt neurogenesis in the visual system of vertebrates and *Drosophila*.

(A, B) Magnified view of schematic sections of the ciliary marginal zone (A; redrawn from Xue and Harris, 2012) and tectum marginal zone (B; redrawn from 43). Both CMZ and TMZ can be further subdivided, which is indicated by color coding. At their peripheral edge, the TMZ and CMZ contain stem cells (yellow). Away from this edge one finds the intermediate TMZ (TMZi) and intermediate CMZ (CMZi), both of which have fast amplifying progenitors (light green). Dark green indicates neural precursors exiting the cell cycle. In dark blue are differentiated neurons. (C) Magnified view of schematic section of *Drosophila* outer optic anlage, generating medulla neurons in a conveyor belt mechanism (for spatial orientation, see Fig.5E). Stippling indicates neuropil. (D) Simplified depiction of neurulation, illustrating inverse apico-basal axis of neuroepithelium in vertebrates and *Drosophila*. In vertebrates, after invagination of neural tube (bottom panel), apical surface of neuroepithelium containing neural progenitors (yellow) faces inward (ventricular lumen). Postmitotic neurons (blue) and neuropil (stippled) accrete at outer (=basal) surface of neural tube. In *Drosophila*, optic lobe neuroepithelium, following invagination, does not form a lumen, and apical surface points outward.
Figure 4-5.

Development of the visual system in insects.

(A) Microphotograph showing frontal view of embryonic head of the crustacean. An integrated growth zone (PZ1/2; blue label demarcates proliferating cells) generates the eye and outer optic lobe (lamina, medulla), following a temporal gradient. Early born cells (“e”) are located posteriorly, late born cells (“l”) anteriorly. (B, C) Schematic lateral views of Drosophila early larva (B) and late larva (C), showing growth zones in eye imaginal disc and optic lobe. (D, E) Schematic section of optic lobe of early larva (D) and late larva (E; based on 34, 56, 57, 58). In (B-E), epithelial optic anlagen and eye disc (giving rise to retina) rendered in yellow; neuroblasts forming from anlagen in light green, neural progeny dark green. Optic anlagen of the early larva are formed by symmetrically dividing neuroepithelia (B, D). In late larva, epithelia convert directionally into asymmetrically, rapidly dividing neuroblasts (E; arrow “a” in inset indicates directionality of epithelium>neuroblast conversion). Neuroblast produce ganglion mother cells (GMCs)/neurons (arrow “b” in inset to E). The eye disc also undergoes directed growth (arrowheads in C, E). Abbreviations: e early born cells; IOA inner optic anlage; l late born cells; OOA outer optic anlage, OOAl lateral domain of outer optic anlage, OOAm medial domain of outer optic anlage.
Figure 4-6.

**Conveyor belt neurogenesis in the bilaterian ancestor.**

Schematic representation of the neuroectoderm of hypothetical primitive bilaterans (top left, bottom center). The inception of the conveyor belt-mode of neurogenesis (top right) in discrete domains of the neurectoderm of the last common ancestor of chordates and arthropods allowed for a more efficient, protracted and temporally coordinated generation of photoreceptors and their target neuropils. The resulting evolution of complex visual systems in the arthropod clades (bottom, left) and chordate clades (bottom, right) greatly enhanced the role of visual input in controlling the locomotion, and thereby the spectrum of visually guided behaviors.
References


Concluding Remarks
The Drosophila optic lobe has become an attractive model system to study many aspects of neuronal circuit formation, from the control of neural progenitor proliferation to the establishment of connectivity. The reason for its attractiveness lies in the significant similarities, which in some cases may represent true homologies, between fly and vertebrate visual system. My thesis work is aimed at elucidating the steps of Drosophila optic lobe morphogenesis, and highlighting where potentially important similarities to vertebrate systems may exist.

1. Controlling speed and pattern of proliferation: conveyor belt neurogenesis

During mammalian neurogenesis, progenitor cells divide with the mitotic spindle oriented parallel to the surface of the neuroepithelium, resulting in symmetric division. Symmetric division allows for an exponential increase of progenitor pools in early neurogenesis (Rakic 1995). This is followed by asymmetric division with the mitotic spindle oriented perpendicularly to the neuroepithelial surface (Chenn and McConnell, 1995; Noctor et al., 2004). In the visual system, symmetrically and asymmetrically dividing progenitors are spatially organized into elongated growth zones surrounding the periphery of the retina and tectum, forming a conveyor belt-like structure that adds neurons in a continuous central to peripheral gradient. My work emphasized that similar arrangement of progenitors, and temporally ordered production of neurons, exists in the Drosophila optic lobe (Ch. 3-4). We identified Jak/Stat and Notch to control the speed of the conveyor belt mechanism, by negatively regulating the neuroepithelial-to-neuroblast conversion (Ngo et al., 2010). This discovery adds to a number of other signaling pathways that have been identified in recent years to regulate proper neuroblast conversion (Egger et al., 2007, 2010; Reddy et al., 2010; Yasugi et al., 2008, 2010).

Notch and EGFR signaling appear to occupy a central role in the network of regulatory interactions controlling the conveyor belt, but many questions remain unanswered and invite further studies. Notch is activated by Serrate from glial cells bordering the neuroepithelium and
inhibits conversion of the epithelium to neuroblasts by activating unique E(spl) target genes (Perez-Gomez et al., 2013). On the other hand, Delta becomes expressed in a narrow transitional zone at the edge of the neuroepithelium. What is the immediate effect of Delta? Does it engage Notch in the neighboring epithelium, or in the neuroblasts, or in both? How does Delta expression remain restricted to the transitioning neuroepithelial progenitors? Turning to the conveyor belt mechanism in the vertebrate visual system (e.g., zebrafish), It would be interesting to investigate whether Notch, EGFR or Jak/Stat plays a similar role in regulating the conveyor belt mechanism in vertebrates.

2. Temporal and molecular gradients in establishing neuronal connectivity

The role of temporal gradients in establishing the retinotopic map have been investigated in great detail. In early Xenopus development the retina grows by concentric accretion in which cells are added to the periphery where cell division is maintained in the ciliary margin (Hollyfield 1971; Straznicky and Gaze, 1971). In Xenopus, RGCs along the anteroposterior (nasotemporal) direction projects in the rostrocaudal sequence; whereas RGCs along the dorsoventral axis projects in the mediolateral direction. Similar central-to-peripheral birth order of retinal cell types have also been reported in chicks, goldfish, other frog species, cat, and rat (Mednik and Spring, 1988; Drager 1985; Reh and Constantine-Paton, 1983; Rager 1980; Hollyfield 1968, 1971, 1972; Fujita and Horii, 1963; Angevine and Sidman, 1961). The location and birthdate of a retinal ganglion cell is reflected in the position of its axon within the optic nerve and optic tract (chronotopy), and this position contributes to the eventual wiring of the axon to a target neuron in the tectum. In addition to chronotopic mechanisms, molecular gradients play an important role in controlling the development of the organization of neural connections (McLaughlin and O’Leary 2005). One prime example is the role of graded Ephrins as guidance molecules for dorsal-ventral and medio-lateral mapping of retinal ganglion cells to tectal target neurons (Huber et al., 2003; Horck et al., 2004).
Temporal gradients also play an important role in regulating connectivity in invertebrates. This has been directly confirmed in classical ablation experiments in the small crustacean Daphnia (Macagno 1978, 1979, 1981), where retinal axons form connections with target neurons in the lamina in the order in which they arrive.

A number of studies have demonstrated the reiterative use of gradients for defining positional information in the Drosophila visual system. Early studies in the eye and lamina show a temporal order from anterior-to-posterior. We observed similar gradients in the deeper compartments of the optic lobe along either the mediolateral or anteroposterior axis: L3/4 and Tm/TmY neurons in the medulla; and IPCl derived proximal medulla and lobula plate neurons. These gradients are reflective of the neuronal birthdates in both the OPC and IPC. This discovery prompts experimental genetic studies, whereby, for example, temporal gradients are modified, or the population sizes of individual neuron classes (e.g., lamina neurons) is changed.

How does a smaller number of lamina neurons occupy the normally sized target space in the medulla? What is the effect on connectivity if the temporal order in which lamina neurons are born is changed?

Similar to the vertebrate visual system, molecular gradients play an important role in controlling connectivity in the Drosophila visual system, among them the Robo/Slit system (Pappu et al., 2011) and most likely also the Ephrin system. It is surprising to note that, in comparison to vertebrates, so little detail has been worked out about the nature of these molecular gradients. It is possible that in Drosophila, chronotopic mechanism (i.e., the strictly correlated birth order of neurons and their targets), as well as local interactions between fiber systems (i.e., axons following a strictly parallel course, retaining their relative position, when passing from one neuropil to the next) play a much stronger role compared to vertebrates. This hypothesis invites numerous studies that can be carried out in the fly visual system. First and foremost, more systematic attempts need to be started to identify the signaling systems.
involved. One would expect that such signaling systems show graded expression patterns. Following a candidate approach, it is possible to identify signals/receptors with a strongly differential expression in anterior (late born) vs posterior (early born) populations in the individual optic lobe compartments (e.g., medulla).

3. Lineage-based and extrinsic mechanisms specifying neuronal fate

In the Drosophila central brain, lineages derived from individual neural progenitors are fixed and form structural modules of connectivity. Each neuron in the lineage is “aware” of its birth date and relationship to its “siblings”, and adopts a specific physiology, shape and connectivity. This is different in vertebrates, as well as in the optic lobe of flies. Here, neurons appear more “free”, deciding upon their fate in response to their environment (e.g., other neurons/glia/endothelial cells/systemic signals they come in contact with). However, a certain degree of order is imposed on them regarding cell type and connectivity. Thus, as well documented in the vertebrate retina, different cell types are born at different time points. Retinal ganglion cells, for example, are always born first. This phenomenon is also observed in the Drosophila optic lobe where, for example, certain types of medulla interneurons are born in the first round of neuroblast divisions, followed by other cell types (Hasegawa et al., 2011). In other words, most neuroblast lineages in the medulla generate different cell types as “pools” of cells at defined time points, just as in the vertebrate retina. From these pools, the proper numbers required for the individual units (e.g., medulla column) are recruited. But what element of the unit acts as the recruiter? In case of the medulla, the R7/R8 axons are the prime candidates, because they are the first elements of a medulla column to appear. We speculate that R7/8 grow into the pools of newly born medulla neurons, in which all cell types of a medulla column are represented, and emit signals that locally (for each emerging column) determine how many neurons of each pool will make it into a given column. This hypothesis invites many studies. First, one needs to confirm that it is indeed R7/8 that is responsible for the recruiting signal. Instrumental genetics (ablation
of the eye at different time points) would open the door to getting to the bottom of this question.

Next, the signals involved into the recruitment process need to be established. In the eye, Egfr signaling plays a pivotal role (reviewed in Malartre 2016), and this is the first candidate that should be scrutinized. In addition, systematic screens can be conducted, which is possible, given the genetic toolkits developed for Drosophila, as well as the in depth knowledge about cell types and cell numbers present in the fly visual system.

References


and Notch signaling pathways regulates proneural wave progression in the *Drosophila* optic lobe. Development 137(19), 3193-3203.

Appendix.

Appendix 1.

Postembryonic lineages of the *Drosophila* brain: I. Development of lineage-associated tracts
Postembryonic lineages of the Drosophila brain: I. Development of the lineage-associated fiber tracts

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ABSTRACT

Neurons of the Drosophila central brain fall into approximately 100 paired groups, termed lineages. Each lineage is derived from a single asymmetrically-dividing neuroblast. Embryonic neuroblasts produce 1,500 primary neurons (per hemisphere) that make up the larval CNS followed by a second mitotic period in the larva that generates approximately 10,000 secondary, adult-specific neurons. Clonal analyses based on previous works using lineage-specific Gal4 drivers have established that such lineages form highly invariant morphological units. All neurons of a lineage project as one or a few axon tracts (secondary axon tracts, SATs) with characteristic trajectories, thereby representing unique hallmarks. In the neuropil, SATs assemble into larger fiber bundles (fascicles) which interconnect different neuropil compartments. We have analyzed the SATs and fascicles formed by lineages during larval, pupal, and adult stages using antibodies against membrane molecules (Neuroactin/Neuroglian) and synaptic proteins (Bruchpilot/N-Cadherin). The use of these markers allows one to identify fiber bundles of the adult brain and associate them with SATs and fascicles of the larval brain. This work lays the foundation for assigning the lineage identity of GFP-labeled MARCM clones on the basis of their close association with specific SATs and neuropil fascicles, as described in the accompanying paper (Wong et al., 2013).

Introduction

The central brain and ventral ganglion of Drosophila is formed by an estimated 30,000 neurons which are generated from a pool of embryonically-derived stem cells, called neuroblasts, in a fixed lineage mechanism. This means that each neuroblast represents a genetically-distinct cell, characterized by the expression of a specific set of transcription factors (Doe, 1992; Urbach et al., 2003; Urbach and Technau, 2003a, 2003b). Each neuroblast gives rise to a group of neurons that is consistent in type and number across all individuals. Embryonic neuroblasts undergo several (5–10) rounds of asymmetric divisions, generating lineages of primary neurons that differentiate and make up the functional larval CNS (Larsen et al., 2009). After a period of mitotic quiescence that extends from late embryogenesis to the end of the first larval instar, neuroblasts enter a second, longer phase of proliferation which gives rise to adult-specific secondary neurons. Lineages constitute units, not only in terms of development (shared gene expression with the parent neuroblast), but also in terms of morphology. In most cases, all neurons of a given lineage extend their axons as one or two coherent fiber bundles along invariant trajectories in the brain neuropil and innervate a specific set of neuropil compartments (Hartenstein et al., 2008; Itô and Awasaki, 2008). Well-described examples are the four mushroom body lineages (Gritsenden et al., 1998; Itô and Itô, 1997) and the four lineages that interconnect the antennal lobe (olfactory center) with the mushroom body input domain, the calyx (Das et al., 2008, 2013; Lai et al., 2008; Stacker et al., 1990; Yu et al., 2010). The development and anatomical projection of most lineages remains largely unknown; ascertaining this knowledge and using it to generate an accurate map of Drosophila brain circuitry at the level of neuron populations (“macro-circuitry”) is an important project followed by us and others over the past several years.

Previous studies have provided detailed analyses of the lineages of the central brain, ventral ganglion (“ventral nerve cord”), and optic lobe at the embryonic and late larval stage, as well as of specific neural subtypes in the adult CNS (Bausenwein et al., 1992; Fischbach and Dittrich, 1988; Helfrich-Förster et al., 2007; Huser et al., 2012; Kuz et al., 2012; Mao and Davis, 2009; Peremura and Hartenstein, 2006; Schmidt et al., 1997; Seibert and

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rather, for many lineages, it decreases, due to cell death (Boeker and Truman, 1987a; Jiang and Reichert, 2012; Kumar et al., 2009).

As a result, the brain cortex becomes thinner and the clusters formed by individual lineages change in shape from radially-oriented "cylinders" to horizontally-flattened "plates" (Fig. 2A-C, e.g. DPLa1, #3). Depending on their position, some lineages are affected more than others by this flattening process. The cortex of the adult brain varies in diameter; it is thick at some locations where two outward-bulging compartments meet and deep "crevices" filled with neuronal cell bodies are formed (Fig. 2D, large arrow) or it is very thin or absent over the convexity of many different compartments (Fig. 2D, small arrow). A general morphological change is that the increase in neuropil volume causes line segment entry points and SATs to move away from each other (shown for DPLa1, #3; DPLd, #4 in Fig. 2E-H). However, the position of most lineages relative to each other remains constant, which is the prerequisite for following SATs throughout metamorphosis. Two processes, the separation of pairs of lineages (presumably hemineuromes) and the extension of additional fiber bundles, complicate the issue of identifying SATs during pupal stages for a number of lineages. In the larval brain, the cell body clusters of lineages and the entry points of their HSAVs are directly adjacent. During metamorphosis, hemineuromes are drawn apart to a varying extent. In most cases, they remain close; in a few cases, they become far removed from each other (Fig. 3A, e.g. DPLb2/3). The example shown in Fig. 3B-G is the paired lineage DPLb2/3, whose HSAVs at the larval and early pupal stages enter together at the dorso-posterior neural surface (Fig. 3B and D). During the course of metamorphosis (Fig. 3E and F), one hemineurom becomes posteromedial, the other one moves anterolaterally, resulting in two separate cell body clusters and two distinct entry points in the adult (Fig. 3C and G). This extreme separation of hemineuromes, typically occurring between P12 and P40, affects several other lineages as well (see Table 1). Development of nascent fiber bundles from a main SAT is the second mechanism by which the overall SAT structure of lineages is altered. As a rule, most lineages have fully extended their SAT (or HSAV/SSATs) by the late larval stage. For example, lineages of anterior lobe projection neurons, whose cell bodies are located in the anteromedial brain close to the antennal lobe, extend their axons far posterior to the calyx (Das et al., 2013; Perena and Hartenstein, 2006). During pupal development, terminal arborizations sprout from these fiber bundles and accumulate in the anamnial lobe and calyx/lateral horn. However, a number of lineages deviate slightly in that their SATs/HSATs acquire one or more major side branches, typically around 24–48 h of pupal development (P24–P48). This is shown in Fig. 4 for BAn1, which in the late larva forms a dorsally-directed and a posteriorly-directed HSAT (HSATd and HSATp, Fig. 4B and D). Beginning around P24, the dorsal HSAT emits a laterally-directed branch (SATd, data not shown for P24). By P22, the aforementioned branching for the BAn1 becomes more apparent (Fig. 4E), where the SATd reaches the VLPa compartment. The terminal arborization of SATd into the VLPa compartment is also observed in the adult stage (Fig. 4C and F). The most likely explanation is that branches added during the pupal period are formed by the axons belonging to a group of late-born neurons. In the larval brain, these cells would not yet have extended an axon contributing to the larval SAT. When they extend their axons in the pupa, these fibers might not all follow the pre-existing larval SAT, but establish a novel trajectory (SATd_new). Table 1 lists lineages forming prominent SAT branches during metamorphosis.

The pattern of fiber bundles in the brain neuropil

In the following presentation of lineages, SATs will be assigned to anatomically defined systems of fiber bundles (fascicles) in the brain neuropil. Fascicles are easily distinguished in the context of commonly used synaptic markers (e.g. Bruchpilot, nc82; N-Cadherin, NCad; Syntaxin, 8C3) which label most neuropil regions in the brain because they appear as domains of low signal, since synapses are scarce or absent in fascicles. Components of most adult fascicles can also be positively labeled by BP104 (this work). The most prominent fascicles can be generally grouped into longitudinal, transverse, and vertical bundles, which are based on the cardinal axis they travel along. Most of these bundles extend along the surface of the inferior protocerebrum, which is the brain domain surrounding the peduncle and lobes of the mushroom body (Perena et al., 2010a; Fig. S). For a more comprehensive description of fascicles and neuropil compartments, visit our website, the Drosofila Brain Lineage Atlas: https://www.mcb.ucsf.edu/Research/Hartenstein/dbb/. Along the boundary between superior and inferior protocerebrum, one further distinguishes a lateral and medial longitudinal superior fascicle (lIsL, lIsM3, and posterior component, lIsM5p (Fig. SC, D and E). Among the transverse fascicles, we distinguish an anterior, intermediate, and
after puparium formation (F4) the neuropil takes up less than 25% of the overall brain volume; around 48% this fraction has raised to almost 50% and at eclosion it is 53%.

Throughout metamorphosis in the pupal brain, secondary axon tracts defining the adult brain remains intact as cohesive fiber bundles and can be visualized using antibody markers against neuronal membrane molecules, such as Neuntactin or Neuromelanin (Pereanu et al., 2010). We present in this paper a detailed map of all SATs for the larva, pupa, and adult. The practical importance of this map is two-fold. First, the SAT/ neuropil fascicles, together with the neuropil compartments, help to define an anatomical framework to which smaller structural units (individual neurons, synapses), functional phenomena, or mutant phenotypes can be related. Second, SATs represent the hallmarks by which MARCM clones of lineages can be identified. To-date, only a small minority of lineages that continuously express a known Ga4-driven in the brain have been followed throughout development. Several groups (Ito et al., 2013; Yu et al., 2013; Wong et al., 2013) have now generated collections of lineage-specific MARCM clones, induced at the early larval stage, thereby marking all secondary neurons of a particular lineage. In all clones, neuronal cell bodies and their fiber tracts are easily visible, making it possible to assign a given done to the lineage it represents.

Materials and methods

Fly stocks

Flies were grown at 25°C using standard fly media unless otherwise noted. For Figs. 8 and 11, 1407-Gal4 (Mz407; Bloomington #8751), mapping out to the insc locus, was used as a driver line to visualize all secondary lineages at various stages of development ranging from L3 to L48.

Markers

The Bruchpilot (Bp) antibody (Developmental Studies Hybridoma Bank, DSHB; n:82) labels synapses and served as a marker for neuropil. It is a mouse monoclonal antibody from a large library generated against Drosophila head homogenates. The antibody recognizes the active zone protein Bp, which forms protease bands of 190 and 170 kDa in Western blots of homogenized Drosophila heads (Wagh et al., 2006).

The Cadeninin antibody (DSHB; DN-EX No.8), another marker for neuropil, is a mouse monoclonal antibody raised against a peptide encoded by Exon 8, amino acid residues 1210-1272 of the Drosophila Cadin gene. The antibody detected two major bands of 300-1Da and 200-kDa molecular weights on Western blot of S2 cells only after transfection with a cDNA encoding the N-Cadherin protein (Iwai et al., 1997).

The Neurotactin antibody (DSHB; BP106) is a mouse monoclonal antibody generated in a screen for novel antigens expressed on the surface of developing neurons in the Drosophila embryo (Patie et al., 1987). The antibody was used to screen a 9-12 h embryonic Drosophila phage-t11 CDNA library (Snow et al., 1987) that identified two phases containing a 435-bp EcoRI fragment that did not include the full open reading frame. A radiolabeled probe derived from this fragment was used to screen the CDNA library and identify a large open reading frame (Hortsch et al., 1990). The deduced amino-terminal sequence of this cDNA (11 amino acids) is identical to protein microsequence data from affinity-purified Neurotactin protein (de la Escaler et al., 1990).

The Drosophila antibody (DSHB; BP104) labels secondary neurons and axons in the adult brain. It is a mouse monoclonal
antibody from a library generated against isolated Drosophila embryonic nerve cords (Bieber et al., 1989).

**Immunohistochemistry**

Samples were fixed in 4% methanol-free formaldehyde in phosphate buffer saline (PBS, Fisher-Scientific, pH = 7.4; Cat No. #BP399-4). Tissues were permeabilized in PBT (PBS with 0.3% Triton X-100, pH = 7.4) and immunohistochemistry was performed using standard procedures (Aschner, 1989). The following antibodies were provided by the Developmental Studies Hybridoma Bank (Iowa City, IA): mouse anti-Neurotactin (BP106, 1:10), rat anti-DM-CD80 (DN-EX #8, 1:20), mouse anti-Neunl (BP104, 1:30), and mouse anti-Bruchpilot (nB82, 1:30). Secondary antibodies, IgG, (Jackson Immunoresearch; Molecular Probes) were used at the following dilutions: Alexa 546-conjugated anti-mouse (1:500), Dylight 649-conjugated anti-rat (1:400), Alexa 568-conjugated anti-mouse (1:500).

**Clonal analysis**

Clones were generated by FLP-mediated mitotic recombination at homologous FRT sites. Larval neuroblast clones were generated by MARCM (Lee and Luo, 2001; see below) or the FLP-out construct (Zeca et al., 1996; Ito et al., 1997).

**Mitotic clone generation by Flip-out**

To generate secondary lineage clones in the larva using the Flip-out technique: flies bearing the genotype:

1. hsFLP; elavGR::Gal4/+; UAS-FRT-mCD8::CFP
2. hsFLP; Act5C-FRT-stop-y-FRT::Gal4, UAS-tauUasZ/UAS-src::3GFP

Briefly, early larval with either of the above genotype were heat-shocked at 38°C for 30-40 min. elavGR::Gal4 is expressed in neurons as well as secondary neuroblasts. Third instar larval and adult brains were dissected and processed for immunohistochemistry (as described above).

**Mitotic clone generation by MARCM**

Mitotic clones were induced during the late first instar/early second instar stages by heat-shocking at 38°C for 10 min to 1 h (approximately 12-44 h AEL). GFP-labeled MARCM clones contain the following genotype:

*Adult* MARCM clones:

1. hsFLP++; FRT101, UAS-mCD8::CFP/FRT101, tub-GAL80; tub-Gal4/+
   or
2. FRT101 GAL80, hsFLP; UAS-mCD8::CFP; elavGR::Gal4, FRT101; UAS-mCD8::CFP

*Larval* MARCM clones:

hsFLP, elavGR::Gal4, FRT101, UAS-mCD8::CFP/Y or hsFLP, elavGR::Gal4, FRT101, UAS-mCD8::CFP/FRT101; FRT42D, tub-GAL80/FRT42D.

**Confocal microscopy**

Staged Drosophila larval and adult brains labeled with suitable markers were viewed as whole-mounts by confocal microscopy (LSM 700 Imager M2 using Zen 2009 (Carl Zeiss Inc.), lenses: 40 × oil (numerical aperture 1.3)). Complete series of optical sections were taken at 2-μm intervals. Captured images were processed by ImageJ (National Institutes of Health, http://rsweb.nih.gov/ij/) and Adobe Photoshop.

**2D registration of clones to standard brain**

Brains with MARCM clones were labeled with DN-cad and BP104 to image the SAT and projection envelope relative to the BP104-positive fascicles and DN-cad-positive neuropil compartments. Fasciculation of the SAT of a clone with a fascicle allowed for its identification with a lineage, or lineage pair. To generate the figure panels 2-projections of the individual MARCM clones were registered digitally with 2-projections of a standard brain labeled with DN-cad ("2D registration"). Additional details are provided in the accompanying paper (Wong et al., 2013).

**Generation of three-dimensional models**

Digitized images of confocal sections were imported into FIJI (Schindelin et al., 2012; http://fiji.sc/). Complete series of optical sections were taken at 2-μm intervals. Since sections were taken from focal planes of one and the same preparation, there was no need for alignment of different sections. Models were generated using the 3-dimensional viewer as part of the FIJI software package. Digitized images of confocal sections were imported using TrakEM2 plugin in FIJI software (Cardona et al., 2012). Surface renderings of larval and adult brains stained with anti-Bruchpilot were generated as volumes in the 3-dimensional viewer in FIJI. Cell body clusters were indicated on surface renderings using TrakEM2. Digital atlas models of cell body clusters and SATs were created by manually labeling each lineage and its approximate cell body cluster location in TrakEM2.

**Results**

The development of secondary lineages during metamorphosis

At the late larval stage, secondary lineages comprise elongated, radially-oriented clusters of approximately 150 cells that tile the brain cortex. Each cluster produces an axon bundle (secondary axon tract: SAT) whose entry point into the neuropil and pathway followed within the neuropil is distinctive and highly invariant (Fig. 1A and B). Pathways of most SATs can be individually followed within the neuropil in some cases, two or more lineages form a bundle in which the individual SATs cannot be distinguished (Fig. 1C-E; Table 1). A number of neuroblasts generate lineages which give rise to two dissimilar SATs; these are assumed to be the axon bundles belonging to two homologous (HSAVs; Fig. 1A-E; Table 1). Finally, the large type II lineages, numbering eight in total (reviewed in Brand and Livelys, 2011), are composed of multiple sub-lineages, each emitting a separate axon bundle (SSAs; Fig. 1F-J). Only the most conspicuous of these fascicles can be followed and are listed in Table 2.

Global neuronal markers such as Neuroglian (hereafter referred to as BP104) and, to a lesser extent, Neurotactin (in the following called BP106) remain expressed post-embryonically, making it possible to follow lineages and their SATs from the larval to the adult stage (Fig. 2). The analysis presented in this paper is based on the reconstruction of lineages from BP104- and BP106-labeled brains of staged pupae fixed at close intervals, including P6, P12, P18, P24, P32, P40, P48, and P72. Whereas the relative position of SAT entry points and pathways within the neuropil remains fairly constant, a number of morphogenetic changes can be observed for most lineages. These will be discussed in the following paragraphs, before focusing on individual lineages.

During the time that secondary neurons differentiate and generate axonal and dendritic branches the neuropil volume increases (eg growth of the SLP compartment, Fig. 2E-H). At the same time, the number of neuronal cell bodies does not increase;
### Table 1

List of abbreviations of neuropil fascicles (left), compartments (center), and entry portals of lineage-associated tracts (right)

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Anterior-dorsal commissure</td>
<td>ADC</td>
<td>Antennal lobe</td>
<td>AL</td>
<td>Dorsal entry portal of the AL</td>
<td>pAL d</td>
</tr>
<tr>
<td>Antennal lobe commissure</td>
<td>ALC</td>
<td>Antenna-mechanosensory</td>
<td>AMMC</td>
<td>Lateral entry portal of the AL</td>
<td>pAL l</td>
</tr>
<tr>
<td>Inner antennio-cerebral tract</td>
<td>iACT</td>
<td>and motor center</td>
<td>VENT</td>
<td>Ventral entry portal of the AL</td>
<td>pAL v</td>
</tr>
<tr>
<td>Medial antennio-cerebral tract</td>
<td>mACT</td>
<td>Anterior periosophagial neuropile</td>
<td>PONPa</td>
<td>Ventrolateral entry portal of the AL</td>
<td>pAL vl</td>
</tr>
<tr>
<td>Outer antennio-cerebral tract</td>
<td>oACT</td>
<td>Posterior periosophagial neuropile</td>
<td>PONPp</td>
<td>Ventromedial entry portal of the AL</td>
<td>pAL vm</td>
</tr>
<tr>
<td>Cervical Connective</td>
<td>CCT</td>
<td>Fan-shaped body</td>
<td>FB</td>
<td>Lateral entry portal of the CA</td>
<td>pCA l</td>
</tr>
<tr>
<td>Commissure of the lateral accessory lobe</td>
<td>LALC</td>
<td>Protocerebral bridge</td>
<td>PB</td>
<td>Medial entry portal of the CA</td>
<td>pCA m</td>
</tr>
<tr>
<td>Dorsolateral root of the fan-shaped body</td>
<td>dIFB</td>
<td>Inferior protocerebrum</td>
<td>IP</td>
<td>Ventral entry portal of the CA</td>
<td>pCA v</td>
</tr>
<tr>
<td>Horizontal VLP tract</td>
<td>hVLP</td>
<td>Anterior IP</td>
<td>IPa</td>
<td>Ventro-lateral entry portal of the CA</td>
<td>pCA vl</td>
</tr>
<tr>
<td>Frontomedial commissure</td>
<td>FrMC</td>
<td>Lateral IP</td>
<td>IPl</td>
<td>Anterior entry portal of the LH</td>
<td>pLH a</td>
</tr>
<tr>
<td>Great commissure</td>
<td>GC</td>
<td>Medial IP</td>
<td>IPm</td>
<td>Posterior entry portal of the LH</td>
<td>pLH p</td>
</tr>
<tr>
<td>Intermediate superior transverse fascicle</td>
<td>(superficial/deep)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>trSis/d</td>
<td>Posterior IP</td>
<td>IPp</td>
<td>Anterior entry portal of the ML</td>
<td>pML a</td>
</tr>
<tr>
<td></td>
<td>LE</td>
<td>Lateral accessory lobe</td>
<td>LAL</td>
<td>Dorso-lateral entry portal of the ML</td>
<td>pML dl</td>
</tr>
<tr>
<td></td>
<td>LEA</td>
<td>Lateral horn</td>
<td>LH</td>
<td>Dorso-medial entry portal of the ML</td>
<td>pML dm</td>
</tr>
<tr>
<td></td>
<td>LEP</td>
<td>Mushroom body</td>
<td>MB</td>
<td>Dorso-lateral entry portal of the PB</td>
<td>pPB dl</td>
</tr>
<tr>
<td></td>
<td>LEF</td>
<td>Calyx of MB</td>
<td>CA</td>
<td>Dorso-medial entry portal of the PB</td>
<td>pPB dm</td>
</tr>
<tr>
<td></td>
<td>LEFa</td>
<td>Medial lobe of MB</td>
<td>ML</td>
<td>Ventral entry portal of the PB</td>
<td>pPB v</td>
</tr>
<tr>
<td></td>
<td>LEFp</td>
<td>Peduncle of MB</td>
<td>P/PED</td>
<td>Lateral entry portal of the PLP</td>
<td>pPLP l</td>
</tr>
<tr>
<td></td>
<td>IoSL</td>
<td>Spur of MB</td>
<td>SP</td>
<td>Postero-inferior portal of the PLP</td>
<td>pPLP pi</td>
</tr>
<tr>
<td></td>
<td>MEF</td>
<td>Vertical lobe of MB</td>
<td>VL</td>
<td>Postero-superior entry portal of the PLP</td>
<td>pPLP ps</td>
</tr>
<tr>
<td></td>
<td>mIFB</td>
<td>Anterior optic tubercle</td>
<td>OTJ</td>
<td>Anterior entry portal of the SLP</td>
<td>pSLP a</td>
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<tr>
<td></td>
<td>IoSM</td>
<td>Posterior lateral protocerebrum</td>
<td>PLP</td>
<td>Lateral entry portal of the SLP</td>
<td>pSLP l</td>
</tr>
<tr>
<td></td>
<td>IoSMa</td>
<td>Subesophageal ganglion</td>
<td>SOG</td>
<td>Posterior entry portal of the SLP</td>
<td>pSLP p</td>
</tr>
<tr>
<td></td>
<td>IoSMp</td>
<td>Superior protocerebrum</td>
<td>SP</td>
<td>Postero-lateral entry portal of the SLP</td>
<td>pSLP pl</td>
</tr>
<tr>
<td></td>
<td>MBDL</td>
<td>Superior intermediate protocerebrum</td>
<td>SLP</td>
<td>Postero-medial entry portal of the SLP</td>
<td>pSLP pm</td>
</tr>
<tr>
<td></td>
<td>obP</td>
<td>Superior lateral protocerebrum</td>
<td>SLP</td>
<td>Dorsal entry portal of the SP</td>
<td>pSP d</td>
</tr>
<tr>
<td></td>
<td>pPLPC</td>
<td>Anterior SLP</td>
<td>SLPa</td>
<td>Ventral entry portal of the SP</td>
<td>pSP v</td>
</tr>
<tr>
<td></td>
<td>PLF</td>
<td>Posterior SLP</td>
<td>SLPp</td>
<td>Dorso-medial entry portal of the VL</td>
<td>pVL dm</td>
</tr>
<tr>
<td></td>
<td>trSP</td>
<td>Superior medial protocerebrum</td>
<td>SLP</td>
<td>Lateral entry portal of the VL</td>
<td>pVL l</td>
</tr>
<tr>
<td></td>
<td>trSPI</td>
<td>Inferior Ventro-lateral cerebrum</td>
<td>VLCi</td>
<td>Ventral entry portal of the VLCi</td>
<td>pVLc v</td>
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<tr>
<td></td>
<td>trSPm</td>
<td>Ventro-lateral protocerebrum</td>
<td>VLP</td>
<td>Antero-dorsal entry portal of the VLP</td>
<td>pVLP ad</td>
</tr>
<tr>
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<td>sPLPC</td>
<td>Anterior VLP</td>
<td>VLPa</td>
<td>Inferior dorso-lateral entry portal of the VLP</td>
<td>pVLP dl</td>
</tr>
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<td>Subellipsoid body commissure</td>
<td>SuEC</td>
<td>Posterior VLP</td>
<td>VLPp</td>
<td>Superior dorso-lateral entry portal of the VLP</td>
</tr>
<tr>
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<td>Superior arch commissure</td>
<td>SAC</td>
<td>Ventro-medial cerebrum</td>
<td>VMC</td>
<td>Dorso-medial entry portal of the VLP</td>
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<td>Subesophageal-protocerebral system</td>
<td>SPS</td>
<td>Anterior VMC</td>
<td>VMCa</td>
<td>Ventro-lateral entry portal of the VLP</td>
</tr>
<tr>
<td></td>
<td>Supraellipsoid body commissure</td>
<td>SEC</td>
<td>Infracommissural VMC</td>
<td>VMCi</td>
<td>Ventro-medial entry portal of the VLP</td>
</tr>
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<td>Longitudinal ventral fascicle</td>
<td>IoV</td>
<td>Postcommissural/Posterior VMC</td>
<td>VMCpo</td>
<td>Entry portal of the VMCpo</td>
</tr>
<tr>
<td></td>
<td>Intermediate IoV</td>
<td>IoVI</td>
<td>Precommissural VMC</td>
<td>VMCpr</td>
<td></td>
</tr>
<tr>
<td>Structure</td>
<td>Abbreviation</td>
<td>Description</td>
<td>Type</td>
<td></td>
<td></td>
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<td>-------------------------------</td>
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<td></td>
<td></td>
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<tr>
<td>Lateral loV</td>
<td>loVL</td>
<td>Supracommissural VMC</td>
<td>VMCs</td>
<td></td>
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<td>Medial loV</td>
<td>loVM</td>
<td>Ventro-lateral protocerebrum</td>
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<td>Posterior-lateral loV</td>
<td>loVP</td>
<td>Anterior VLP</td>
<td>VLPa</td>
<td></td>
<td></td>
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<tr>
<td>Vertical VLP tract</td>
<td>vVLPT</td>
<td>Posterior VLP</td>
<td>VLPp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vertical tract of the SLP</td>
<td>vSLPT</td>
<td></td>
<td></td>
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Fig. 2. Secondary lineages during metamorphosis. (A) Cell body clusters (CC) of lineages evolve from a columnar shape to a flattened shape in the adult due to a thickening of the cortex, but the general morphology of the SAT does not change dramatically (B) and (C). Z-projections of a MARM clone of the DPL1/1 (CC3) lineage at the 13 larval stage (B) and the adult stage (C) The SAT of this lineage extends along a crescent-shaped trajectory around the anterior tip of the superior lateral protocerebrum (SLP). In the adult, terminal arbors (TA) of DPL1/1 and several other lineages result in a growth of this compartment while the SAT remains relatively unchanged. General changes include movement of the cell clusters (CC) from a more dorsal position to a more lateral position, decreases in cell number (most likely due to cell death) flattening of the CC, and elongation of the SAT crescent to extend around the ventral anterior surface of the SLP. (D) and (E) 3D and 2D projections of DPL1/1 represented by MARM neuronal clones induced during larval development. (D) Confocal sections of adult brain hemispheres (anterior leaf) double-labeled with N-Cadherin (purple, neurite) and BrdU (green, cortex). Illustrating variations in the diameter of the cortex (CC) at different locations. Small arrow points to a dorsal region where the cortex is thick; large arrow points to a region with thick cortex in the crescent formed between the Abramian lobe (AL), lateral accessory lobe (LAL); anterior optic tubercles (AOT); mamillo-basal body (MBB), and anterior ventral lateral protocerebrum (VLP) (E) (E)-(H) Z-projections of confocal sections of MP4/2/8-labeled brains (P4, P3, P4, adult) show the cell body clusters of DPL1/1 (CC 33). In addition, the distance between DPL1/1 and an adjacent lineage, DLPL1 (D42) increases as a result of the growth of the SLP compartment compared to the location of R33 and D42. From (E) to (H). Scale bars: 10 μm in (C)-(H) and 25 μm in (B) and (D).

Fig. 3. Hemilineage cell clusters and SAT neurite entry points migrate away from each other during metamorphosis. (A) Cartoons depiction of the behavior seen in secondary lineages containing hemilineages. Hemilineage cell body clusters (CC) shown as spheres and their corresponding axon tracts initially form adjacent to one another as seen at the late larval stage (L3). By adulthood, many hemilineages completely separate (CCs and HSATs) to form morphologically distinct elements (generically termed as HLa and HLb, shown in orange and green, respectively). The extent to which hemilineages migrate apart varies between lineages. (B)-(G) Metaphasemorphosis of the hemilineages of the DLPL1/2/3 secondary lineages (B) and (C) are 3D projections of confocal sections of single brain hemispheres containing gap-labeled DLPL3/2 neurite clones, induced in the early larval period and fixed in the late larval (B) or adult (C). Neurite (in purple) is labeled by N-Cadherin (D)-(C) Z-projections of contiguous confocal sections of BP1060/BP104-labeled brains (BP1060 in (B)-(E); BP104 in (F) and (G)). Confocal stacks used in (B)-(G) were digitally rotated 90° to show the DLPL3/2 lineages from a lateral view. (E) (E) At the 13 and early proplab stage, DLPL1/2 appears as a pair of cell clusters (CC) whose axons come very close to each other and form a single SAT. This SAT splits into a posterior ventral (HSATp) and an anterior dorsal (HSATd). These hemilineage tracts extend around the dorsal and lateral surfaces of the growing superior lateral protocerebrum (SLP) compartment during metamorphosis. (E)-(G) As the SLP grows (I), P4 (F), P4 (G): (C); adult), the hemilineage clusters HLa and HLb of the DLPL3/2 lineage move away from each other. From P4 onward the clusters and HSATs are completely separated. Scale bars: 50 μm in (B)-(G).
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Column A: lineage names based on topology (Pereanu and Hartenstein, 2006). Shading indicates paired lineages with common tract. For lineage pairs shaded lightly, different MASCM clones were identified (see accompanying paper by Wong et al., 2013); dark shading indicates pairs for which only a single type of clone was found. B: Number identifying lineage-associated tracts (SATs). C: Tracts; in lineages with multiple heterogeneous tracts or sublineage tracts, these are individually listed (e.g. dorsal heminegative tract of BI4k is identified as “Sd”, ventral heminegative tract as “Sv”). C: Markers for lineages. References: 1 reviewed in Spindler and Hartenstein (2010). 2 Ute et al. (2011). 3 Lichtenstein et al. (2008). 4 Weisse et al. (2008). D: Entry portal of lineage-associated tracts (for abbreviations, see Table 1). E: Separation of lineages during metamorphosis. Lower case “s” indicates that heminegative clusters and entry portals remain adjacent; lower case “v” indicates that heminegative clusters separate; capital “S” stands for extensive shift of one or both lineages (separation of clusters > 20 µm in adult). F: Neuroep fascicle joined by lineage-associated tract. For abbreviations of fascicle names, see Table 1. “S” indicates that tract does not form part of any designated fascicle. G: Traceability of lineage-associated tracts in Bp104-labeled adult brain specimen. First letter refers to neuroript entry point, second letter represents proximal tract (< 20 µm away from entry point), third letter distal tract. Some tracts branch off another tract (e.g., BAmv/1#5dfn branches off #15a); in these cases, letter representing neuroript entry point is omitted. In cases where lineage associated tract is short (e.g. BA1m/10), third letter indicating distal tract is omitted. “S” stands for “solid visible”, “V” for “faint visible”, “O” for “not visible”. “T” signifies that tract forms part of fascicle in which it cannot be distinguished from other components. H: Commurine joined by lineage-associated tract. For abbreviations, see Table 1. In cases where distal tract is not visible in adult brain (e.g. iBAK/#5s), entry into commurine is inferred from earlier, pupal specimens.

rather, for many lineages, it decreases, due to cell death (Bolek and Truman, 1987a; Jang and Reichert, 2012; Kumar et al., 2009b). As a result, the brain cortex becomes thinner and the clusters formed by individual lineages change in shape from radially-oriented “cyinders” to horizontally-flattened “plates” (Fig. 1A-C, e.g., DPL1.1, #33). Depending on their position, some lineages are affected more than others by this flattening process. The cortex of the adult brain varies in diameter: it is thick at some locations where two outwardsbulging compartments meet and deep “crevices” filled with neural cell bodies are formed (Fig. 2D, large arrow) or it is very thin or absent over the convexity of many different compartments (Fig. 2D, small arrow). A general morphological change is that the increase in the volume causes lineage entry points and SATs to move away from each other (shown for DPL1.1, #33; DPLd, #42 in Fig. 2E–H). However, the position of most lineages relative to each other remains constant, which is the prerequisite for following SATs throughout metamorphosis. Two processes, the separation of parts of lineages (presumably heminegatives) and the extension of additional fiber bundles, complicate the issue of identifying SATs during pupal stages for a number of lineages. In the late larva, the cell body clusters of heminegatives and the entry points of their HSATs are directly adjacent. During metamorphosis, heminegatives are drawn apart to varying extent. In most cases, they remain dose; in a few cases, they become far removed from each other (Fig. 3A, e.g., DPL1/2/3). The example shown in Fig. 3B–G is the paired lineage DPL1/2/3, whose HSATs at the larval and early pupal stages enter together at the dorsal-posterior neuropil surface (Fig. 3B and D). During the course of metamorphosis (Fig. 3E and F), one heminegative remains posteriorly, the other one moves anteriorly, between two separate cell body clusters and two distinct entry points in the adult (Fig. 3C and G). This extreme separation of heminegatives typically occurring between P12 and P40, affects several other lineages as well (see Table 1).

Development of nascent fiber bundles from a main SAT is the second mechanism by which the overall SAT structure of lineages is altered. As a rule, most lineages have fully extended their SAT (or HSATs/SSATs) by the late larval stage. For example, lineages of antennal lobe projection neurons, whose cell bodies are located in the antennal ventral brain close to the antennal lobe, extend their axons far posterior to the calyx (Das et al., 2013; Pereanu and Hartenstein, 2006). During pupal development, terminal arborizations sprout from these fiber bundles and accumulate in the animal lobe and calyx/lateral horn. However, a number of lineages deviate slightly in that their SATs/HSATs acquire one or more major side branches, typically around 24–48 h of pupal development (P24–P48). This is shown in Fig. 4 for BAmv1, which in the late larva forms a dorsally-directed and a posteriorly-directed HSAT (SAMT and HSAT, Fig. 4B and D). Beginning around P24, the dorsal HSAT emits a laterally-directed branch (SAMT, data not shown for P30). By P32, the aforementioned branching for the BAmv1 becomes more apparent (Fig. 4E), where the SATp reaches the VLPa compartment. The terminal arborization of SATp into the VLPa compartment is also observed in the adult stage (Fig. 4C and F). The most likely explanation is that branches added during the pupal period are formed by the axons belonging to a group of late-born neurons. In the late larva, these cells would not yet have extended an axon contributing to the larval SAT. When they extend their axons in the pupa, these fibers might not all follow the pre-existing larval SAT, but establish a novel trajectory (SATp instead). Table 1 lists lineages forming prominent SAT branches during metamorphosis.

The pattern of fiber bundles in the brain neuropil

In the following presentation of lineages, SATs will be assigned to anatomically defined systems of fiber bundles (fascicles) in the brain neuropil. Fascicles are easily distinguished in the context of commonly used synaptic markers (e.g. Bruchpilot, nC82; N-Cadherin, NCad; Syntaxin, 8C3) which label most neuropil regions in the brain because they appear as domains of low signal, since synapses are scarce or absent in fascicles. Components of most adult fascicles can also be positively labeled by BP104 (this work). The most prominent fascicles can be generally grouped into longitudinal, transverse, and vertical bundles, which are based on the cardinal axis they travel along. Most of these bundles extend along the surface of the inferior protocerebrum, which is the brain domain surrounding the peduncle and lobes of the mushroom body (Pereanu et al., 2010, Fig. 5). For a more comprehensive description of fascicles and neuropil compartments, visit our website, the Drosophila Brain Lineage Atlas: https://www.mcb. ucla.edu/Research/Hartenstein/dbla/. Along the boundary between superior and inferior protocerebrum one further distinguishes a lateral and medial longitudinal superior fascicle (lO5L and iO5M, respectively; Fig. 5A–E; for alphabetical list of abbreviations of fascicles and compartments, see Table 2). The lO5M can be subdivided into an anterior component, iO5Ma (Fig. 5A, B and E) and posterior component, iO5Mp (Fig. 5C, D and E). Among the transverse fascicles, we distinguish an anterior, intermediate, and
posterior superior transverse fascicle (trsA, trsI, and trsP, respectively; Fig. 5B-E). More ventral and anterior is the lateral ellipsoid fascicle (lEf, lEPf; Fig. 5A) that passes obliquely underneath the mushroom body medial lobe (Fig. 5A and E) and connects to the central complex. Fiber bundles entering the central complex from posterior form the medial and dorsolateral roots of the fan-shaped body (mFB, dFB), as well as part of the medial equatorial fascicle (MEF; Fig. 5D and E; see below).

Longitudinal fascicles extending at the ventral surface of the inferior protocerebrum are the medial equatorial fascicle (MEF), lateral equatorial fascicle (lEF), and posterior lateral fascicle (PLF; Fig. 5C, D, and F). The lEF is subdivided into anteriorly- and posteriorly-directed tracts, lEFa and lEPf (Fig. 5A-C, F, and C-D, respectively). Further ventral is the ventral longitudinal fascicle (vLoV). Anteriorly, this massive fiber system has three components: the medial, intermediate, and lateral LoV (mLoV, iLoV, oLoV, respectively; Fig. 5A-C and F). All three components converge and form a conspicuous confluence of fibers in the middle of the ventral cerebrum (Fig. 5B and F; white and black arrowheads), the ventral fibrous center (VFC). Beyond this confluence, the ventromedial fascicle continues and passes posterior-medially into the cervical connective (CC) that joins the brain with the thoracic ganglia (Fig. 5D and F). A more laterally-located fascicle, the postero-lateral component of the LoV (oLoV), moves nearly straight posterior, ending near to the posterior neuropil surface (Fig. 5C, D, and F).

The conspicuous fiber systems that connect ventral and dorsal regions of the brain are the medial antennal lobe tract (mALT), the median bundle (MBdL), and the central descending protocerebral tract (dCP). The mALT primarily carries ascending fibers from the antennal lobe, travels dorso-posteriory along the central complex, and turns laterally towards the calyx and lateral horn (Fig. 5A-E). The MBdL contains numerous ascending and descending fibers connecting the superior medial protocerebrum (SMIP) with the subesophageal ganglion (SEG) and tritocerebrum (Fig. 5A). The dCP arises in the superior protocerebrum, passes the peduncle medially, and aims for the ventro-medial cerebrum (VMC) and SEG (Fig. 5B and E).

Bundles of commissural fibers interconnected the two brain hemispheres are grouped around the central complex. Dorsally, one can distinguish four main commissures, including (from anterior to posterior; for nomenclature see Strausfeld, 1976) the anterior-dorsal commissure (ADC, dorsal of the medial lobe of the mushroom body; Fig. 5A); the fronto-dorsal commissure (FDC, between the medial lobe and ellipsoid body; not shown); the supra-ellipsoid body commissure (SEBC, dorsal of the ellipsoid body; Fig. 5A and E); the superior arch commissure (SAC, dorsal of the fan-shaped body; Fig. 5B and E); and the superior commissure of the postero-lateral protocerebrum (SLPC, dorso-posterior of the fan-shaped body; Fig. 5D and E). Commissures passing ventral of the central complex are (from anterior to posterior) the antennal lobe commissure (ALC, ventral of the medial lobe; Fig. 5A and F), the commissure of the lateral accessory lobe and sub-ellipsoid commissure (LAC, and SaEC, ventral of the ellipsoid body; Fig. 5A and F), the great commissure (GC, ventral of the fan-shaped body; Fig. 5C and F), and the posterior commissure of the postero-lateral protocerebrum (pPLPC; Fig. 5D and F).

Several shorter fiber bundles entering the center of neuropil compartments (rather than extending along compartment boundaries) can be distinguished. As points of reference in this and the accompanying paper (Wong et al., 2013) are the vertical tract of the superior lateral protocerebrum (sLPT), penetrates the SI from antero-dorsal; Fig. 5B), the vertical posterior tract (VP), projecting between the lateral horn and posterior lateral protocerebrum (not shown), the vertical tract of the ventral-lateral protocerebrum (vLPT), enters the VLPs from ventral; Fig. 5B), and the horizontal tract of the ventral-lateral protocerebrum (hLPT), enters the VLPs from lateral; Fig. 5A).

Classification of lineages

In the previously published map of secondary lineages a nomenclature based on topology was introduced (Cardona et al., 2010b; Dumstrei et al., 2003a; Pereanu and Hartenstein, 2006). Using the easily identifiable mushroom body and antennal lobe as points of reference, twelve groups were defined, including the mushroom body (Fig. 6). Groups BA (basal anterior), DAL (dorsal anterior lateral), and DAM (dorsal anterior medial) have entry points at the anterior brain surface. BA lineages enter in close proximity to the antennal lobe (blue arrow in Fig. 6A; antennal lobe indicated by red “A” in Fig. 6B-C); the DAL lineage enters anterior and lateral of the mushroom body vertical lobe (purple arrow in Fig. 6A) shown in shades of purple in Fig. 6B); tip of vertical lobe indicated by red “V” in Fig. 6B-C; and DAM lineages enter anterior and medial of the mushroom body vertical
lobe (yellow arrow in Fig. 6A; shown in shades of yellow in Fig. 6B–G). SAT entry-points of the groups DPL (dorsal posterior lateral) and DPM (dorsal posterior medial) are to be found at the dorsal brain surface. DPL is postero-lateral of the vertical lobe and antero-lateral of mushroom body calyx (DPL; turquoise arrow in Fig. 6A; shown in shades of cyan-turquoise in Fig. 6D–I; calyx indicated by red “C” in Fig. 5D–I); DPM is postero-medial of the vertical lobe and medial of the calyx (DPM; orange arrow in Fig. 6A; shown in shades of orange in 6D–I). The four lineages producing the mushroom body (MB), as well as CP (central posterior) and CM (central medial) lineages, enter at the posterior brain surface; CPs are located ventro-lateral of the mushroom body calyx (maroon arrow in Fig. 6A; maroon in Fig. 6H–I); and CMs ventro-medial of this structure (magenta arrow in Fig. 6A; magenta in Fig. 6H–I). Finally, the BL (basal-lateral) lineages converge on the lateral brain surface, surrounding the broad connection between the optic lobe and central brain (green arrows in 6A; shown in shades of green in 6B–I; optic lobe indicated by red “O” in Fig. 6B–I). BLA lineages enter from anterior (Fig. 6B–G), BLD lineages enter from dorsal (Fig. 6B–I), the BI2 group enters from posterior (Fig. 6F–I), and BIL lineages enter from ventral (6D–I). Most of these main groups were further subdivided into smaller units of lineages entering the neuropil densely together, in the case of the BA lineage group, BAI, BAlp, or BAm (Fig. 6B–G). As evident from Fig. 6, the position of SAT entry-points in relationship to each other and to the neuropil compartments is very similar in the larva and adult, if one takes into account the previously discussed growth of certain compartments, in particular the antennal lobe, optic lobe, and the superior protocerebrum, that occurs during metamorphosis.
In the remaining sections of this paper and in the accompanying paper (Wong et al., 2013), the above topological classification will be used to order the description of secondary lineages and their projections (Figs. 7–13). In the first set of figures, we describe the axonal projections of the adult secondary lineages, starting with lineages entering the anterior brain surface (BA; Fig. 7; DAL and DAM; Fig. 9), followed by those of the dorsal surface (DPL; Fig. 10), posterior surface (DPM, CM, CP; Fig. 12), and finally, lateral surface (BLA, DLD, BLP, BLV; Fig. 13). In each of these figures, the left column of panels shows projections of frontal sections of left brain hemispheres, ordered from
posterior (top) to anterior (bottom). Each z-projection represents a brain slice of approximately 15-20 μm thickness in which segments of SALs, labeled by BP104, are visible. The panels on the right hand side of Figs. 7, 9, 10, 12, and 13 represent semi-schematic 3D maps of the group(s) of lineages shown in the corresponding figure. Lineages are represented as a sphere (location of SAT entry point into neuropil) and line (SAT trajectory in neuropil). In panels at the bottom, neuropil entry points are projected on a 3D volume rendering of the neuropil surface, which illustrates the position of the lineage in relation to prominent surface landmarks (e.g. antennal lobe, anterior optic tubercle, mushroom body). The large right panel at the top schematically shows the trajectories of SALs in the neuropil. A second set of figures (Figs. 8 and 11) document SALs of the eleven lineage groups at different developmental stages, including larvae (LA), pupa (P12, P24, P32, P48), and adult. Fig. 8 shows lineages located in the anterior part of the brain while Fig. 11 shows posterior lineages. To complement this paper as well as the accompanying paper (Wong et al., 2013), we have developed an online tutorial, the Drosophila Brain Lineage Atlas, which provides a three-dimensional description of adult secondary lineages (highlights neuropil entry points, SAT trajectories, and axonal projection patterns): https://www.ncd.biu.ac.il/Research/Hartenstein/ atlas.

The BA lineages (#1–17)

The BA group comprises lineages associated with the ventral brain compartments (antennal lobe, antennomechanosensory and motor center, ventro-medial cerebrum, ventro-lateral cerebrum, lateral accessory lobe). BA cell body clusters are grouped around the antennal lobe (AL). Four lineages, BAAla1–4 (#1–4), form the antero-lateral BA subgroup whose SALs enter the neuropil in the niche formed between the ventral AL and antennomechanosensory and motor center (AMMC), the compartment receiving input from the auditory Johnston’s organ and other mechanosensory bristles of the head (entry portal pAL vl; Figs. 7A–C; BAIC (#5d)/v; corresponding to the group of neurons called the lateral cluster in the literature, and labeled by the marker GMR4a4-GAL4; Lai et al., 2008) enters the lateral surface of the AL (pAL vl; Figs. 7A, B and C). SATs of the postero-lateral BA group [BAlp-1 (#6–9), BAlp-10] reach the neuropil further posteriorly, in the niche formed between the AL, ventral lateral protocerebrum (VLP) and AMMC (pVLP vmp; Figs. 7A–C). The pair of medial ascending lineages, BAm1 and 2 (#11–12), are located ventrally-medially of the AL and project their SALs dorsally into the median bundle (pAL vm; Figs. 7A–C; BAm1 and 2 (#13–14; Figs. 7A–C) are located dorsally of the AL. The two separate hemlineage clusters of BAm1 flank the mushroom body medial lobe; the dorsal HSAT (#13d) enters dorsally of the medial lobe (pVM vl; Figs. 7A and B), the ventral HSAT (#13v) passes between the medial lobe and antennal lobe (pAL vl; Figs. 7A). The SAL entry point of BAm2 is obscured by the fibers of the median bundle and antennal nerve in the adult brain. BAm3 (15–17) form a compact group of SALs at the dorso-lateral surface of the AL in the larva. Whereas BAm3 (the entry point into the AL is also obscured by antennal nerve afferents) maintain this position (entry point pAL d; Figs. 7A), the entry points of BAm1 and BAm2 come to lie at the ventral surface of the adult AL (pAL v; Figs. 7A–C; see below).

Four BA lineages, BAAla1 (#1, labeled by per-Gal4; Spindler and Hartenstein, 2010; Spindler and Hartenstein, 2011), BACk (#5d, dorsal hemelineage; labeled by GMR4a4-GAL4; Stocker et al., 1997), BAAP4 (#9), and BAm3 (#13, labeled by GMR4a4-GAL4; Stocker et al., 1997) include all of the projection neurons connecting the AL and superior protocerebrum (calyx and lateral horn; Das et al., 2013; Lai et al., 2008) via the antennal lobe tracts (ALT; Fig. 7G); for a detailed description of the distinct entry points of these lineages into the AL, see Das et al., 2013). The ventral HSAT of BAlc forms the intermediate bundle of the ioV fascicle (ioV) that extends posteriorly into the inferior ventro-lateral cerebrum (VLC; #5v in Fig. 7A, D and C). BAm1 and BAm2 (#13–14) have commissural tracts. The dorsal HSAT of BAm1 (#13d) projects medially directly behind the medial lobe and crosses in the fronto-dorsal commissure. Shortly after its entry point (#13d in Fig. 7A), the trajectory of the HSAT becomes obscured by the dense labeling of the mushroom body medial lobe; the tract is visible until mid-pupal stages (Fig. 8A). The ventral HSAT of BAm1 projects diagonally through the AL to cross in the antennal lobe commissure (#13v in Fig. 7A, D and C). BAm1 (marked by per-Gal4; Spindler and Hartenstein, 2010; Spindler and Hartenstein, 2011) and BAm2 form the ioV fascicle that passes underneath the AL and extends posteriorly through the ventro-medial cerebrum (VM; #15–16 in Fig. 7A, D and C). A major dorsal branch of BAm1 (BAm1v; #15d) curves dorsally towards the central complex, forming the posterior portion of the lateral ellipsoidal fascicle (Lep; Fig. 7D and G). Tracts of BAlp2 and BAlp3 form the lateral ioV fascicle (ioV; #7–8 in Fig. 7A, D and C). The BAlp2 tract gives off a dorsal branch that extends along the lateral surface of the lateral accessory lobe (LAB; #7d in Fig. 7D).
BAla3 (#3, Fig. 7A; marked by et-Gal4; Kumar et al., 2009a), BAla4, BAlp1, and BAlv have single SATs that enter from a position lateral of the AL. BAla3, BAla1, and BAlp1 project medially towards the ventro-medial cerebrum (VMC), with BAlp1 crossing the IoVM fascicle at its dorsal surface (#6 in Fig. 7D), and BAla3-4 crosses the medial IoV (IoVM) at its ventral surface (#3 in Fig. 7D). BAlv has a short SAT that contacts the inferior ventro-lateral cerebrum from ventral (VLCi; #10 in Fig. 7D and G).

Of the BA lineages, nine (BA1c, BAlp1-4, BAlv, BAmd1, and BArm1-2) can be individually followed from their point of entry deep into the neuropil throughout metamorphosis (Fig. 8; Fig. S1). Six BA lineages (BAla1-2, BAla3-4, BArms1-2) form pairs whose
SATs are closely associated. The paired SATs of these lineages (indicated by the number corresponding to the first lineage of the pair followed by an asterisk; for example, "#3" for the pair "BalAa-4"; Fig. 7D) can also be followed throughout metamorphosis (Fig. 8), but lineages within each pair are distinguishable only on the basis of clones or genetic markers. The points of entry of two BA lineages (BaAm-3, #17; BaAm-2, #14) become indistinct at later pupal stages because of strong labeling of anatomical features (Fig. 8). BaAm-3, marked by the GHA46-Gal4 driver (Stocker et al., 1997), enters the AL from dorsal (Fig. 7B-G, Fig. 8A), BaAm-2 (#14), clearly visible until P24, enters near the midline in between the two brain hemispheres (Fig. 8A-B, C-D and E-F); the SAT joins the ventral HSAT of BAm1, crossing in the antennal lobe commissure (ALC). In addition, BaAm-2 has an ipsilateral branch that is fairly thin in the larva and early pupa, but increases in diameter and forms a visible tract in the late pupa and adult stages (#14) in Fig. 7D, Fig. 8 and I.

Changes in the position of BA lineages are mainly brought about by the general expansion of the anterior brain neuropil compartments, notably the AL, AMMC, and anterior ventrolateral protocerebrum (VLPs; see panels of left column of Fig. 8; Fig. 8A, C and E). The AMMC formed along the mechanosensory component of the antennal nerve during metamorphosis, has no larval counterpart; it grows and expands in a region between the BAp lineages (dorsolateral of the AMMC) and BAl lineages (ventro-medial of the AMMC) starting around P32 (Fig. 8C, I and K). Furthermore, the hemilines of clusters of BAlc (#5, Fig. 5C; white arrows) and BAm1d (#13, Fig. 5C; white arrows) and their HSAT entry points move slightly apart. However, the relative positions of these and all other BA SAT entry points remain constant; with the notable exception of all the BaAm1 and BaAm2 (#15–16) lineages which undergo an interesting switch in position relative to the AL (compare the yellow and orange squares in the top two panels of Fig. 5A). In the larva, the SATs of BaAm1-2 enter dorsal of the AL (Fig. 8A, blue arrow); in the adult, they are ventral (Fig. 8K, blue arrow). This change occurs as a result of the metamorphic decay of the larval AL (AL(lar)) and the formation of the adult AL (AL(ad)). The AL(ad) primordium is visible in the larva as a small domain of dense Ncad-labeled at the dorsal edge of the AL(lar) (Fig. 8A). The AL(ad) domain expands throughout papal development (Fig. 8C and E) and acquires a glomerular texture by P32 (Fig. 8C). At the same time, the glomerular composition of the AL(lar) decays and becomes inviable by P32. The neuropil entry point of BaAm1 and 2 in the larva is position dorsally of the AL(lar) adjacent to the small AL(ad) primordium (Fig. 8A). As the AL(ad) primordium grows (P12, P34), it pushes the BAm1-2 entry point ventro-midially (Fig. 8C and E, blue arrow). Note that at this transitional stage, the entry point is still dorsal of the decaying AL(lar) (Fig. 8C). Finally, by P48, the BAm1-2 entry point ventral of the AL(ad).

DAL lineages (#18–32)
DAL lineages occupy a posterior dorsal of the BA group, surrounding the spur (SP) and lobes of the mushroom body (medial lobe ML; ventral lobes VL; see Table 2). Neurons of the first subgroup, DALCl and DALCe2 (#18–19), encircle the anterior optic tubercle (AOTU, a distinct compartment receiving input from the optic lobe via the anterior optic tract; Strausfeld, 1976; Fig. 9B and C). DALCl enters the neuropil at the junction between the mushroom body spur (SP) and vertical lobe (VL) (entry ports at pSP d and pSP v; Fig. 9A and G). The second subgroup, DALCl-2 and DALC (#20–22), is located dorso-medial of the DALC lineages (Fig. 9A–C); its entry into the neuropil close adjacent to the DALC lineages, turning two entry points that flank the base of the V1 medially and laterally (pVM v and pVL v; Fig. 9C and G). The third of the third subgroup, DALCl3-1 (#25–27), located ventrally of the ML, pass underneath the SP and ML (pSP v; Fig. 9A–D and G). Further laterally, DALCl and DALC (#23–24) enter the anterior surface of the ventro-lateral protocerebrum (VLP), lateral adjacent to the SP (pVLP dm; Fig. 9A–D).

DALCl1 and DALCl2 each have two hemilines whose diverging HSATs, in a “pincer-like” manner, enclose the SP (#18v and #18v’; Fig. 9A, Fig. 5B and D). The ventral HSAT of DALCl1/2 pass underneath the SP and continue medially. Ventral DALCl1 (#18v) crosses the midline in the subellipsoid commissure (SUFC) ventral DALCl2 (#19v) joins the lateral ellipsoid fascicle (LE), along with DALv2 and DALv3 (see below), and projects to the central complex (#19v in Fig. 9A, D, E and G). The dorsal HSATs of both DALCl lineages curve over the dorsal surface of the SP and peduncle (P) and project towards the central complex, lateral accessory lobe (LAL), and superior medial protocerebrum (SMPC; #18v’ in Fig. 9A, D, E and G).

DALClm1 and DALClm2 have two hemilines forming two paired HSATs. The medial HSAT passes behind the medial lobe into the medio-medial commissure, following the dorsal HSAT of BAm1d (RMc, #20rn in Fig. 9A and G). As in the case of BAm1d (#13d), the medial HSAT of DALClm1 that passes through the ML is clearly demarcated in the pupa, however it is indistinct in adult brains (Fig. 8C and F). The lateral HSATs of DALClm1-2 (#20rn), accompanied by the single SAT of DALClm2 (#22), pass through the ethmoid formed by the VL and peduncle before turning ventrally (Fig. 9A, D–E, G, Fig. 5B). These tracts constitute the descending central protocerebral tract (dCPT) that projects towards the ventral brain, including the VM, VLc, and SEC (Perez-Franco et al., 2008). DALCl1 has a prominent SAT that projects straight posterior in between the ventro-lateral protocerebrum (VLP) and lateral accessory lobe (LAL) compartments (#25 in Fig. 9A, D and G), forming the anterior component of the lateral equatorial fascicle (LEF; Perez-Franco et al., 2010). The LEF bifurcates more posteriorly and enters the ganglionic mass (GM, Fig. 9F and G; Fig. 5C and D). DALv2 (marked by EB1-Gal4 and per-Gal4; Spindler and Hartenstein, 2010, 2011) and DALv3 (marked by en-Gal4; Kumar et al., 2009a) send their SAT dorso-medially, forming the anterior component of the lateral ellipsoid fascicle (LEf) that passes underneath the ML towards the central complex (#25n in Fig. 9A, D and G; Fig. 5D–E). DALv2 projects into the ellipsoid body, forming the I-neurons of this compartment; DALv3 is branched, crossing the ellipsoid body dorsally and ventrally in the superalloblissoid commissure (SEF; #27d) and subellipsoid commissure (SUFC; #27v, Fig. 9D; SD2-E), respectively.

DALCl1 and DALCl2 are located laterally of the SP. DALCl2 projects a short SAT into the anterior part of the anterior ventro-lateral protocerebrum (VLPs; #24 in Fig. 9A and G); DALCl1 has a long tract that passes posteriorly (#25 in Fig. 5B–D) and, after giving off a branch ventrally towards the posterior lateral protocerebrum (PLP), makes a 180 degree turn back towards anterior to reach the anterior optic tubercle (AOTU). The recurrent leg of the DALCl1 SAT can be followed in the larva and early pupa (not shown), but is indistinct in the adult.

Changes in DAL lineage topology during metamorphosis occur when the emerging AOTU, which has no larval counterpart, pushes in between DALCl1–2 (#18–19), lateral and DALClm1–2 (#20–21), medially; (Fig. 8C–G, E–E’ and G–G’). During this period, HSATs of these two pairs move slightly apart (arrows in Fig. 5D–E). These changes aside, all DAL lineages maintain their relative position. Most of the SATs or HSATs of the DAL group, including the ventral HSAT of DALCl1/2 and the SATs of DALCl, DALCe1, and DALCe2 (#23, #25), can be individually followed throughout development (Fig. 8). The dorsal HSATs of DALCl1/2 (#18rn), as well as both HSAT of DALClm1/2 (#20rn, 2010v) form pairs; DALv2 and DALv3 have tracts that are close together and cannot be separated (#25n). Furthermore, these paired tracts become fairly indistinct in BF104-labeled brains of late pupae (>P48); as mentioned above, medial DALCl cannot be followed beyond its entry point into the neuropil of the adult brain.
Clusters and SAT entry points of all five DAM lineages (#28–32) are located close to the brain midline, medial of the VL (entry portal: pVL: d.r.; Fig. 9B–D). All DAM lineages have single SATs: tracts of DAMd2–3 (#29–30) and DAMv1–2 (#31–32) form pairs whose SATs cannot be separated from each other in the neuropil. DAM tracts can be clearly followed throughout pupal development (compare panels Fig. 8A, D, F, H, J and L; Fig. S2A–B). The DAMd1 SAT (#28) projects medially and crosses the midline in the anterior dorsal commissure (ADC, Fig. 9D and G). The paired DAMd2–3 tract (#29*) projects posteriorly, forming the thick isSM fascicle (Fig. 9D–G). The DAMv1–2 pair (#31*), located ventrally adjacent to DAMd2–3, forms a short tract that is directed dorso-posteriorly and terminates near the surface of the superior medial protocerebrum (SMP; Fig. 9D and G). The only developmental change affecting the DAM lineages is a dorsal shift in location, subsequent to the growth in volume of the ML and the surrounding anterior inferior protocerebrum (Ipa)/SMP compartments (compare panels Fig. 8D, H).

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Fig. 8. Larval to adult development of lineages of the anterior brain. Panels of each side of this split figure are arranged in three rows and three columns. Each row represents one stage, indicated at the top left corner (L3, A–B; P12, C–D; P24, E–F; P32, G–H; P48, I–J; Adult K–L). All panels show z-projections of contiguous, confocal sections of a brain hemisphere labeled with BP104 or BP104 and N-Cad, representing brain slits of 15–25 μm thickness. Z-projections of the first and second column (A–H; C–F; G–I; K–L) correspond to an anterior level (mushroom body lobes). Both BP104 labeling (secondary neurons, SATs and fascicles; green) and N-Cad labeling (neuropil; red) is shown in left panels; middle panels show BP104 labeling only (white, A–C, E–G, I–K). Panels of the right column (C–D, F–H; J, L) represent a "sub anterior" level (ellipsoid body/morphodorsal ellipsoid body). Compartmental visibility at the anterior neuropil surface are annotated (white lettering, panels of left column; see Table 2 for complete listing of abbreviations). SATs and SATs of individual lineages are annotated with a unique numerical identifier (see Table 1). Numbers follow by an asterisk indicate tracts formed from more than one SAT (typically two SATs) which cannot be followed separately. For example, “20*” stands for “20 and 21”. Lower case letters (‘a’, ‘b’, ‘c’, ‘d’, ‘e’, ‘f’, ‘g’, ‘h’), indicate SATs formed by individual hemilines within a particular lineage. The red circle in each panel marks the location of the peduncle. The blue arrow in (A), (C), and (E) marks the entry point for the SATs of Blum et al. Scale bar: 10 μm.
DPL lineages (33–50)

DPL lineages are clustered over the dorso-lateral brain surface. The lineage subgroups DPLa1-3, DPLam, and DPLd are located anteriorly in the crevice between the anterior optic tubercle (AOTU) and superior lateral protocerebrum (SLP). All other DPL subgroups are located more posteriorly, surrounding the mushroom body calyx (CA): dorso-medially (DPLa1-5), dorsally (DPLa1-6), and dorso-laterally (DPLa1-2, DPLpv) (Fig. 10A–C and F). SATs of DPLa1-3 (33–35) form a conspicuous, crescent shape bundle that defines the anterior transverse superior fascicle (trSA).

From the DPLa entry point, located at the lateral base of the SLP (ptSLP I; Fig. 10C–C'), this bundle curves ventro-medially, turns, and then continues dorso-medially to terminate superficially within the superior lateral protocerebrum compartment (SLP; Fig. 10A and G). A second, more ventral bundle curving around the peduncle at its ventral side branches off the trSA. According to the larval and adult data of DPLa lineages (Wong et al., 2013), one of them (DPLa1, #33) is restricted to the trSA, whereas two (DPLa2-3) contribute to both the trSA and a ventral branch (#34d* and #34d* in Fig. 10A and G, Fig. 5A–B). DPLam (36), marked by the e-Gal4 driver (Kumar et al., 2009a), is located mediolaterally adjacent to the DPLa lineages (Fig. 10A–C'). The DPLam SAT enters between the AOTU and SLP (ptSLP a; Fig. 10C'), projects straight postero-ventrally, through the center of the hemi-circle formed by the trSA fascicle (Fig. 10A) and terminates in the inferior protocerebrum (purple line in Fig. 10G), laterally adjacent to the peduncle (Fig. 10D and G, Fig. S2C–D). We call this projection the ventral tract of the superior lateral protocerebrum (vSLP; Fig. 10D). DPLd fibers (#42) enter laterally of the vertical lobe of the mushroom body (pVL dl; Fig. 10A–C*). DPLd projects two HSABs: one is directed medially (#42m), curving around the anterior surface of the VL and entering the anterior dorsal commissure (ADC in Fig. 10A and G); the second one (#42p) projects posterolaterally, forming an anterior component of the loSL fascicle (Fig. 10A, D and G; Fig. S2D–E).
Fig. 9. Trajectories of SATs formed by the DAL and DAM lineage groups. The composition of this figure follows the same plan explained for Fig. 1, with sets of z-projections (A), (B)–(F) illustrating segments of the SATs and fascicles at different anterior-posterior levels. Neuron surface views showing location of SAT entry points (B and C) and neuron cut-away diagrams depicting SAT trajectories (G). Note that some of the panels on the left ((E) and (F)) do not show the same z-projection as those depicted in Fig. 7 (e.g., F represents a level on slightly posterior to that shown in (E); (F) corresponds to the fan-shaped body/axial commissure level, see white arrows pointing out anterior-posterior levels of the z-projections). Panels B and C show anterior-lateral view of right hemisphere (B) and dorsal anterior-lateral view of both hemispheres (C). White lettering in C and G annotates neuropil compartments (left side of C) and SAT entry portals (right side of C); yellow lettering in G indicates fascicles (for alphabetical list of abbreviations, see Table 1). Scale bars: 25 μm ((A), (B)–(F)); 50 μm ((B) and (C)).
(#41) converge upon a point directly lateral of the conspicuous IsoSM fiber system (green lettering in Fig. 10E), which at this level contains numerous bundles of the DPM and CM lineages (see below). From this point, DPLc1 and DPLc5 curve in a crescent shaped path underneath the IsoSM (medial component of the transverse posterior fascicle of the superior protocerebrum, trSPm; Fig. 10E and G, Fig. 5A and B). The SAT of DPLc3 (#39) projects straight anteriorly, rather than medially as with DPLc1 or DPLc5 (Fig. 10E and G, Fig. 5A and B). DPLc5 has a second SAT (#41p in Fig. 11B and D; not distinguishable in BP106/BP104-labeled
Fig. 11. Larval-to-adult development of lineages of the posterior brain. The composition of this figure follows the same plan explained for Fig. 8, with panels of each side of this split figure arranged in three rows and three columns. Rows represent stages, indicated by the upper-left corner of A, C, E, G, I, and K. Projections of the first and second column (A, A'; C; C'; G; G'; I; I') correspond to a posterior level (mushroom body calyx, protocerebral bridge) where SADs approach the neuropil surface. Panels of the right column (B, D, F, H, J, L) represent a "subposterior" level (posterior surface of fan-shaped body/primordium of fan-shaped body). Compartments visible at the posterior neuropil surface are annotated (white lettering, panels of left column; see Table 2 for complete listing of abbreviations). SADs and HADs of individual lineages are annotated with a numerical identifier (see Table 1). Scale bar: 30 μm.
Fig. 12. Trajectories of SANs formed by the BPM, CM, and CP lineage groups. The composition of this figure follows the same plan explained for Fig. 7, with sets of z-projections (A, D, F) illustrating segments of the SANs and fascicles at different antero-posterior levels, neuropil surface views showing location of SAT entry points (B) and (C), and neuropil cut-away diagram depicting SAT trajectories (G). Position of A, D, and E along the antero-posterior axis is indicated by white arrows. Panel II shows posterior view of both brain hemispheres. C presents enlargement of posterior view of right hemisphere. White lettering on right side of B and G annotates neuropil compartments; hatched circles and white lettering on left side of B indicates SAT entry portals; yellow lettering in G indicates fascicles (for alphabetical list of abbreviations, see Table 2). Scale bars: 25 μm (A, D), 10 μm (B and C).

preparations past P24), which enters at the posterior brain surface and projects anteriorly as part of the LoSM system (see below), SATs of DPLc2 (#38) and DPLc4 (#40), located laterally adjacent to DPLc1/3/5 (#37, #39, #41), form a paired tract which extends ventrally and medially, curving around the LoSM parallel to, but slightly more ventro-posteriorly than the crescent formed by DPLc1/5 (Fig. 10E and G, Fig. 53A–B). DPLm1 (#46) and DPLm2 (#47) enter the posterior surface of the superior lateral protocerebrum, just dorsal of the calyx (ptSLP p, Fig. 10B and F). The SAT of DPLm1 projects anteriorly into the
SLP: DPLm2 turns laterally towards the lateral horn (LH; Fig. 10E and G; Fig. S3A and B). DPLm2 has a second tract (#44p) in Fig. 11D), no longer marked by BP14/BP106 after P12, which leaves the brain and projects to the ring gland.

SATs of DPL1-3 (#43–45) enter at the junction between the SLP and LH (psSLP pl; Fig. 10B and F). DPL2 and DPL3 form a pair with two hemlineages each. The posterior HSATs of DPL2-3 (#44p) are directed anteriorly, forming a thick bundle that constitutes the loSL fascicle (Fig. 10D–G, Fig. S4A). The anterior DPL2-3 hemlineages (#44p*) shift far anteriorly during metamorphosis (Fig. S8B, D, F, H, J and L); forming a paired HSAT that enters the SLP at its anterior surface close to DPLam (psSLP a), and projecting parallel to DPLam ventrally into the inferior protocerebrum (Fig. 10A, B, D, and G; Fig. S4A). DPL1 (#43) enters next to the posterior HSATs of DPL2-3.
(Fig. 10B), but extends medially, forming a thin fiber bundle, the tSPI, which converges upon the fiber tracts formed by the DPL group (Fig. 10D-G; Fig. 35A).

The last DPL subgroup, DPLp (#48-50), includes clusters of neurons lying at the posterior surface of the lateral horn, lateral of the calyx (PH; CA; Fig. 10E and F). DPLp1 and DPLp2 form a pair with two HSATs. One HSAT (#484m) enters via the entry portal ptCA I and projects medially, crossing over the peduncle and forming the most posterior of a dorsal set of commissures (pPf; Fig. 10B, E and G; Fig. 3C and D). The second HSAT (#484a) is a short, anteriorly directed tract that enters at the junction between the SLP and LH (pSLP p) and terminates in the LH (Fig. 10B, E and G; Fig. 3C and D). An additional fiber bundle (#48v*) that branches off the anterior tract projects ventrally along the posterior ventral fascicle (Fig. 10G). DPLp (#50) lies far more ventral, flanking the posterior surface of the posterior lateral protocerebrum (PLP; Fig. 10C and E-G). Its single SAT enters via the pPf portal and projects anteriorly as part of the fiber system called the posterior lateral fascicle (PLF; Fig. 10B, E and G; Fig. 3C and D). In the larva and early pupal stages, one can recognize a lateral branch of the DPLp SAT that projects toward the optic lobe (blue arrowhead in Fig. 11A; C and E).

Of the 18 DPL lineages, nine (DPLam, DPLd, DPLc, DPL3, DPLc5, DPLc1-2, DPLp1, DPLpv) can be individually followed throughout pupal development (Figs. 8 and 11). DPLa1-3 form a trident tract; DPLc2 and DPLc4, as well as DPLp-3 and DPLp-2 form paired tracts, all of which are clearly visible from larval to adult stages (Figs. 8 and 11). The cell body cluster location of DPL lineages is affected strongly by the expanding superior protocerebrum, which pushes many of the lineages farther posteriorly. Thus, DPLc1-2 are more anterior in the larva (Fig. 1A and B), at the level of the VL and end up anteriorly in the adult (Fig. 3A and B). Cell bodies of DPLpv move from a posterior-dorsal position in the larva to a ventro-posterior one in the adult (#50; Fig. 11A; C, E, F, G, H and K; Fig. 3C and D).

Four lineages, DPLc5, DPLm2, and DPLc-2-3 deserve special mention because of the separation of hemlineage cell bodies and their HSATs. In the case of DPLc5, one HSAT follows the other DPLc tracts medially, whereas the other HSAT, clearly visible only in the larva and early pupa (#41p; Fig. 11B and D) first extends ventro-posteriorly and then turns anteriorly into the loSM, following the general trajectory of DPL lineages (see below). For this reason, it was previously named “DPLm3” (Preanu and Hartenstein, 2006), but more aptly deserves the group designation DPLc, because it’s anterior HSAT (and its clone geometry; see accompanying paper by Wong et al., 2013) shares more commonalities with DPLc lineages. The DPLc5 hemlineage generating the posterior HSAT separates from the anterior hemlineage, moving far posteriorly and ventrally (see accompanying paper). The paired DPLc-2-3 also represent a case where hemlineage clusters become far removed from each other as discussed previously (see above).

Finally, in the case of DPLm2, two hemlineages also seem to exist, one of which loses expression of the Neuregulin protein. Aside from the short, laterally-directed tract that is clearly visible in the larva, pupa, and adult, a second, thinner tract (#47p) is visible only in the larva and early pupa (compare #47 to #47p in Fig. 11D). The DPLm2 HSAT extends ventro-medi ally, exits the brain, and projects to the ring gland. DPM lineages (#51-59)

Cell body clusters of the DPM group are situated at the dorso medial surface of the brain, medially of the calyx. We distinguish a more dorso-anterior (DPMm1-2, 53-54), ventro-posterior (DPMp1-2, 58-59), and lateral (DPMc1, 51; DPMc1-3, 55-57) subgroups (Fig. 12A-C). DPMp1-2 (55-56) form a pair whose SATs enter the neuropil medially adjacent to the calyx (ptCA m) and continue antero-dorsally following the loSM fascicle (Fig. 12A, B and D-F). DPMp3 (#57), located further ventrally, forms an SAT that enters via the conspicuous pFB v portal at the tip of the protocerebral bridge (PB) and projects along the MEF fascicle (Fig. 12A, B and D-F). DPMc1 (#53) and DPMp1-2 (#58-59) represent three of the large and fast-cycling type II lineages that have been previously described to innervate the central complex (Belle et al., 2008; Boone and Doe, 2008; Igorzina et al., 2008). Of the eight type II lineages, six of the dorsal-posteriorly located type II lineages (CM1, CM3, DPMm1 and DPMp1-2) are marked by expression of Dm14C-Gal4 (Jergini et al., 2008) and earmuff (91D1-Gal4; Bayraktar et al., 2010). There are no known markers for the remaining two lineages (CM3’ and DPMm3). CM1, DPMp1-2, and DPMm1-2 include sub-lineages whose main SATs, destined for the central complex (53b, 58b, 59b), characteristically enter the neuropil in contact with the protocerebral bridge (entry portals pPB dm and pFB dl respectively; Fig. 12B). As a result, the SATs of the dorsal-medial root and dorso-lateral roots of the fan-shaped body (mRFb, 53b; 58b; 59b) project antero-posteriorly into the neuropil of the fan-shaped body (FB), respectively (Fig. 12D-F; Fig. 54B-C; Preanu et al., 2010).

Additionally, DPMp2 gives off an SSAT (#59a) projecting more dorsally, as part of the loSM (Fig. 12D-F); DPMm1 has another SSAT (#58a) following the mAL anterior-posteriorly (Fig. 12F). DPMm1 has multiple other SSATs; the four most prominent ones are a tract joining the loSM (#53a), one crossing the posterior brain surface towards the contralateral hemisphere (#53d), and two (#53c, e) extending ventrally and anteriorly along the ventro-medial surface of the fan-shaped body (Fig. 12E). #53c turns ventrally into the ventro-medial commissure (VMC; Fig. 12E and F); #53e continues anteriorly (not shown). DPMm2 (#54) is located laterally adjacent to DPMm1 and has a single SAT that enters the superior medial protocerebrum vertically via the pFB d portal (Fig. 12B, D). The distal SAT of DPMm2, which expresses BP106 only until P12 (Fig. 11D), turns anteriorly, extends over the moi of the central complex, and crosses to the contralateral hemisphere in the anterior chiasm that lies immediately posterior of the median bundle (MBDch in Fig. 12F). DPM1 (#51), located next to DPMp13 (#57) as it enters the MEF (Fig. 12C), has a long descending SAT that projects toward the subesophageal ganglion (SEG; Fig. 12F). The distal SAT is visible with BP106 only until stage P22 (Fig. 11B and D).

Cell body clusters and SATs of all DPM lineages perform a shift ventro-posteriorly as the superior medial protocerebrum and inferior protocerebrum expand during metamorphosis (Fig. 11B, D, F, H, J and L; Fig. 54B-C). Of the eight DPM lineages, four (DPMm1-2, DPMp1-2) can be individually followed throughout metamorphosis (Fig. 11A-L). During the later stages of pupal development, axons formed by the three type II lineages (DPMm1, DPMp1-2) break up into multiple thinner, parallel bundles that flank the protocerebral bridge (PB; #53, 58, #59 in Fig. 11C-G). The paired tract formed by DPMp1-2 travels in the loSM fascicle and is also visible throughout development (#55* in Fig. 11A-L). The distal DPM1 and DPMp13 lose expression of BP104 Neuregulin during later pupal stages and can only be identified in the adult brain on the basis of GFP-labeled MAXCM clones. For all other DPM lineages, it is only possible to follow the SATs into the larger fascicle (e.g. loSM, MEF) to which they contribute; thus, a lineage trajectory can only followed when it is labeled by GFP (see accompanying paper by Wong et al., 2013).

CM lineages (#60-63)

CM lineages occupy the postero-medial brain cortex, ventromedially of the calyx (CA; Fig. 12A-C). Three CM lineages (CM1,
CM3, CM4 are large type II lineages (called DM6, DMS and DM4, respectively, in Bello et al., 2008). Each of these forms multiple sub-lineages, and possesses a complex array of sub-lineage tracts (SSATs). Only a subset of these, the "main" SSATs, can be followed using global markers like BP106 or BP104. CM1 (#60) forms two main SSATs; CM4 (#62) possesses three; and CM3 (#61) has four (Fig. 12F). One SSAT of each of these three lineages enters at a ventral level into the ventro-medial cerebrum (pV McPco) and projects forward in the loVp fascicle (#60v* in Fig. 12A, B, D and F). The CM3 SSAT enters at the tip of the protocerebral bridge (pPb b v) and projects straight anteriorly in the MEF (#60a* in Fig. 12A, B, D and F). CM3 and CM4 form an additional main SAT turning dorsally into the loSM (#61a* in Fig. 12D and F). CM5 (#63), located close to the brain midline, projects a single SAT forward in the MEF (Fig. 12A-D and F). The CM5 SAT then leaves the MEF and turns ventro-posteriorly, exiting the brain towards the subesophageal ganglion (SEG; Fig. 12F; visible in MARCM labeled CM5 done; see accompanying paper by Wong et al., 2013).

Whereas CM5 with its single SAT can be identified throughout metamorphosis (#63; Fig. 11A-L), the more complex CM1, CM2, and CM4 (#60-62) with their multiple SSATs present difficulties. In the larva, SSATs of each lineage still form one bundle. CM4 (#62) is situated further dorsally and its SSAT bundle enters right at the tip of the protocerebral bridge, where it splits to send one main SSAT dorsally into the loSM, one straight anteriorly into the MEF, and one ventrally into the loVp (#62; Fig. 11A). CM3 lies ventrally of CM4; its SSAT bundle projects dorsally, then splits into similar components as those of CM4 (#61; Fig. 11A). CM1 lies medially adjacent to CM3 (#60; Fig. 11A) and sends a dorsal SSAT into the MEF and a ventral one into the loVp. Already by P2, the cell body cluster of CM4 has split into two, one dorsal component connected to the SSAT that enters the loSM and MEF and one ventral component projecting as part of the loVp (Fig. 11C). These separate clusters move further apart towards later stages of metamorphosis (Fig. 11E, Fig. 54D and E). CM3 and CM1 undergo a similar change, but the dorsal and ventral components stay closer together than those of CM4 (Fig. 54C and D). From P3 onward, individual subcomponents are no longer clearly distinguishable: diffuse dorsal cell body groups with fibers coalescing into bundles joining the MEF and loSM with the ventral groups projecting into the loVp (Fig. 11G-L).

CP lineages (#64-67)

The CP group includes four lineages, CP1-4, located ventro-laterally of the calyx (CA; Fig. 12A-C). CP2 (#65) and CP3 (#66) form a paired lineage with joined tracts that cannot be distinguished from each other. CP1 (#64) and CP2/3 (#65-66) possess two hemineurones and form two SSATs each; CP4 (#67) has one. The dorsal HSATs of CP1-3 (#64d, 65d*) joined by CP4 (#67), enter via the entry portal ptpA1 and ptpA4 and project straight anteriorly, forming the oblique posterior fascicle (obP; Fig. 12A, B, D and F). The obP is a conspicuous lamina tract that crosses over the peduncle immediately in front of the CA and then turns anteriorly to merge with the loSM (Fig. 12D-F). Within the obP, the paired HSAT of CP2/3 is located further dorsally; tracts of CP1 (#64) and CP4 (#67), which are close and form a single bundle, are more ventral, nearby "touching" the peduncle (Fig. 12D). The ventral HSATs of CP2/3 (#65v*) enter through the ptpLP ps portal and project forward as part of the posterior lateral fascicle (PLF). The running ventro-laterally of the peduncle (Fig. 12A, B and D-F). The ventral HSAT of CP1 (#64v) joins the posterior component of the lateral equatorial fascicle (LEFp) that extends ventrally and parallel to the peduncle (Fig. 12D).

All CP tracts can be followed throughout metamorphosis (Fig. 11A-L). As described above, for the CM group, hemineurones of CP1-3 (#64-66) move apart from P4 onward (Fig. 11E, G; and F).

BLA lineages (#68-75)

The BLA lineages fall into two subgroups, a dorsal one (BLAd1-4, BLAI) located laterally of the superior protocerebrum, and a ventral one (BLAv1-2, BLAvm) with cell bodies anteriorly and laterally of the anterior ventro-lateral protocerebrum (VIPs; Fig. 3A-F and I). BLAd1-4 (#68-71) and BLAI (#72) are neighbors of the anterior DPLA lineages (#68*, Fig. 13B). The SATs of the quartet BLAd1-4 (#68-71) coalesce into one thick bundle that passes medially underneath the anterior optic tract (AOT) and enters via the ptlLP portal, then turns upward to form the trs fascicle (yellow arrowhead in Fig. 13F) that extends into the superior lateral protocerebrum (Fig. 13B, D, F, E, F and I). BLA, ventrally adjacent to the BLAd cluster, has two hemineurones (#72). One HSAT (#72m; Fig. 13I) is directed antero-medially, extending over the dorso-anterior surface of the VIPs, parallel to the AOT; this SAT is distinguishable in 8P106 labeled brains until P24 (Fig. 5B, D and F) and later becomes indistinct. The second, dorso-posteriorly directed HSAT (#72d; Fig. 13I) extends upward along the lateral surface of the VIP, joining the superficial component of the trs fascicle formed by BLD1-4 (see below; Fig. 13E, F and I). The three ventral BLAvs (BLAv1-2, #73-74; BLAvm, #75) have two hemineurones each with one of their HSATs directed medially, the other posteriorly. BLAv2 (#74), located further dorsally in the BLA group, projects its medial HSAT (#74m) through the ptLP dl portal into the VIPs, where it forms the horizontal VIP tract (hVIP; Fig. 13A, B, D, E and I). The posterior HSAT of BLAv2 (#74p) extends backward over the lateral surface of the VIPs and then turns medially through the ptlH a portal towards the great commissure (GC; Fig. 13E, F and I). BLAv1 (#73) forms a similar pattern, with one HSAT (#73m) projecting medially over the surface of the VIPs, close to the AOT and the BLAI towards the pt VIP dm portal (see above; Fig. 13A, B, D, E and I) and a second HSAT (#73p) extending posteriorly and then ventrally, through the pt VIP dl portal into the GC (Fig. 13A, B, D, E and I). BLAvm (#75), the ventral-most BLA lineage, sends one HSAT dorsomedially over the surface of the VIPs, and up into the anterior VIP (75m; Fig. 13A, B and I); the posterior HSAT (#75p) projects along the lateral surface of the VIPs (Fig. 13F and I).

The tracts formed by all BLA lineages, with the exception of BLAI and the posterior HSAT of BLAvm, which are no longer visible after P32, can be followed throughout metamorphosis. Dorsal BLA clusters and SAT entry points maintain their position (Fig. 8A-I; Fig. 55). Ventral BLA clusters and their HSAT entry points move apart, following the enormous growth of the VIPs compartment (Fig. 8F, G; Fig. 8A-I; Fig. 55C-D). The hemineurones of BLAv1 and BLAv2 become separately, with the medial cluster moving upwards towards the dorsal tip of the VIPs while the posterior hemineurones move laterally (Fig. 13D, Fig. 53C and D). The posterior HSATs of both BLAv1 and BLAv2 acquire an additional branch: one BLAv2 HSAT projects dorsally towards the lateral horn (LH; Fig. 13E, green arrowhead), while the BLAv1 HSAT projects ventrally, into the inferior ventral lateral cerebrum (ILC; Fig. 13B, green arrowhead; Fig. 53C and D).
Discussion

SATs and neuropil fascicles can be followed throughout brain development

In the present work, we have assigned all lineage-related tracts of the Drosophila supraesophageal ganglion to fiber bundles that can be visualized by global markers, including antibodies directed against neuronal membrane proteins (BP; BD, resulting in positive labeling of bundles), as well as synaptic proteins (NCad/nd2; negative labeling of bundles). Lineage-related tracts arise in the larva when secondary neurons extend their axons, forming cohesive fiber tracts that we termed secondary aon tracts (Durnstrei et al., 2003; Pererau and Hartenstein, 2006). By the end of the larval stages, most secondary aon tracts of the supraesophageal ganglion have reached their full length. This is to say, tracts that in the mature (adult) brain will reach from the anterior neuropil (e.g., the antennal lobe) to the posterior neuropil (e.g., calyx or lateral horn) do so already in the larva. Based on the appearance of dunes induced at later larval stages, it is evident that SATs of the late larva have not yet reached their full size in terms of number of axons: neurons born during late larval stages in many cases have only short axons or no axons (J.L., O., and V.H., unpublished). These late-born neurons then differentiate during early pupal stages and fasciculate with the earlier formed axons, leading to an increase in diameter of SATs. Furthermore, we show that only in a minority of lineages do axons that grow out in the pupa (presumably axons of late-born neurons) form novel branches that split off the pre-existing SAT (e.g., BAnv1).

SATs and the fascicles they form in the neuropil remain visible as conspicuous landmarks throughout metamorphosis and in the adult brain. Variations in the labeling properties of different SATs and fascicles around P24 when the differentiation of adult neurons is initiated. Some fascicles maintain a strong signal along their entire length; in other cases, labeling becomes fainter (e.g., lower signal-to-noise ratio, see Table I). Typically, we observed that the most proximal segment of the tract remains strongly labeled, allowing one to follow its entry into the neuropil. More distally, however, the intensity of the labeling declines. Although the overall labeling of fascicles within the neuropil remains strong, it becomes very difficult to distinguish between individual SATs within a fascicle (formed by multiple lineages) by the late pupa (after P48).

Hemineurones and sub-lineages form separate tracts

In the majority of lineages (type 1 lineages), neuroblasts divide asymmetrically into next-generation neuroblasts and ganglion mother cells (GMCs). GMCs undergo one division, producing two neurons which typically follow two different fates (a and b). Recent work has shown that all neurons sharing the a fate or the b fate group together as a hemineurone. The two hemineurones formed by one neuroblast typically form two separate tracts (HSATs). In a good number of cases, one of the hemineurones undergoes cell death, leaving only one hemineurone and its HSAT (Truman et al., 2010). Other lineages maintain both hemineurones and HSATs. At the larval stage, HSATs of a lineage enter the neuropil very close to each other. This close proximity is maintained throughout metamorphosis in the majority of lineages possessing two hemineurones, including DAlc11/2, DAlc11/2, DPla12/3, DPla4, and BLav2. In a number of cases, hemineurones and their HSATs move slightly apart (BAlc, BAm1, CP2/3, BLa, BLav1); in other cases (DPLC3, DPla12/3, BLa, BLavm, BLD1, BLD3, BLvPl/2), the separation of hemineurones is more extreme, leading to HSAT entry portals being on opposite sides of the neuropil. In most of these cases (DPla12/3, BLavm, BLvPl/2), the gradual separation can be directly followed by analyzing the SAT patterns at successive, closely spaced pupal stages (P12, P18, P24, P32; see Fig. 3). In several other cases, notably concerning lineages located close to each other in the dorsolateral cortex (BLD1, BLD3, BLAI, DPla2, DPla3), the separation only became clear with the help of MARCM clones, where the two hemineurones were always labeled concurrently (see accompanying paper by Weng et al., 2011).

What is the mechanism behind the “movement” of hemineurones? We surmise that the SAT, or HSAT, is “anchored” in the neuropil where it interacts via a multitude of adhesion molecules with its neighboring cells, be they primary axons or glia cells. That would imply that the position of the entry portal of an SAT does not move relative to the neuropil. By contrast, the position of the cell bodies, distal to the entry portal, is more flexible. Large scale morphogenetic processes, like the unfolding of the optic lobe at the lateral surface of the brain, may lead to displacements of the cell bodies relative to their original position at the neuropil surface. In the case of two hemineurones whose cell body clusters are “pulled apart” we speculate that, although both HSATs initially contact the neuropil surface very close to each other, only one HSAT enters the neuropil. The other HSAT does not enter, but follows the neuropil surface tangentially, entering at a distant location through a separate, anterior portal. For both DPla12/3 and BLa, this type of behavior can be observed. During metamorphosis, the cell body clusters of the anterior hemineurones move forward, reaching a position close to the anterior entry portals.

Neuroblasts of type II lineages follow a different pattern of proliferation from type I neuroblasts. Rather than forming ganglion mother cells, they produce more neuroblasts (“intermediate progenitors”; for review, see Brand and Liebesin, 2011). An intermediate progenitor then behaves like regular (type I) neuroblast, giving rise to GMCs which divide into two neurons, thereby forming a sub-lineage. Even though more detailed work is required to establish the precise pattern of sub-lineages generated by DPM1/2, DPM1/2, and the other type II lineages, it seems likely that at least some of the sub-lineages also generate their own aon bundles (SSAT). For example, the three type II lineages DPM1, DPM1, and DPM2 each have a tract that passes on either side of the protocerebral bridge and then projects straight forward, forming the dorsomedial and dorsolateral roots of the fan-shaped body. These tracts most likely correspond to the X, Y, Z fiber systems which carry the columnar neurons of the central complex (Hanesch et al., 1989). Columnar neurons connect in a topographically ordered manner small segments of the protocerebral bridge with columns of the fan-shaped body and/or sectors of the ellipsoid body, but have no projections outside the central complex. We speculate that the columnar neurons form one, or several, sub-lineages of DPM1, DPM1, and DPM2, while other neurons of these lineages, projecting outside the central complex via different tracts, represent other sub-lineages.

Comparison of protocols for visualizing fiber assembly and commissural remodelling

In various invertebrate brains, such as the house fly Musca, both tracts and fasciciles have been defined, providing the most comprehensive treatment of gross anatomical fiber systems (Strasfeld, 1976). In this and other classical works on insect neuroanatomy, brain sections were stained with the reduced silver technique developed by Power and Chen (Chen and Chen, 1968; Power, 1943). This technique labels individual fibers and authors have noted that according to precise conditions, the labeling intensity varies according to cell type and depends strongly on parameters such as fiber diameter (Strasfeld, 1976). How can one compare the assemblies of fibers that have been defined as tracts/ fasciciles in previous studies of silver-stained fly brains with
BLD lineages flank the lateral surface of the superior lateral protocerebrum and the lateral horn, posteriorly adjacent to the dorsal BLA lineages discussed above (SLP; LH; Fig. 13C and D). Four BLD lineages, BLD1-4 (#77–80), have tracts that enter at the base of the superior lateral protocerebrum (ptSLP), male a characteristic 180 degree turn around the anterior optic tract (AOT) and backward, dorso-posteriorly along the surface of the SLP, forming the superficial component of the trS1 fiber system (Fig. 13D). F–J; Fig. 56A, B). This conspicuous fiber system (red arrowheads in Fig. 13F) lies posterior to and superficially of the similarly shaped tract carrying the BLAd SATs (see above; yellow arrowhead in Fig. 13F). It also contains the dorsal HSAT of BLAd (see above). BLD1 (#77), BLD3 (#79), and BLD4 (#80) consist of two hemilineages. Aside from the HSAT that joins the trS1 (#77, #79d, #80c; Fig. 13E, F and I), they have a ventrally directed HSAT. In the case of BLD4, located more anteriorly, this tract (#Bdv) grows ventrally into the anterior ventro-lateral protocerebrum (VLPa; Fig. 13E and I). The ventral HSAT of BLD1 (#77p), located further posteriorly, forms the surface of the posterior ventral VLP (vpLP) compartment ventrally, then splits into a medial branch that enters the vpLP and a lateral one which extends towards the lobula of the optic lobe (Fig. 13G, I). BLD5 (#82d) and BLD6 (#82e) are located in the posterior brain, flanking the lateral horn ventro-posteriorly (LH; Fig. 13D, G and H). Best visible in the larva and early pupa (Fig. 11B, D and E), both BLD5 and BLD6 form characteristic l-shaped SATs that initially project ventrally along the surface of the posterior lateral protocerebrum (PLP) and then turn medially (Fig. 13I–J) to enter via the pPLP portal (Fig. 13D, G and H). Both send a short lateral branch into the lobula at this medial turn (not shown). The SAT of BLD5 (#82d, marked by expression of aro-Gal4; Hassan et al., 2000; Spindler and Hartenstein, 2010, 2011) continues across the midline in the great commissure (GC); the short SAT of BLD6 (#83) terminates in the ventral VPbLP (Fig. 13L, Fig. 56C and D). Anterior BLD lineages (#77–83, BLD1–4) can be individually followed only throughout early stages of pupal development. Even at these early stages, it is difficult to separate the SATs in confocal sections taken in the (typical) frontal plane. However, BLD1–4 can be separated from each other by their characteristic additional branches (described above). This also makes it possible to assign MARCM clones to their proper lineages (see accompanying paper by Wang et al., 2013). Cell body clusters of the anterior BLD lineages maintain their antero-dorsal position (Fig. 9F and G; Fig. 56A and B). The analysis of GFP-labeled clones (see accompanying paper by Wang et al., 2013) indicates that the hemilineages of BLD1 (#77) and BLD2 (#79) move apart. In the case of BLD1, the hemilineage producing the ventral HSAT comes to lie posterior of the hemilineage projecting into the trS1; with BLD3, it is the other way around (Fig. 56B). Clusters and SATs of BLDS and BLD6 are easy to follow throughout metamorphosis (Fig. 11R, D, F, H, J and L); clusters of these lineages also move posteriorly and ventrally, to end up in the niche between the posterior lateral protocerebrum (PLP) and the lobula (Fig. 13D).

BLP lineages (#84–87)

The BLP lineages form two pairs, BLP1-2 (#84/85) and BLP3-4 (#86/87), whose cell body clusters are located posteriorly of the lateral horn (LH) and posterior lateral protocerebrum (PLP; Fig. 13C, D, H and I). Each BLP lineage projects one SAT. The paired SAT of BLP1-2 (#84#) enters via the PLP portal and extends antero-ventrally, following the lateral surface of the posterior lateral protocerebrum (PLP) and posterior ventro-lateral protocerebrum (VLPb), and projecting into the anterior ventro-lateral protocerebrum (VLPa; Fig. 13E–J). The paired BLP3/4 (#86#) tract enters the neuropil at the boundary between the lateral inferior protocerebrum (LIP) and the LH and then turns dorso-laterally into the LH (plLH p) where it ends (Fig. 13H, green arrowheads; Fig. 13L). The BLP3/4 (#86–87) tract is similar in entry and trajectory to the anterior HSAT pair formed by DLP1/2 (#48–49), which lies mediolaterally of it (Fig. 13L, blue arrowhead; see above).

The two paired BLP tracts (#84/85 and #86/87) are clearly visible throughout metamorphosis (Fig. 11A, C, E, G, I and K). The BLP3/4 cluster shifts upward and, in the P44 pupa and adult, comes to lie dorsally of BLP1/2 (Fig. 11I; K; Fig. 56C and D). Aside from BLP1-4, two other BLP lineages, BLP5 and BLP6, were described in the larval brain (Cardona et al., 2010a). Located ventrally of the other BLP lineages, the tracts of BLP5 and BLP6 extend anteriorly, passing ventrally of the transverse fiber systems connecting the lobula and the ventro-lateral protocerebrum (VLp). The BLP5–6 tracts are no longer distinguishable from P24 onward, and we recovered no MARCM clones (see accompanying paper by Wang et al., 2013). We speculate that these tracts, visible in the larva, belong to groups of neurons of the lobula.
Discussion

SATs and neuropil fascicles can be followed throughout brain development

In the present work, we have assigned all lineage-related tracts of the Drosophila supraesophageal ganglion to fiber bundles that can be visualized by global markers, including antibodies directed against neuronal membrane proteins (BP1046; resulting in positive labeling of bundles), as well as synaptic proteins (NCad/mcd2; negative labeling of bundles). Lineage-related tracts arise in the larva when secondary neurons extend their axons, forming cohesive fiber tracts that we termed secondary axon tracts (Dunstrei et al., 2003b; Pemar and Hartenstein, 2006). By the end of the larval stage, most secondary axon tracts of the supraesophageal ganglion have reached their full length. That is to say, tracts that in the mature (adult) brain will reach from the anterior neuropil (e.g., the antennal lobe) to the posterior neuropil (e.g., calyx or lateral horn) do so already in the larva. Based on the appearance of cones induced at later larval stages, it is evident that SATs of the late larva have not yet reached their full size in terms of number of axons: neurons born during late larval stages in many cases have only short axons or no axons at all (J., O., and V.H., unpublished). These late-born neurons then differentiate during early pupal stages and fasciculate with the earlier formed axons, leading to an increase in diameter of SATs. Furthermore, we show that only in a minority of lineages do axons that grow out in the pupa (presumably axons of late-born neurons) form novel branches that split off the pre-existing SAT (e.g., BAmv1).

SATs and the fascicles they form in the neuropil remain visible as conspicuous landmarks throughout metamorphosis and in the adult brain. Variations in the labeling properties of different SATs and fascicles arise around P4 when the differentiation of adult neurons is initiated. Some fascicles maintain a strong signal along their entire length; in other cases, labeling becomes fainter (e.g., lower signal-to-noise ratio, see Table 1). Typically, we observed that the most proximal segment of the tract remains strongly labeled, allowing one to follow its entry into the neuropil. More distally, however, the intensity of the labeling declines. Although the overall labeling of fascicles within the neuropil remains strong, it becomes very difficult to distinguish between individual SATs within a fascicle (formed by multiple lineages) by the late pupa (after P4).

Hemineurones and sub-lineages form separate tracts

In the majority of lineages (type 1 lineages), neuroblasts divide asymmetrically into next-generation neuroblasts and ganglion mother cells (GMCs). GMCs undergo one division, producing two neurons which typically follow two different fates (a and b). Recent work has shown that all neurons sharing the a fate or the b fate group together as a hemineurone. The two hemineurones formed by one neuroblast typically form two separate tracts (HSTs). In a good number of cases, one of the hemineurones undergoes cell death, leaving only one hemineurone and its HST (Truman et al., 2010). Other lineages maintain both hemineurones and HSTs. At the larval stage, HSTs of a lineage enter the neuropil very close to each other. This close proximity is maintained throughout metamorphosis in the majority of lineages possessing two hemineurones, including DALc11/2, DALcn1/2, DPLc13/2, DPLc3, and BLAv2. In a number of cases, hemineurones and their HSTs move slightly apart (BAlc, BAm1, CP2/3, BLAl, BLAv1); in other cases (DPLc5, DPLc2/3, BAlc, BLAvm, BLD1, BLD3, BIVp2), the separation of hemineurones is more extreme, leading to HST entry portals being on opposite sides of the neuropil. In most of these cases (DPLc1/2, BAm2, BIVp1), the gradual separation can be directly followed by analyzing the SAT patterns at successive, closely spaced pupal stages (P12, P16, P24, P32; see Fig. 3). In several other cases, notably concerning lineages located close to each other in the dorsolateral cortex (BLD1, BLD3, BLAl, DPLc5), the separation only became clear with the help of MARCM clones, where the two hemineurones were always labeled concurrently (see accompanying paper by Wong et al., 2013).

What is the mechanism behind the "movement" of hemineurones? We surmise that the SAT, or HST, is "anchored" in the neuropil where it interacts via a multitude of adhesion molecules with its neighboring cells, be they primary axons or gial cells. That would imply that the position of the entry portal of an SAT does not move relative to the neuropil. By contrast, the position of the cell bodies, distal to the entry portal, is more flexible. Large scale morphogenetic processes, like the unfolding of the optic lobe at the lateral surface of the brain, may lead to displacements of the cell bodies relative to their original position at the neuropil surface. In the case of two hemineurones whose cell body clusters are "pulled apart" we speculate that, although both HSTs initially contact the neuropil surface very close to each other, only one HST enters the neuropil. The other HST does not enter, but follows the neuropil surface tangentially, entering at a distant location through a separate, anterior portal. For both DPLc1/2 and DPLc2/3 this type of behavior can be observed. During metamorphosis, the cell body clusters of the anterior hemineurones move forward, reaching a position close to the anterior entry portals.

Neuroblasts of type II lineages follow a different pattern of proliferation from type I neuroblasts. Rather than forming ganglion mother cells, they produce more neuroblasts ("intermediate progenitors"; for review, see Brand and Lisevesy, 2011). An intermediate progenitor then behaves like regular (type I) neuroblast, giving rise to GMCs which divide into two neurons, thereby forming a sub-lineage. Even though more detailed work is required to establish the precise pattern of sub-lineages generated by DPMm1, DPMm2, and the other type II lineages, it seems likely that at least some of the sub-lineages also generate their own axon bundles (SAT). For example, the three type II lineages DPM1, DPM2, and DPM3 each have a tract that passes on either side of the protocerebral bridge and then projects straight forward, forming the dorso medial and dorsolateral roots of the fan-shaped body. These tracts most likely correspond to the X, Y, Z fiber systems which carry the columnar neurons of the central complex (Hanesel et al., 1989). Columnar neurons connect in a topographically ordered mannersmall segments of the protocerebral bridge with columns of the fan-shaped body and/or of the ellipsoid body, but have no projections outside the central complex. We speculate that the columnar neurons form one, or several, sub-lineages of DPM1, DPM2, and DPM3, while other neurons of these lineages, projecting outside the central complex via different tracts, represent other sub-lineages.

Comparison of protocols for visualizing fiber assembly and commissural nomenclature

In various invertebrate brains, such as the house fly Musca, both tracts and fascicles have been defined, providing the most comprehensive treatment of gross anatomical fiber systems (Strasfield, 1976). In this and other classical works on insect neuroanatomy, brain sections were stained with the reduced silver technique developed by Power and Chen (Chen and Chen, 1969; Power, 1943). This technique labels individual fibers and authors have noted that according to precise conditions, the labeling intensity varies according to cell type and depends strongly on parameters such as fiber diameter (Strasfield, 1976). How can one compare the assemblies of fibers that have been defined as tracts/fascicles in previous studies of silver-stained fly brains with the
pattern of SATs/fascicles based on immunofluorescence of adhesion molecules and synaptic protein localization?

Thin axons, like those making up SATs in the larva and adult (~0.2 μm diameter; Cardona et al., 2010b), seem to show little if any labeling following silver impregnation. Fiber systems like the peduncle, or posterior roots of the fan-shaped body, appear mostly signal-negative (Strausfeld, 1976). On the other hand, many terminal axons, whose presynaptic sites are concentrated on thick "varicosities" or "boutons" (Cardona et al., 2010b; Prokop and Meinertzhagen, 2006; Watson and Schürmann, 2002) are labeled strongly (Strausfeld, 1976; Strausfeld and Li, 1990; Strausfeld et al., 2005). As a result, the pattern of fiber systems visible on sections using antibodies against neuronal molecules, such as Neurontacin (labeling axons) or Bruchpilot (labeling active zone at presynaptic sites), appears very different from what is visible on a silver-impregnated brain sections (e.g. the lightly stained SATs and SAT-based neuropil fascicles are barely visible on these sections, being overshadowed by the prominently labeled thick fibers). It is therefore difficult to reconcile the nomenclature of fascicles proposed on the basis of silver-impregnated sections of the Musca brain (Strausfeld, 1976) with the naming of fiber systems visible in brain confocal sections labeled with neuronal or synaptic antibodies.

A clear exception to this general rule are the commissures, most can be recognized in the silver-impregnated Musca brain and the immunofluorescently-labeled Drosophila brain. For these fiber systems, the original topology based nomenclature proposed by Strausfeld (1976) was taken over or modified slightly (Pereanu et al., 2010; Tanaka et al., 2012). For example, the "inferior interantennal connective" is now named the "antennal lobe commissure," the "subellipsoid connective" is called the "subellipsoid commissure," the "inferior ventral body connective" the "accessory lobe commissure" (the "accessory lobe of the dorsal body was "ventral body") and the "arched connective of the ventral body" the "supraellipsoid commissure." In the posterior brain, the "superior arch commissure" and "great commissure" have maintained their name; the "commissure of the lateral horn" is renamed the "dorsal commissure of the posterior lateral protocerebrum."

Among the longitudinal fascicles introduced for Drosophila, only the antennal lobe tract (formerly "antennal-glomerular tract") and median bundle are easily recognizable in silver-stained and immunofluorescently-labeled sections. In addition, in the superior protocerebrum, the SiGM defined here follows a similar trajectory as the "posterior division of the median fascicle" of Strausfeld (1976). More ventrally, the medial and lateral equatorial fascicles (MEF, LEF) are located at a position corresponding to the "equatorial horizontal fascicle" in Musca. The oblique posterior fascicle (OPF) may correspond to the "lateral horn-medial protocerebral tract" of Strausfeld (1976). None of the transverse fiber systems formed by the SATs (tr1A, tr3A, tr5P) appear as named entities in the Musca Atlas; although many tracts and fascicles defined for Musca have no obvious counterparts in the Drosophila brain using global markers like RP104 (anti-Neurolig). One can hope that the great anatomical detail revealed by silver impregnation techniques in the Musca Atlas and in numerous previous studies can be eventually "translated" to confocal microscopy, using appropriate markers for individual neurons or subsets of neurons.

As previously discussed, we propose that the system of tracts and fascicles that is positively or negatively labeled by antibody labeling against neuronal proteins (e.g. Neurotactin, Neurolig, N-Cadherin, and Bruchpilot) will provide a helpful anatomical framework for neurobiological studies dealing with specific neuronal subsets. These antibodies are readily available, and can be used in the background of specific neuronal markers (e.g. promotor or enhancer-driven Gαδ drivers combined with membrane-localized fluorescent reporters); thereby allowing the observer to directly relate the neuronal subset to SATs and neuropil fascicles. As shown in the accompanying paper (Wong et al., 2013), it is a straightforward task to determine the lineage identity of MARCM clones based on their characteristic fasciculation with specific SATs. Thus, this work serves as a foundation for assigning secondary neuron populations (visualized by stable GFP markers or as single-cell clones; Chiang et al., 2011; Jefferis et al., 2001; Bai et al., 2005; Lin et al., 2012) to a comprehensive map of well-defined SATs, taking us a step closer towards reconstructing the brain macrocircuit.

Tracts and fascicles across insect taxa

It has been generally assumed that, for the last several decades, the basic pattern of lineages is conserved among different insect groups (Boyant and Ball, 1993; Duman-Scheel and Patel, 1999; Jarvis et al., 2012; Thomas et al., 1984). This strong statement is based on the observation of conserved patterns of neuroblasts and various subsets of neurons. The map of neuroblasts of an individual segment of the ventral nerve cord in Drosophila (Brodar et al., 1995; Doe, 1992; Hartenstein and Campos-Ortega, 1984; Truman and Bate, 1988) and grasshopper (Bate, 1976; Doe and Goodman, 1985) contains the same number of columns (4) and rows (7) of neuroblasts. Lineages including specific subsets of neurons are found at identical positions within the Drosophila and grasshopper neuromere (Bosming et al., 1996; Schmid et al., 1999; Taglert et al., 1992; Udeloph et al., 1993), whereas their neuroblast maps have also been constructed (Urbach et al., 2003; Urbach and Teichau, 2003a, 2003b; Williams and Boyan, 2005; Youanisi-Hartenstein et al., 1996; Zacharias et al., 1993), showing significant similarities in cell number and arrangement. As lineages of the Drosophila brain form tracts and fascicles with characteristic trajectories, it is reasonable to assume that this will be the case for other insect brains as well. Thus, it follows that neuropil fascicles should adhere to a pattern that can be recognized in other insect groups as well, which, if true, would be a great advantage for comparative insect neuroanatomy. Specific antibodies for neuronal proteins have not been produced in many insects, but some of the antibodies raised against Drosophila (as well as vertebrate) proteins cross-react with epitopes in other taxa, and numerous groups use these as markers in their (confocal) study of various insect brains (e.g. Boyant et al., 2003; Dryer et al., 2010; Huettenh et al., 2010; Ignell et al., 2005; Mysoe et al., 2011; Rybek et al., 2010). Several studies prepared recently (JE. and VH. pers. comm, Lansen et al., 2008; Mysoe et al., in prep; Pereanu et al., 2010) including this work, strongly suggest that the pattern of neuropil fascicles labeled negatively when using antibodies cross-reacting with synaptic epitopes shows significant similarities to the Drosophila pattern described previously. In this work (Fig. 7), we compare the different neuromeres, taken at corresponding anter-posterior levels (fan-shaped body), of four different insects, including Drosophila, Aeles aegypti (Diptera), Tribolium castaneum (Coleoptera), and Cardiostylops obscurus (Hymenoptera). Many fascicles, including the ISGM and ISOl of the superior protocerebrum, the MEF and LEF of the inferior protocerebrum, the lnV separating ventrolateral and ventromedial cerebral, the great commissure of the ventral cerebral; and the dorsal and ventral commissure of the suboesophageal ganglion can be tentatively identified on the basis of their entry points into the neuropil, and their positions relative to the surrounding compartments. It will be crucial to develop antibody markers that allow for the positive labeling of tracts and fascicles. Once that goal has been achieved, it will be possible to embark on a lineage-based, and thereby much more detailed comparative-evolutionary analysis of insect brain structure. It is likely, as proposed in previous studies (reviewed in Parris, 2005; Galiati and Resler, 2010; Homberg, 2008; Strausfeld et al., 2009), that changes in brain anatomy of different insects (and animals in general) occurred by varying a basically conserved pattern of lineages. For
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Appendix 2.

Postembryonic lineages of the *Drosophila* brain: II. Identification of lineage projection patterns based on MARCM clones.
Postembryonic lineages of the Drosophila brain: II. Identification of lineage projection patterns based on MARCM clones

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ABSTRACT

The Drosophila central brain is largely composed of lineages, units of sibling neurons derived from a single progenitor cell or neuroblast. During the early embryonic period, neuroblasts generate the primary neurons that constitute the larval brain. Neuroblasts reactivate in the larva, adding to their lineages a large number of secondary neurons which, according to previous studies in which selected lineages were labeled by stably expressed markers, differentiate during metamorphosis, sending terminal axonal and dendritic branches into defined volumes of the brain neuropil. We call the overall projection pattern of neurons forming a given lineage the “projection envelope” of that lineage. By inducing MARCM clones at the early larval stage, we labeled the secondary progeny of each neuroblast, for the supraesophageal ganglion excluding mushroom body (the part of the brain investigated in the present work) we obtained 81 different types of clones. Based on the trajectory of their secondary axon tracts (described in the accompanying paper, Lovic et al., 2013), we assigned these clones to specific lineages defined in the larva. Since a labeled clone reveals all aspects (cell bodies, axon tracts, terminal arborization) of a lineage, we were able to describe projection envelopes for all secondary lineages of the supraesophageal ganglion. This work provides a framework by which the secondary neuron (forming the vast majority of adult brain neurons) can be assigned to genetically and developmentally defined groups. It also represents a step towards the goal of establish, for each lineage, the link between its mature anatomical and functional phenotype, and the genetic makeup of the neuroblast it descends from.

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Introduction

In the field of developmental biology, the concepts of cell determination and cell lineage are fundamental to our understanding of the formation of complex tissues and organs. When talking about a cell lineage, we are referring to the genealogy (family tree) of groups of cells. The lineage produced by a progenitor cell is generally used synonymously with the progeny descending from this cell. During early stages of development, a progenitor cell initiates a genetic program that controls the later fate of this cell and its progeny. The genetic program of the progenitor is defined by the expression of cell fate determinants, typically transcription factors, that either remain active in the progeny or trigger the expression of a next tier of factors impacting the fate of the progeny (Guillenot, 2007; Shirasaka and Pfaff, 2002; Sleath and Thor, 2003). Thus, when embarking on the analysis of a complex organ, one of the assumptions that guides our research is that cells which possess a similar phenotype do so because they are part of a lineage produced by a common progenitor which, early on, expresses a set of transcription factors (“intrinsic determinants”) controlling the fate of its lineage. Of course, this assumption has to always be tested against the alternative: extrinsic signals from the environment into which cells are placed trigger a genetic switch in these cells which controls their fate. In this scenario, the family tree of the cells is not important.

The fate of cell lineages in the Drosophila nervous system is heavily influenced by intrinsic determinants. A number of pioneering experiments in which neural progenitors (neuroblasts) were cultured in vitro revealed that, even when removed from their natural environment in the early embryo, neuroblasts are capable of dividing and producing progeny in the same number and cell type (assayed by expression of neurotransmitters) (Huff et al., 1989). Later studies identified many specific transcription factors expressed in neuroblasts (Doe, 1992; Urbach and Technau, 2003). Furthermore, it was shown that the timing of expression of a transcription factor is able to influence the fate of subsets of neurons (“sublineages”) forming part of a lineage (Brody and
Odenwald, 2000; Ishikii et al., 2001; Kambadur et al., 1998; Pearson and Doe, 2004). Thus, a neuroblast (N) divides asymmetrically, with one daughter cell (N) remaining in the state of a dividing neuroblast, whereas the other daughter cell (ganglion mother cell) after an additional round of division, becomes postmitotic and differentiates into two daughter cells (neurons or glia). The asymmetric division allows for a mechanism by which transcription factors are differentially inherited by daughter cells. The general model is that transcription factor (A), expressed during a specific time interval, will be inherited by one daughter cell or sublineage (a). Eventually, (A) is no longer expressed and a second one (B) turns on. All neurons born after (A) is turned off now inherit (B) and become a second sublineage (b). Several transcription factors were identified that are expressed in a sequential manner during neuroblast proliferation in the embryo and were shown, using molecular markers as a read-out, to influence the fate of embryonic-born (primary) neurons (Itskhaki et al., 2001; Kambadur et al., 1998). However, it should be emphasized that many transcription factors are active in neuroblasts from before they are mitotically active through to a later developmental period, and that this window of expression varies for each transcription factor (Doe, 1992; Kumar et al., 2009a, 2009b; Lichtenecker et al., 2008; Urbach and Technau, 2003); these factors would be predicted to have an impact on the fate of an entire lineage.

Analyses of a few select lineages in the larval and/or adult brain support the idea that neuronal fate is controlled by factors inherited by entire lineages and by specific sublineages, which may manifest itself in a lineage’s overall structure. Thus, lineages appear as “morphological units,” with all axons forming one or two (in the case of hemilineages; Truman et al., 2010) bundles and terminal arborizations focusing on a discrete neuropil territory. For example, four lineages (MB1–4) form the mushroom body (Crittenden et al., 1998; Ito et al., 1997) and three lineages (vNvB, INvB/INvA, and DNvB/DNvA) include the majority of projection neurons connecting the antennal lobe with the protocerebrum (Lai et al., 2008). Endings of all secondary neurons of MB1–4 are confined to the calyx, peduncle, and lobes of the mushroom body; the antennal lobe-associated lineages innervate three compartments, namely the antennal lobe, calyx, and lateral horn (Das et al., 2013; Lai et al., 2008; Stocker et al., 1990). We will use the term “projection envelope” to describe the overall neuropil volume that is innervated by neurons of a lineage. Individual neurons in a lineage form a longitudinal arbor that target smaller volumes within the projection envelope. For example, the neurons produced by MB1-4 in the late embryo/early larva fill the γ-lobe, they are followed by neurons forming the α/β lobes, and finally by neurons of the α/γ lobes (Ito et al., 1997; Kunz et al., 2012). In the case of DNvB/INvA3, most neurons innervate a single glomerulus of the antennal lobe and project to discrete regions within the calyx and lateral horn (Jeffries et al., 2001; Yu et al., 2010), it is reasonable to assume that the projection envelope of a lineage, which is shared by all neurons of that lineage, is determined to some extent by transcription factors expressed earlier in development and are common to the neuroblasts of that lineage. In addition, other factors expressed later at defined temporal intervals, thereby only reaching neurons born during that interval, may be responsible for more specific structural and functional characteristics that set neurons of a lineage apart from each other.

 Whereas both expression of molecular determinants of cell fate and the phenotypic elements of cell fate (e.g., shape of neuronal arbor, choice of pre and postsynaptic partners, physiological characteristics) can be studied in great detail, the complex cascade of molecular events linking the two levels has remained elusive. What mechanism acts on outgrowing axons and guides restricts them to a specific compartment? How is this mechanism encoded in the cell fate determinants expressed in the neuroblast?

Drosophila offers a favorable system to address these questions: its nervous system is built by a relatively small number of lineages (previous descriptive maps yielded approximately 100 lineages per central brain hemisphere and 25 lineages per ventral nerve cord hemineuromere; Doe, 1992; Urbach and Technau, 2003; Younessi-Hartenstein et al., 1996). Lineages can be globally and/or individually labeled by antibodies for various neuronal proteins (e.g. mushroom body-specific antibodies; Crittenden et al., 1998; neuropeptide pigment-dispersing factor or PDF; Helfrich-Förster et al., 2007; neuropeptide IPMamide: Shafer et al., 2006) and reporter constructs (Gal4 and Gal4-based; e.g. en-Gal4, Kumar et al., 2009a; Th-Gal4, Mao and Davis, 2008; Gal4 lines expressed in ellipsoid body neurons, Renn et al., 1999). Maps of the expression of transcription factors in the neuroblasts, as well as the anatomical pattern of lineages at the larval stage, have been generated (Cerdon et al., 2010; Pereanu and Hartenstein, 2006; Truman et al., 2004; Urbach and Technau, 2003; Urbach and Technau, 2004). In the accompanying paper (Lovick et al., 2013) we had mapped the association between lineages and neurophil fascicles and followed these fascicles throughout metamorphosis into the adult. In this paper, we have analyzed individual lineages at the adult stage by the MARCM technique (Lee and Luo, 2001), where a GFP reporter gene is activated by somatic recombination in neuroblasts shortly before they enter their larval phase of proliferation. In this manner, all secondary neurons produced by these neuroblasts (the “secondary lineages”) are labeled as “clones.” A clone includes the cluster of cell bodies derived from the larval neuroblast, as well as the axons and terminal arborizations of these cell bodies. Based on the trajectory of their axon bundles, we are able to assign clones to their respective lineages. We analyzed a total of 814 clones located in the supraesophageal ganglion, the largest part of the brain. Excluded from this study are clones in the optic lobes, whose modular (and probably not lineage based) structure has been described previously (Bauenwein et al., 1992). Excluded are also clones representing the four well known lineages of the mushroom body (Crittenden et al., 1998; Ito et al., 1997; Kunz et al., 2012), and the lineages of the subesophageal ganglion, which will be analyzed in an upcoming study (Kuett et al., in preparation). Clones fell into 81 groups, where each group corresponded to a known lineage or lineage pair. We provide a brief description of the projection envelopes for all lineages. The complexity of these lineages clearly warrants a much finer level of analysis, taking into account aspects like overlap of terminal arborizations of different lineages, precise relationships between arborizations and compartment boundaries, and variations in the size and location of cell bodies. These investigations, which require that specimens with different clones are digitally registered to a “standard brain,” will be presented in a series of upcoming studies. Note that numerous aspects of lineage analysis has been recently published in two large, independent studies where MARCM clones of secondary lineages were generated (Ito et al., 2013; Yu et al., 2013). The main purpose of the present work is to identify clones with defined lineages, contributing to the ultimate goal of linking the mature anatomical and functional phenotype of a lineage and its constituent neurons with the specific genetic make-up of the embryonic neuroblast that produces the lineage.

Material and methods

Fly stocks

Flies were grown at 25°C using standard fly media unless otherwise noted. Fly stocks used are the ones detailed in the “Clonal Analysis” section.
Immunohistochemistry

Samples were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS, Fisher-Scientific, pH=7.4; Cat No. #BP399-4). Tissues were permeabilized in PBT (phosphate buffer saline with 0.3% Triton X-100, pH=7.4) and immunohistochemistry was performed using standard procedures (Ashburner, 1989). The following antibodies were provided by the Developmental Studies Hybridoma Bank (Iowa City, IA): mouse anti-Neurotactin (BP106, 1:10), rat anti-DCN-adenin (DN-EX #8, 1:20), mouse anti-Neurolan (BP104, 1:30). Secondary antibodies, IgG (Jackson Immunoresearch; Molecular probes), were used at the following dilutions: Alexa 546-conjugated anti-mouse (1:500), Dynalight 649-conjugated anti-rat (1:400).

Clonal analysis

Clones were generated by FLP-mediated mitotic recombination at homologous FRT sites. Larval neuroblast clones were generated by MARCM (Lee and Luo, 2001; see below) or the FLP-out construct (Zecch et al., 1996; Ita et al., 1997).

Mitotic clone generation by FLP-out

To generate secondary lineages dyes in the larva using the FLP-out technique; flies bearing the genotype:

1. hslp, elav<sup>Gal4</sup>/+, UAS-FRT-C218, y, stop-FRT-mCD8:GFP
2. hslp, Act5C-FRT-stop+y, FRT-Gal4, UAS-tau-lacZ/UAS-src:GFP

Briefly, early larva with either of the above genotype were heat-shocked at 38°C for 30-40 min. elav<sup>Gal4</sup> is expressed in neurons as well as secondary neuroblasts. Third larval and adult brains were dissected and processed for immunohistochemistry (as described above).

Mitotic clone generation by MARCM

Mitotic clones were induced during the late first instar/early second instar stages by heat-shocking at 38°C for 30 min to 1 h (approximately 12-44 h AUE). GFP-labeled MARCM clones contain the following genotype:

Adult MARCM clones:

1. hslp/+; FRT13G, UAS-mCD8GFP/FRT13G, tub-GAL80, tub-Gal4/+ or
2. FRT19A GAL80, hslp, UAS-mCD8GFP/elav<sup>Gal4</sup>-GAL4, FRT19A; UAS-C6D8GFP/+;

Larval MARCM clones:

hslp, elav<sup>Gal4</sup>-GAL4, FRT13 B, UAS-mCD8GFP/Y or hslp, elav<sup>Gal4</sup>-GAL4, FRT13 GAL80, UAS-mCD8GFP; FRT42D, tub-Gal80/FRT42D

Confocal microscopy

Staged Drosophila larval and adult brains labeled with suitable markers were viewed as whole-mounts by confocal microscopy [LSM 700 Imager M2 using Zen 2000/ Carl Zeiss Inc.; lens: 40 x oil (numerical aperture 1.3)]. Complete series of optical sections were captured images were processed by Image J (National Institutes of Health, http://rswebb.nih.gov/ij) and Adobe Photoshop.

2D registration of clones to standard brain

Brains with MARCM clones were labeled with DN-cad and BP104 to image the SAT and projection envelope relative to the BP104-positive fascicles and DN-cad-positive neuropil compartments. Fascination of the SAT of a clone with a fascicle allowed for its identification with a lineage, or lineage pair (see accompanying paper by Lovick et al., 2013). To generate the figures of this paper, z-projections of the individual MARCM clones were registered digitally with z-projections of a standard brain ("2D registration"). To this end, the standard brain was subdivided along the anteroposterior axis into six slices of approximately 20 µm thickness. These slices, each one characterized by one or more easily recognized landmark structures (antennal lobe, optic tubercle, elliptoid body, fan-shaped body, lateral bend of antennal lobe tract, calyx), are introduced in Pereuan et al. (2010), and are depicted throughout the figures of this and the accompanying paper (Lovick et al., 2013). The process of 2D registration involved the following steps:

1. The confocal stack depicting a given clone was imported into the FIJI program (National Institutes of Health, http://rswebb.nih.gov/ij and http://fiji.sc/) and digitally oriented such that the peduncle was aligned with the z-axis of the stack.
2. A z-projection of the entire clone (e.g., all sections of the green channel showing label) was generated.
3. In the case of brains containing more than one clone, background fluorescence and/or fluorescence from other clones were digitally removed to allow visualization of a single clone.
4. This z-projection was merged with a z-projection of the red channel (BP104 or DN-cad) derived from the confocal sections of one slice. The chosen slice is dependent upon the corresponding clone's SAT location. For example, consider the lineage BALP4, whose SAT enters the antennal lobe posterolaterally. In terms of anteroposterior brain slices, this SAT forms part of the second slice ("level anterior optic tubercle").
5. Both the anterior optic tubercle slice of the standard brain and the brain specimen containing the BALP4 clone were imported as two layers into a file generated by the Adobe Photoshop program. Using few standard landmarks (location of the peduncle, tips of the MB vertical and medial lobe, vertical midline), the layer containing the clone (rendered temporarily semitransparent) was optimally fitted to the underlying layer representing the standard brain.
6. The optimally-fitted layer containing the clone was re-opened in FIJI, and then merged with the red channel (BP104 or DN-cad) of the standard brain. For the panels of the figure set depicting clone SATs relative to BP104-positive fascicles (Figs. 4, 6, 9, 12, 15 and 17), the red channel (BP104) was rendered white in Adobe Photoshop. For the figure set depicting the projection envelopes of clones (Figs. 2, 3, 5, 7, 8, 10, 11, 13, 14 and 16), the red channel (DN-cad) was rendered magenta by duplicating it in the blue channel.

Results

MARCM clones reveal the projection envelope of secondary lineages

We analyzed a total of 814 secondary clones, distributed over 493 brains. About half of the brains had a single clone, the other half had two or more (up to five) brains containing in excess of five clones were discarded). Aside from clonal labeling of individual secondary lineages, most brains also contained labeled-endings of afferents from the optic lobe and/or antennal nerve. All clones could be assigned to a specific secondary lineage (or lineage pairs) based on the entry point and trajectory of the SAT, defined as the fiber bundle that directly emerges from the cell body cluster and enters the neuropil (Fig. 1A and B; BALP4 clone assigned to BP104-labeled SAT). Given the number of clones analyzed, most secondary lineages were represented by more than one clone. We observed a wide range, with some lineages represented more than 20 times
and others less than five times (average 10 clones per lineage; Table 2).

Based on our analysis of SAT development (see accompanying paper by Lovick et al., 2013), 56 lineages defined in the late larva have SATs that can be individually followed within the neuropil throughout development (Table 2). Within this group, we could identify clones in all cases except one, DALv3. The projection pattern of DALv3 has been characterized previously (Kumar et al., 2009a). A second group of 30 lineages (e.g., B/Anas1/2; DALcm1/2) have SATs that form pairs or form a quartet (the four BIAd lineages). In these cases, it is not possible to predict whether the two lineages forming the pair (or quartet, in the case of BIAd1-4) will have projection envelopes that are identical or different. Within the group of 30 lineages, four pairs (B/Anas1/2; DPLp1/2; CP2/3; BLP3/4) were obtained that had clones with significantly different arborization patterns. This suggests that paired lineages with identical SATs form distinct arborization patterns (e.g., B/Anas1/2; Fig. 4C11–12). In three pairs within this group (DPLp1/2, BLP3/4, BLP3/4), the patterns were very similar, but the trajectory of part of the SATs differed consistently (e.g., DPLp1/2 in Fig. 10B and F). In the case of the BLAd-4 quartet we isolated three different classes of clones in the eight remaining pairs (BAl8/4, DAllcm1/2, DAMd2/3, DAMv1/2, DPLp2/3, DPLp1/2, BLP3/4). Only one type of clone was recovered, suggesting that these lineages form identical arborization patterns. Alternatively, it is possible that we could recover clones for only one member of the pair, which is unlikely given the fact that an average of 10 clones per lineage was obtained for all other lineages.

A significant fraction of lineages form more than one SAT. In cases where these tracts separate from the very beginning where axon tracts enter the neuropil we tentatively assume they represent two separate hemineuronal lineages (or sublineages, in case of type II lineages), ultimate proof for their status as "true" hemineuronal lineages would have to come from experimental studies such as those done for the thoracic lineages (Truman et al., 2010) or the small number of engrailed-positive brain lineages (Kumar et al., 2009a). As described in the accompanying paper by Lovick et al. (2013), hemineurones move apart during metamorphosis in a number of cases. GFP labeled clones provide confirmation for this movement of hemineurones. All except one (BLAvm) of the lineages in question, notably BAlc, BAnm1, DPLp2/3, CP2/3, BLAI, BLAvm, BLP1/2, were represented by more than five clones; for BLAvm we have three clones. In all cases, GFP labeling invariably marked both hemineurones simultaneously, whereas other independent lineages could be represent by a clone in some cases, but not in

Fig. 1. Secondary lineages: SATs and projection envelope. (A–C) Assignment of clones to their respective secondary lineages is based on the entry point and trajectory of the SAT. Panel (A) shows a 3D projection of an adult brain labeled with BP014 (white), containing a MARCM clone of the BAlp4 lineage (green). The SAT of BAlp4 is shown in orange. Panel (A') shows BP014 channel of the same 3D projection. Green arrow in (A–B) point at the SAT of the BAlp4 clone, as visible by green arrow in (A), the SAT follows one of the BP014-positive tracts that can be followed back through metamorphosis in lineage BAlp4. Panel (B) represents a 3D projection of the standard brain used in this paper, at an antero-posterior level corresponding to the one used for (A/A'). Note invariant pattern of BP014-positive fiber bundles in (A/A') and (B) (green arrows; BAlp tract; red arrow: BAlp1/2 tract). (C–D) Relationship of individual neurons and projection envelope. Schematic representation of a lineage (pink) with a projection envelope including compartments "a" and "b". Individual neurons of the lineage could (all or in part) form arborizations throughout all compartments of the projection envelope [represented by red neuron in panel (D)], or could project to one or the other compartment [blue and green neuron in (D)]. (E) Classification of lineages based on the contour of projection envelope. Shown are four classes of lineages. In "PD" (proximal-distal) lineages, a long segment of the SAT connects proximal to distal arborizations. In "C" (commissural) lineages, hippocampal inputs at or regular intervals along the entire length of the SAT. Two subtypes of commissural lineages, local ("O") and widefield ("Cw") are illustrated. In "D" (distal) lineages, terminal arborizations are delimited to the distal end of the SAT. Bar: 25 μm. 201
others. In cases of several lineages for which the movement of SATs and H5 SATs was difficult to follow (BLD1, BLD3, BLD4, DPLC5), the existence of two separating hemilineages was confirmed. In three cases, BAm2, DPLm2, and DPM1, the analysis of MARM clones made it possible to identify the proper lineage in the adult brain. Thus, the SAT of BAm2 cannot be followed beyond P24 because its entry and proximal SAT is masked by the arrays of antennal afferents surrounding it. A clone with the characteristic SAT entry point and crossing in the antennal lobe commissure confirmed BAm2 for the adult brain. The same applied for DPM1, whose characteristic descending SAT is not visible beyond P24. DPLm2 represents a unique case where the MARM clone united two clusters of cells.

Fig. 2. (A-H) Clones representing lineages of the BA group (#1/BAla1, #9/BAll4) in the larval and adult brain. This and the following Figs. 3, 5, 7, 8, 10, 11, 13, 14, 16 are designed in the same manner: Each lineage is represented by a panel showing a z-projection of a larval brain hemisphere on the left, and of an adult hemisphere on the right. Number and abbreviation of the lineage is given at the bottom left of the panel. Next to the abbreviation, the type of lineage (C, PD) is indicated. Images were generated by registering full z-projection of clones (that is, a z-projection containing all sections of a stack showing GFP label) to a z-projection of a "slice" of the DIn-cad labeled standard brain, as described in the "Methods and materials" section. DIn-cad visualizes neuropil compartments, annotated by white letters on part of panels showing adult brain. Compartments receiving major innervation by the lineage shown on a given panel are annotated by colored letters. Intervened compartments contained within the brain slice shown by DIn-cad are in orange; compartments located significantly anterior or posterior to the slice shown appear in blue letters. For example, #1/BAla1 (A) is represented by a clone registered to an anterior brain slice (level opt: tubercol VU/mushroom body medial lobe [MB/M]). Major compartments visible at that level are annotated by small white letters. Within this slice, only the antennal lobe is innervated by BAla1; it is annotated by orange letters. BAla1 projects posterior-laterally towards the lateral horn, which is located in a slice posterior to the one shown. The lateral horn (LH) is therefore annotated by blue letters. For alphabetical list of all abbreviations see Table 1.

Bar: 50 μm.
that had been previously considered to represent two separate lineages. Thus, in our larval analysis DPM2 with a characteristic centrifugal axon bundle projecting to the ring gland was considered as a separate lineage (Pereanu and Hartenstein, 2006). MARCM clones showed that the ring gland associated axons form part of DPM2 instead.

Classification of lineages based on the geometry of their projection envelope

The GFP-labeled clone, when superimposed on a backdrop of an adult brain labeled with a neuropil marker (anti-DN-cadherin; from here on called DN-cad) or axonal marker (anti-Neurotactin, from here on referred to as BP104), allows one to map the neurite arborizations of all neurons of a single lineage (the "projection envelope") with respect to brain neuropil compartments (Fig. 1C). Note that the relationship between the projection envelope of a lineage and the projection of an individual neuron forming part of a lineage is not simple (Fig. 1D). For example, when an envelope includes two neuropil compartments, a and b, there are two possibilities: (1) each individual neuron may have arborizations in a and b (Fig. 1D, red neuron); or alternatively, a subset of neurons might only project to a or to b (Fig. 1D, green neuron and blue neuron, respectively). Nonetheless, documenting the projection envelope for each lineage represents a significant step towards describing brain circuitry. In this paper we will provide an overview of the projection envelopes for each lineage of the supraesophageal ganglion, following the same

Fig. 1. (A–H) Clones representing lineages of the BA group (#10/BA1v–#17/BAmv3) in the larval and adult brain. For description of how panels are made and displayed, see legend of Fig. 2. Only one larval close is shown for the pair BA/as1/2 (panels B and C). For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.
Fig. 4. Clones representing lineages of the IA group in the adult brain. This figure, as well as the following Figs. 6, 9, 10, 12, 15, and 17, is all designed in the same manner. Large panels on the left (A, B in case of Fig. 4) show z-projections of frontal confocal sections of adult brain hemisphere labeled with BP104 (white), SATs and neuropil fascicles) and DN-cad (purple; neuropil compartments). Each z-projection represents a brain slice of approximately 15−20 μm thickness. Brain slices correspond to different levels along the antero-posterior (z) axis. Panels A and B of this figure represent the two slices of the brain where IA lineage enters the neuropil (A: level of optic tubercle and mushroom body lobes; B: posteriorly adjacent slice, marked by ellipsoid body). SATs of lineages are annotated by colored numbers; the same color key is used as in the accompanying paper by Javick et al. (2013) in cases where two or more SATs or HSAs come very close and cannot be distinguished, the identifying numbers may be contracted into a single number followed by an asterisk; see, for example, the annotation of the HSAs of the DPla2/3 lineages, #34/35, as "34"*, and "34"** in Fig. 9A. Fascicles with which SATs are associated are annotated by yellow letters. (C) The small panels in section (C) of this figure show z-projections of clones representing lineages of the IA group. Panels were generated by registering a projection of clones to a slice of the BP104-labeled standard brain, as described in the "Materials and methods" section. Each lineage is identified by a number and abbreviation (bottom of its panel) rendered in the same color as that used in panels A and B. For the BP104 channel (white), only slices shown in the left panels (A, B) are used and indicated at the bottom left of the panel ("BP104 A", "BP104 B"). For example, the first small panel depicts lineage #1 (BAla1). The clone is registered to the anterior slice (optic tubercle and mushroom body lobes); shown at higher magnification in panel A), because the proximal SAT of BAla1 is contained within this slice. Panels with other lineages (e.g., #10/34B) use the more posterior slice (the one shown in panel B; ellipsoid body), because the SAT of BAmv is contained within that slice. In each small panel, the orange-colored arrow points at the proximal SATs by which the clone is identified. In terms of position and orientation, the arrow matches the orange line in panel on the left (A or B) which points at the corresponding BP104-labeled tract. Yellow arrows in the C-panel point at neuropil fascicles joined by the SAT. For example, the yellow arrow in the panel #1/BAla1 points at the beginning of the antennal lobe tract (ALT). For alphabetical list of abbreviations see Table 1. Bar: 25 μm.
topology-based ordering used in the accompanying paper (Lovick et al., 2013).

Representative clones and lineage restricted markers used in previous studies suggested that, aside from their topology (spatial relationship of a SAT entry point and projection relative to neuropil track and compartments), lineages can also be classified according to the “geometry”, defined as the distribution of axonal/dendritic branches relative to the main SAT (Larsen et al., 2009). It should be noted that unlike vertebrate neurons, where dendritic branches connected to the cell body are separated from axonal branches, insect neurons have a neurite tree on which dendritic and axonal branches are frequently intermingling (Hartenstein et al. 2008; Watson and Schürmann, 2002). The degree of intermingling, nonetheless, varies for different neurons and presumably different lineages. For example, in the case of the well-characterized lineages of antennal lobe (AL) projection neurons (e.g. Bmnv3, BAlc, and BAlaf), dendritic branches are concentrated along the proximal region of the SAT in the AL, whereas axonal branches are close to the distal tip of the SAT in the calyx (CA) and lateral horn (LH). The long segment of the SAT connecting proximal to distal, called the antennal lobe tract, is devoid of branches. Lineages with this geometry were classified as “PD” (“proximal distal”) lineages (Larsen et al., 2009; Fig. 1E). In other lineages, branches emerged at more or less regular intervals along the entire length of the SAT (“continuous” or “C” lineages), or were all concentrated at its distal tip (“distal” or “D” lineages). Further

Fig. 5. (A-H) Clones representing lineages of the DAL group (#18/DALcl1-#26/DALc2) in the larval and adult brain. For description of how panels are made and displayed, see legend of Fig. 2. For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.
analysis using fluorescent reporters differentially localized to either dendritic or axonal branches (e.g. UAS-ICAM5ΔC:mtRed
and UAS-GFP-KDEL; Nicolai et al., 2010 and Okajima et al., 2005,
respectively) can be used to compare their distribution in the C, D,
and PD lineages.

In the following, clones representing individual lineages will be
described in the order established for the lineage tracts in the
accompanying paper (Lovick et al., 2013). Clones are documented
in three sets of figures. In one set of figures, we show z-projections
of clones with spatial respect to the BP164-labeled scaffold of
neuropil fascicles, starting with lineages entering the anterior
brain surface (BA: Figs. 4; DAL and DMM: Fig. 6), followed by those
of the dorsal surface (DPL: Fig. 9), posterior surface (DPM, CM, CP:
Fig. 13), and finally, lateral surface (BLA, BLD, BLP, BUV: Figs. 15 and
17). These figures illustrate the identification of clones with their
corresponding lineages. The second set of figures show z-projec-
tions of clones registered to DN-cad-labeled brain slices, in the
order as described above (BA: Figs. 2 and 3; DAL: Fig. 5; DMM:
Fig. 7; DPL: Figs. 8 and 10; DPM: Fig. 11; CM and CP: Fig. 13; BLA
and BLD: Fig. 14; BLP and BUV: Fig. 16), illustrating the projection
envelopes of all lineages. In all panels of all these figures, the adult
MARCM clone is paired with a larval clone (MARCM or Flip-out; see
"Materials and methods" for more details) representing the

![Fig. 6. Clones representing lineages of the DAL and DMM groups in the adult brain. (A, B) z-projections show brain slices at level of optic stalk/ellipsoid body lobes (A) and ellipsoid body (B) (C) z-projections of clones representing lineages of the DAL and DMM group. For a description of how panels are made and displayed, see legend of Fig. 4. For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.](image-url)
Fig. 2. (A–D) Clones representing lineages of the DAL group (#27/DALv3) and the DAM group in the larval and adult brain. For description of how panels are made and displayed, see legend of fig. 2. For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.

**BA lineages**

The BA group comprises PD, C, and D lineages whose neurons are mostly associated with the ventral brain compartments (Figs. 2–4; Figs. S1, S2). Four PD lineages, BAl1 (#1; Fig. 2A), BAlc (#5; Fig. 2D; dorsal hemlineage), BAIp4 (#9; Fig. 2H), and BAmq3 (#17; Fig. 3A) include all of the projection neurons connecting the antennal lobe (AL) and superior protocerebrum. After forming proximal (dendritic) branches in the AL, neurites of all neurons of these lineages converge and exit the AL posteriorly as the common antennal lobe tract (ALT; yellow arrows in Fig. 4C1, C5, C9, C17). Axons of BAl1 soon leave this bundle and directly head for the lateral horn (LH) via the median–lateral ALT (mALT; Fig. 2A, white arrowhead; Fig. S1). The remaining three SATs (#5, 9, 17) stay together as the median ALT (mALT) which continues dorso-posteriorly towards the calyx (Ca), before bending laterally towards the LH (arrowheads in Figs. 2D, H; 3H; Fig. S1). As published previously (Das et al., 2013), the lineage BAIp4 (#9) forms proximal branches that not only reach the AL, but also part of the ventrally adjacent subesophageal ganglion (abbreviated as SAstr in Fig. 2H; possibly a domain with gustatory input), and projects to the superior lateral protocerebrum (SLP), rather than the Ca and LH (Figs. 2H; S1). The ventral hemlineage of BAAlc (#5L) includes complex projection neurons which are mostly unlinked to the AL. They project ventro-posteriorly, forming the lvII fascicle (Figs. 4A, B; S2; see accompanying paper by Loević et al., 2013). Proximal branches of the lvII arborize in the antennomechanosensory and motor center (AMMC; Fig. 2D). Distally, this tract forms a T-junction, with one branch projecting laterally into the inferior domain of the ventro-lateral cerebrum (VLC) and the other branch crossing the midline via the great commissure to reach the contralateral inferior ventro-lateral cerebrum (VLCG; Figs. 2D, S2). In addition, a thin branch continues dorso-laterally towards the LH; this constitutes the lateral antennal lobe tract (not resolved in Figs. 2D; S1).

Two additional PD lineages, BAmq1 and BAmq2 (#11, #12; Figs. 3B, C), form a connection between the titocerebrum and the superior medial protocerebrum (SMP) and mushroom body, respectively. Thus, proximal branches of BAmq1/2 form dense arborizations in the titocerebrum. The titocerebrum is also called, in a segment-neutral manner, anterior periesophageal neuropil (PaP; Kumar et al., 2009b; Pereanu et al., 2010; Fig. 3B, C). The SATs of BAmq1/2 then project dorsally through the median bundle and reach the SMP (MBD1; Fig. 4C1, C12). Short distal terminal branches of BAmq1/2 here: BAmq1 bends laterally and forms terminal arbors in the ventral lobe of the mushroom body (MV; Figs. 3B, C; S1).

BAmq1 (#13) and BAmq2 (#14) are complex lineages with commissural tracts. The dorsal HSAT of BAmq1 (#13) projects medially directly behind the mushroom body medial lobe (ML) and crosses in the fronto-dorsal commissure; terminal branches innervate the medial lobe of both hemispheres, as well as the anterior inferior protocerebrum on the ipsilateral side (IPA; Figs. 3D; 4A; C13; S3). The ventral HSAT of BAmq2 (#13V) projects diagonally through the AL, crosses in the antennal lobes commissure (Aco), and then bifurcates into a dorsal branch directed towards the superior lateral protocerebrum (SLP) and a ventral branch with a large terminal domain in the lateral accessory lobe (LAL), ventro-medial cerebrum (VMC), and subesophageal ganglion (SEG) (Figs. 3D; 4C13; S4). BAmq2 (#14) enters near the midline, in between the antennal lobes of either side. The SAT bifurcates, with one branch crossing in the Aco (Figs. 4C14; S2). The ipsi- and contralateral branches project in a nearly symmetrical fashion postero-laterally, innervating the inferior ventro-lateral cerebrum (VLI) and posterior lateral protocerebrum (PLP) (Figs. 3E; S2).

Another complex PD lineage, BAmq1 (#15), is marked by the per-Gal4 driver line and has been documented previously (Spindler & Hartenstein, 2010; Spindler & Hartenstein, 2011). The large proximal SAT of BAmq1 (#15P) forms a major component of the roVM that passes underneath the AL into the ventro-medial cerebrum (VMC) (Fig. 4B, C15). The SAT splits into three
Fig. 8. (A–H) Clones representing lineages #33/DPLal1 to #42/DPLd of the DPL group in the larval and adult brain. For description of how panels are made and displayed, see legend of Fig. 2. For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.

major branches: one curving dorsally and medially towards the central complex; the second continuing posteriorly into the VMC; the third one extending laterally towards the ventro-lateral protocerebrum (VLPv) (Fig. S2). Terminal branches innervate the lateral accessory lobe (LAL), the fan-shaped body (FB), the noduli (NO), the VMC, and the VLP (shown in orange letters in Figs. 3F; S2). BArm2 (#16) has a distally branching (type D) single SAT that accompanies BArm1 in the loVLM fascicle #15; Fig 4B, C16). At the level of the great commissure (GC) the tract turns medially and dorsally and splits into an ipsilateral and contralateral component that innervate the VMC surrounding the great commissure (Figs. 3G; S2).

BAmp2 and BAmp3 (#7, #8) are lineages with long C-type SATs that contribute to the lateral component of the ventral longitudinal fascicle (loV; Fig. 4B, C7, C8). The BAmp2 tract splits into a dorsal branch with dense terminal fibers in the lateral part of the LAL compartment and a posterior branch that continues posteriorly (Fig. 2F), innervating the VC1 and PLP (Fig. S2). BAmp3 has a single tract that follows BAmp2 towards the VLM and PLP (Figs. 2G; S2).

BAmp3/4 (#3/4) and BAmp (#10) have C-type SATs, and BAmp1 (#6) has a PD-type SAT that enters from a position lateral of the AL (Fig. 4A, B, C). The first three of these lineages (#3/4, 6) project medially towards the ventro-medial protocerebrum: BAmp1 crosses the loVLM fascicle at its dorsal surface and BAmp3/4 at its ventral surface (Fig. 4A, B, C3/4; C6). BAmp1 has terminal arborizations within the VMC compartment, with ventral branches reaching into the
Fig. 9. Clones representing lineages of the DPL groups in the adult brain. (A, B) z-projections show brain slices at an anterior level (ellipsoid body; A) and posterior level (lateral bend of antennal lobe tract). (C) z-projections of clones representing lineages of the DPL group. For description of how panels are made and displayed, see legend of Fig. 4. The lineage pair BP104/3 is represented by three panels in C. The first two of these (C44/DPL2, C45/DPL3) show the clone registered to the posterior brain slice, to show association of the posterior HSAl with the ceil fibres (orange arrows, compare to panel B). In the third panel (C44/DPL2a) a z-projection of the anterior hemilineage of DPL3 is registered with anterior brain slice, showing entry point of anterior HSAl into dorsal SLP (orange arrow). For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.

SEG/PENPa (figs. 2E; S2). BAal3, marked by the driver en-Gal4 (Kumar et al., 2009b), has widespread terminals in the VMC (Fig. 2C; S2). BAal4 extends alongside BAal3; only a single type of clone was recovered for the BAal3/4 pair. BAal4 (#16) contacts the inferior VLC (VLC) from ventral (Fig. 4B; C10) and has terminal fibers confined to the VLC and neighboring AMMC (Fig. 3A; Fig. S2).
**DAL lineages**

Projections of the DAL lineages are predominantly associated with the medial and vertical lobes of the mushroom body (ML, VL), the central complex, and the adjacent protocerebral compartments (AOTU, SMP, Pa). Most of the DAL lineages, including the DALcl1/2, DALm1/2, DALd, DALU1, and DALU1-3 are PD lineages with long tracts, many of which are commissural.

DALcl1 and DALcl2 (#18, #19; Figs. 5A, B; 6C, 18, C19), located laterally of the mushroom body vertical lobe (VL), form a pair of PD lineages associated with the anterior optic tubercle (AOTU), central complex, and adjoining compartments; each one consists of two hemilineages whose diverging HSATs in a “pincer-like” manner enclose the mushroom body spur (SP; Figs. 6A, C18, C19). Dense proximal branches of DALcl1 and DALcl2 innervate the AOTU (Figs. 5A, B; 6A, S3). The ventral hemilineage tract of DALcl1 (#18v) passes underneath the SP and continues medially, crossing the midline in the subellipsoid commissure (SuEc; Figs. 5A, S3). Terminal arborizations of this tract end bilaterally in the LAL. In addition, on each side, a posteriorly directed branch of DALcl1v
projects along the MEF fascicle towards the posterior VMC compartment (VMCpo; Figs. 5 and 6A). The ventral DALc lineage projects as part of the lateral ellipsoid fascicle (LEf) towards the central complex (Figs. 5B, 6A, C19, S3). Dorsal hemilineages of DALc1/2 (#181/19d) curve over the dorsal surface of the spur (SP) and peduncle. DALc1 projects in a fairly restricted manner to the bulb (BU), a small compartment relaying information towards the ellipsoid body (EB; Figs. 5A, S3); DALc1/2 projects in a more widespread manner, including the BU, adjacent IAL, P, and SMP (Figs. 5B and S3).

DALc (#22) and the DALcm1/2 pair (#20/21) are located medially of the mushroom body vertical lobe (VI; Figs. 5C, D, 6B, C20–22). DALc (#22) constitutes a PD lineage with dense proximal arborizations in the IPa and the superior intermediate protocerebrum (SIP), which surround the medial lobe and vertical lobe, respectively (Figs. 5D and S4). The SAT of DALc (#22) projects ventro-medially, crossing the peduncle, and continues as part of the central protocerebral descending fascicle (dCP; Figs. 6B, C22). Distal arborizations are found in the VMC and SEG (Figs. 5D and S4). The lineage pairs DALcm1/2 each have two PD hemilineages with very similar projection patterns in all recovered clones. This suggests that both lineage pairs possess the same projection envelope. The medial hemilineage tracts pass behind the medial lobe (ML) and enter the frosso-medial commissure (FMC; Figs. 6C20–21 and S1). In terms of projection, arbors are found
Fig. 12. Clones representing lineages of the DPM group in the adult brain. (A, B) z-projections show brain slices at a posterior level (lateral bend of antennal lobe tract, A) and central level (fin-shaped body, great commissure, B). (C) z-projections of clones representing lineages of the DPM group. For description of how panels are made and displayed, see legend of Fig. 4. For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.

bilaterally in the ML and the surrounding IP, AO, SI, SIP, and SMP compartments (Figs. 5C and 5D). The ventral hemilineage tracts of D4Lcm1/2 pass through the elbow formed by the Vl and peduncle before turning ventrally (Figs. 5C and 5D). This projection initially forms part of the deCJ but then separates from the fascicle, extending into the ventral brain as a separate, loose bundle. The proximal terminal branches are found throughout the anterior domain of the SMP, IP, and the distal branches in the VMC (Figs. 5C and 5D).

Two DAL lineages, DAL1 and DAL2 (#23, #24), are located laterally of the DAlc1 group, flanking the anterior VLP compartment (VLPs; Figs. 5E, F and 5C23, C24). DAL1 (#23) is a PD lineage with a conspicuous recurrent projection. The SAT projects posteriorly, splits
into a ventral branch with arborizations in the PLP and adjacent lobula (LO), and a recurrent branch that turns dorsally and anteriorly forming arborizations in the anterior SLP, IP, and AOTU (Figs. 5E, 6B, and 55). DAL2 (#24; Fig. 5F) represents a C lineage. Its short SAT projects into the anterior VLPs where it splits into several groups of terminal arborizations filling much of the anterior and posterior VIP compartment (VIPa/p). A few "outlier" branches continue dorsomedially into the IP and VIP compartments (Fig. 5F).

The DALv group, comprising three PD lineages with commissural connections, is located ventral to the spur (SP). DALv1 (#25) has a long unbranched proximal SAT that forms the anterior component of the lateral equatorial fascicle (LEF; Figs. 5G; 6B, C5) and then bifurcates into an ipsilateral and commissural branch that crosses the great commissure. Terminal arborizations fill the ipsilateral and contralateral posterior VIP and neighboring VLP compartments (Fig. 5G). Ipsi-laterally there is a projection to the posterior SEG (not shown). DALv2 (#26) and DALv3 (#27) are marked by the expression of per-Gal4 and en-Gal4, as described previously (Kumar et al., 2009b; Spindler and Hartenstein, 2010; Spindler and Hartenstein, 2011); proximal SATs form the lateral ellipsoid fascicle (LEF; Figs. 5H; 6A, C26-27; 7A). The DALv2 (#26) lineage forms large proximal arborizations in the bulb (BU), as well as distal, ring-shaped branches of the ellipsoid body (EB; Figs. 5H; 55), that represent the ring (R)-neurons of the EB (Spindler and Hartenstein, 2011). Additional terminal arborizations of
DALv2 are found in the adjacent LAL and IPa (Fig. 5H). DALv3 (#27), marked by the expression of en (Kumar et al., 2009b), projects alongside DALv2 in the LFa fascicle, which then splits into a dorsal and ventral commissural branch (Figs. 6A, C2; S3). DALv3 terminal arborizations are confined to the ipsilateral and contralateral inferior protocerebrum (IPa/m) and the SMP (Fig. 7A; see Kumar et al., 2009b for detail).

**DAM lineages**

The small group of DAM lineages is located in the anterior dorso-medial cortex and has arborizations predominantly associated with the SMP/SIP and adjacent IPn/IPa compartments. DAMd1 (#28), a PD lineage with a unique recurrent commissural projection, first crosses the midline in the anterior-dorsal
commissure (ADC; Figs 6B, C2B; 7B). It forms profuse arborizations in the contralateral SMP, SII, and IPa; and crosses back via the fronto-medial commissure (frMC) to form distal arbors in the ipsilateral SMP and IPa (Figs 6B, C2B; 7B; S5). The DAMd2/3 pair (#29/30) comprises large C-type lineages (Figs 6B, 7C). Among the clones recovered for this pair, only a single type of projection envelope could be observed. The DAMd2/3 tract forms the anterior longitudinal superior medial fascicle (IoSMa), continuously giving
off terminal arborizations throughout the SLP, SMP, and IPm compartments (Figs. 6B, C2–30; 7C; S5). Posteriorly, projections of DAMd2/3 extend ventrally to fill regions of the ipsilateral and contralateral VMQpo (Figs. 7C; S5). The DAMv1/2 (#31/32) paired lineages also possess an indistinguishable projection envelope. The short SAT enters the SMP from anterior (Fig. 6B; C1–32) and splays out into dense terminal arborization, filling much of the SMP compartmen (Figs. 7D; S5).

**DPL lineages**

DPL lineages predominantly innervate the lateral domains of the superior and inferior protocerebrum. Five lineages, DPla1-3, DPlam, and DPlId represent the anterior subgroups, located dorso-laterally of the anterior optic tubercle (AOTu). DPla1-3 (#31–35) are PD lineages recognizable by their crescent shaped SAR which form the anterior transverse fascicle of the superior protocerebrum (trsA; Figs. 8A, B; 9A, C3–35; S5). Proximal arborizations of DPla1 (#33) fill the deep regions of the SLP, LH, and the adjacent lateral IP (IP1); distal arbors innervate the dorso-anterior SLP (Figs. 8A; S5). The DPla23 (#34/35) pair has an indistinguishable projection envelope, each with two terminal sites (Figs. 8B; 9C4, C35). The dorsal hemilinex (#34/25d) resembles DPla1 (#33), forming part of the trsA (Fig. 8A), but arbors more widely than DPla1 in the LH, SLP, SLP, SMP, and much of the IP (IPmI, Fig. 8B). The ventral HSAT forms projections in the medial IP and the
Fig. 17. Clones representing lineages of the ILP and BLV group in the adult brain. (A-C) $z$-projections show slices of the brain at an anterior level (A; level ellipsoid body), central level (B; fan-shaped body and lateral commissure) and posterior level (C; lateral bend of antennal lobe tract). (D) $z$-projections of clones representing lineages of the ILP and BLV group. The lineage pairs BLVa1/2 (89/90) and BLVa3/4 (91/92) each are shown by two panels which show differences in location of cell body clusters. Lineages BLVp1 (84/85) and BLVp2 (86/87) are shown in two panels each. The left panel (84/85 ILVp1 p; 86/87 ILVp2 p) shows the clone registered to the posterior brain slice, to show projection of the posterior HSAT of BLVp1/2 along the PIF fascicle (orange arrow). In the right panels (BLVp1 a, BLVp2 a) the anterior HSATs of these lineages, penetrating the VLP, are shown (orange arrow). For alphabetical list of all abbreviations see Table 1. Bar: 50 μm.
adjacent posterior VLP (VLPp) (Figs. 5B; S5). We recovered one clone where the two HSATs extended at a moderate distance from each other (Fig. 8C); this could represent a random variant, or indicate that DPLLa/DPLb do differ in regard to their exact HAT paracentricity. DPLam (#36) is a C-type lineage marked by the expression of engrailed and has been described previously (Kumar et al., 2008b). Projecting its single SAT ventro-posteriorly via the vertical tract of the superior lateral protocerebrum (vSLP; Figs. 8C, 9A, C36), DPLam arborizes widely in the anterior SIP and the central part of the IP/m and the VLPp (Figs. 8C, S4). DPLd (#42) forms sparse proximal arborizations in the SIP and part of the adjacent SLp (Figs. 8H, S42). The lineage has two HSATs, a medial one crossing the midline in the anterior-dorsal commissure (ADC) and projecting to the commissural SIP (S#2 m; Figs. 8H and 9A), and a posterior one that extends posteriorly along the anterior part of the iOsL fiber system, forming terminal arborizations in the LH and lateral SIP (#42p; Figs. 8H; S42; S5).

The remainder of the DPL group, including DPLc1-5, DPLc1-3, DPLc1-1-2, DPLc1-2, DPLc1-2p, and DPLc1p are located in the posterior brain cortex. DPLc1-5 (#37-41; Figs. 8D, G) enter through a common portal located at the junction between the SIP and SMP components (Fig. 8B; C37-41) and have arborizations focused on the superior and inferior protocerebrum. DPLc1 (#37) is a C lineage with a characteristic crescent-shaped tract that forms part of the medial trSP fiber system (trSPm, Figs. 8D; 9B, C37). Arborizations fill much of the SIP/SMP and the posterior part of the IP/m (Figs. 8D; S5). DPLc2/4 (#38, #40) is a C-type lineage pair that also forms part of the trSP fascicle (Fig. 8B, C38-40). Unlike DPLc1, DPLc2/4 do not curve dorso-medially into the more anterior and dorsal part of the SMP; rather the pair remains close to the IP/m, filling the compartment with widespread terminal arborizations and additional branches in the deep SIP/SMP compartments (Figs. 8E; S5). DPLc3 (#39), another C-type lineage, has a short, anteriorly-directed SAT and arborizes in the central parts of the SIP, SMP, and IP (Figs. 5B; S5). DPLc5 (#41) possesses two hemineurites (#41ap) which, in the adult, are spaced relatively far apart from one another. The anterior hemineurite produces a curved SAT that enters alongside DPLc1-4 (Fig. 9B, C41), extending antero-medially into the anterior SMP and part of the SIP; its dense terminal arborization fills this compartment and the adjacent domains of the IP (Figs. 8G; S5). The posterior DPLc5 hemineurite (#41p) is located at the ventro-posterior brain surface; the SAT projects antero-dorsally, joining the iOsL fascicle and crossing the midline in the ADC commissure (Fig. 9C41). Terminal arborizations overlap with those of the anterior hemineurite in the SMP and IPm (Fig. 8G). DPL11 (#43) and DPL2/3p (#44/45p) enter the posterior-lateral neuropil surface at the junction between the SIP and LH (Fig. 9C3-6). The DPL2/3p pair projects anteriorly, forming the iOsL fascicle (Fig. 8B). From the iOsL fascicle, terminal branches sprout off and innervate the superior brain compartments (LH, SIP, SL, and SMP) and ventrally directed branches also reach into the IPm, VLPp, and IP (Figs. 10B, E; F; S4, S5). While the DPL2/3p hemineurites innervate identical compartments, they have distinct fasciation patterns. Only one of the hemineurites, DPL2p (#4-6p), forms a tight tract; fibers of the other hemineurite (DPL2p, #4-6p) are more loosely aggregated (Fig. 10E, F). This same characteristic holds true for the anterior hemineurites (#44/ 45a). As described in detail in the accompanying paper (Lovick et al., 2013), the anterior hemineurite tracts of DPL2/3 (44/45a) shift forward during metamorphosis and enter the anterior surface of the SIP (Fig. 9A, C4, C45) they project ventrally into the upper part of the VLPp compartment (Fig. 10B,C). In contrast to DPL2/3 (#44a), which forms a thin, compact tract with dense endings in a narrowly defined subdomain of VLPp (Fig. 10C), DPL3a (#45a) axons form a loose tract and extend diffuse terminal arborizations along their entire trajectory from the SIP to the VLPp (Fig. 10D). DPL11 (#43) enters the brain at the same point as DPL2/3 (Fig. 9B, C43), but sends its tract medially via the trSP fascicle, arborizing in the posterior SIP/SMP; a lateral branch innervates the LH/Pp (Figs. 10A; S4, S5). DPL1m and DPLm2 (#46, #47; Fig. 10G, H) are located lateral of the DPLc cluster, dorsal of the mushroom body calyx, DPLm1 (#46) is a C-type lineage and projects anteriorly in the SIP (Fig. 9B, C46), producing branches in the SLp as well as the adjoining IP/l SIP compartments (Figs. 10G; S5). DPLm2 (#47) also innervates the SIP and adjacent IP; in addition, it sends a short SAT laterally (Fig. 9B, C47) to form a terminal arbor in the lateral horn (LH; Figs. 10H; S5). A long thin fiber bundle of DPLm2 leaves the brain and projects to the ring gland (Fig. 10H, arrowhead).

For the pair DPL1p1/2 (#48/49), we were only able to isolate a single clonal type (Fig. 10I). The paired tract enters the posterolateral neuropil surface at the base of the lateral horn (LH) and branches extends in the oblique posterior (obp) fascicle, across the peduncle and the brain midline, forming terminal arborizations along its trajectory in the posterior IP/l SIP of both hemispheres (Figs. 5C48, C49; 10I; S4). The antero-nearly HSAT of DPL1p1 penetrates into the LH and forms profuse terminal branches in this compartment (Figs. 9B; 11E; S4). A massive projection of DPL1p2 is directed ventrally (#48v) along the same tract to the LPL and posterior VLPc compartments (Figs. 10I; S4). The posterior-most of the DPL lineages, DPLpv (#50) enters the posterior neuropil surface ventro-laterally; its SAT follows the posterior lateral fascicle anteriorly (P1F; Fig. 9B, C50). Terminal branches appear along the entire length of the SAT and innervate the P1P/VLPc compartments and the adjacent IP (Figs. 10J; S3).

**DPM lineages**

Located in the postero-medial brain, DPM lineages are primarily connected with the compartments of the central complex and the medial protocerebrum (SMP, SIP, IP). Three of the DPM lineages (DPMm1, DPMmp1, and DPMmp2) are type II lineages which have been recently described (Bayaktar et al., 2010; Bello et al., 2008; Boone and Doe; 2008; Ito and Awasaki, 2008; Izzogina et al., 2008), where they were termed DM1 (DPMmp1), DM2 (DPMmp1), and DM3 (DPMmp2), respectively. Expression of two genes, distalless (Izzogina et al., 2008) and eumoff (Bayaktar et al., 2010), mark the type II lineages. Along with another type II lineage, CM4 (#62, see below; called DM4 in Bello et al., 2008), DPMm1, DPMmp1, and DPMmp2 (#53, #58, #59) include sub-lineages whose SATs characteristically enter through the dorsolateral and medial roots of the fan-shaped body (dHFb, mHFb; Figs. 12A, C53, C58, C59). They form the columnar neurons of the central complex, connecting specific small domains of the protocerebral bridge (BP) in a topographical manner with segments and sectors of the FR and SR, respectively (Ito and Awasaki, 2008; Yang et al., 2013; Figs. 11B, G, H; S3). In addition, these type II lineages have other sub-lineages with widespread terminal arborizations outside the central complex. The most prominent arborizations of DPMm1 (#53) are found in the (1) medial IP and deep layers of the adjacent SMPSLPSL (via SSAT #53a following the bSM1); (2) posterior VMC of both hemispheres (via SSAT #53d); (3) in the LAL, IFa, VLP, VMCI, SEG (via the anterior and descending SSATs #53c; Figs. 11B; 12A, C53; S4, S5). DPMmp1 via its long forward-directed SSAT #53a, has terminal arborizations in the anterior SIP, IPm, and PENP (cerebrom; Figs. 11G and S4). DPMmp2 (#59) arborizes more widely in the superior protocerebrum (SIP, SMP) and mushroom body lobes via its ISM-associated SSAT, #59a (Figs. 11H; S5).
Lineages DPMp1 and 2 (#55, #56) enter the posterior neuropil as the most lateral component of the posterior iO5M fascicle. The tract extends into the superior protocerebrum, with branches all along its length (SMP, SIF, SLP; Figs. 11D, E; 12A, C55, C56; S4; S5). DPMp1 is one of the lineages with a long descending fiber bundle, which leaves the iO5M, crosses in the chiasm of the median bundle (MBd;iCh1), follows the median bundle ventrally, and forms terminal arborizations in the PENpA (tritocerebrum), SEG, and the thoraco-abdominal ganglion (TAG; Figs. 11D; 12A, C55, S4). The SAT formed by DPMp2 (#56) has no descending projections, but, after leaving the iO5M, it continues medially into the FB where it forms wide-field arborizations (Figs. 11E, S3). DPMpB (#57), whose cell bodies are initially located close to those of DPMp1/2 (hence inclusion of this lineage in the same subgroup), but shift ventrally during metamorphosis, project along the MEF fascicle (Fig. 12B, C57) and innervate specific ventral compartments, including the VMpco, VLPp, and VLGc. This lineage also has a strong commissural component, reaching via the great commissure, the contralateral VLGc and VLPp (Figs. 11F, S2).

Two DPM lineages, DPM1 (#51) and DPM2 (#54), innervate the posterior brain compartments and send a descending tract towards the SEG and TAG (Figs. 11A, C; 12C51, C54; S4). DPM1 (#51) arborizes more ventrally than DPM2 (#54), including in the IP (IP; Wmp/p), VMp, VMpco, and SEG (Fig. 11A; S4). DPMm2 also branches in the posterior dilator of the IP (IP; Pmp/p), as well as in the adjoined SMP, SLP, and VMpco (Figs. 11C, C54; S4). DPM2 also has a laterally-directed branch which reaches the lobula (10B).

CM lineages

Three of the four CM lineages (CMI, #60; CM3, #61; CM4, #62; labeled DMS, DM6, and DM4, respectively, in Izergina et al., 2009) are large type II lineages with multiple sub-lineages. Each of the three has one major ventral SAT (the three ventral SATs of CM1–4), forming the iO5p fascicle (Labvick et al., 2013; #60v; in Fig 12A), and arborizes in the postero-ventral brain, including the VMp, VMc, PLP, and VLG compartments (Figs. 11A–C; S2). The ventral SATs of CM3/4 have a commissural component crossing in the pIP/PC commissure and reaching the postero-ventral compartments of the contralateral hemisphere (Figs. 13B, C; S2). The intermediate and dorsal SATs of the lineages CM1–4 (#60d in Fig. 12A) connect the postero-ventral brain to more anterior and dorsal regions of the neuropil. CM1 and CM3 each have one SAT (#60d and 61d2) that travels with the MEF fascicle and arborizes posteriorly (VMpco) as well as more anteriorly (VMc, IP/m, LAL; Figs. 12A, 13A, B; S2). CM1 (#60) has a conspicuous commissural component that interconnects the LAL compartments of either side (Figs. 12A, 13A; S2). CM3 (#61d1) also arborizes throughout the entire FB (Figs. 13B; S3). As described in the previous section, CM4 (#62) is one of the four lineages (besides DPMm1, DPMm2, and DPMp1) which produces columnar neurons of the central complex: the CM4 SAT (#62d) forming these arborizations is uncrossed and innervates the most lateral part of the PB and FR (Figs. 13C, S3). CM3 and CM4 have one other major SAT (#61/62a) that projects dorsally along the iO5M and interconnects dorsal protocerebral compartments along their antero-posterior axis (SIP/SMP; Figs. 13B, C; S5).

CM5 (#63), the most medial member of the CM group, has an SAT that enters the posterior neuropil medially of the MEF fascicle (Fig. 12A, C53). CM5 is the third lineage (besides DPM1 and DPMm2) which has a long SAT descending posteriorly towards the thoraco-abdominal ganglion (TAGc). Its proximal axons are seen on the VMpco compartment (Figs. 13D; S4).

CP lineages

The four CP lineages (CP1-4) are located laterally adjacent to the CM group and form mostly projection neurons associated with the superior and inferior protocerebrum. The CP2/3 pair (#65/66) each produces a dorsal and ventral HSAT (HSATn, HSATp) that have a characteristic spatial relationship to the mushroom body peduncle (Figs. 12A, C65, C66). Even though the two lineages innervate similar neuropil compartments, each shows distinctive differences. The lineage defined as CP2, with its dorsal HSAT (#65d), forms arborizations in the LH, SIF, and SMP and also projects to the mushroom body vertical lobe (VL) and fan-shaped body (FB) where it forms wide-field arborizations (Figs. 13C, S3; S5). The dorsal component of CP3 (#66d) has denser innervations in the LH, but misses the projection to the FB (Fig. 13H). The ventral HSATs of CP2/3 (#65/66v) project along the PLF fascicle that converges upon the peduncle from ventrally (Fig. 12A, C65, C66). They form terminal arbor along their axons in the ventral-lateral and inferior protocerebrum (PLF, VLP, PLp, IP/m; Figs. 13G, H; S2).

CP1 and CM4 (#64, #67) have similar SATs to the HSATs of CP2/3, crossing over the peduncle along the dOBp fascicle. Characteristically, the tracts of CP1 and 4 are closer to the peduncle than those of CP2/3 (compare Figs. 12G4/G7 to C65/C66). Both CP1 and CM4 have dense terminal arborizations in the LH, SIF, and SMP compartments (Figs. 13E, F). CP1 (#64), in the larval larval brain, has a dorsal (blue arrowhead in Fig. 13E) and ventral hemineurome (white arrowhead in Fig. 13E); the HSAT1 projects along the posterior LEF fascicle (LEf). In the adult, the tract of the dorsal hemineurome (#64) can be followed along the iO5M towards the MBXchci, where it runs DPMm2 and DPMm1 to descend towards the SEG/TA/G (Figs. 12G4/45; S4). We identified a total of four clones in different brains for CP1. However, none of them had a ventral hemineurome component, even though a CP104-positive LEf bundle could be clearly distinguished in the adult (Fig. 12A; see accompanying paper by Labvick et al., 2013). One possible explanation is that the ventral CP1 hemineurome undergoes apoptosis during metamorphosis. CP4 has only a dorsal component, both in the larva and adult (Figs. 13P; S5).

BLA lineages

BLA lineages are located at the antero-lateral neuropil surface. A subgroup of five dorsal BLAs (BLA1–4 (#66–71) and BLA1 (#72), forms SATs that converge on one neuropil fascicle, the t5s1 (Figs. 14A, B; 15B, 68–72; S5), which primarily interconnects domains of the superior protocerebrum. For the four BLAs defined in the larva, three types of clones with different projection envelopes were recovered (Fig. 14A); these were assigned arbitrarily to the lineages BLA1 (#68), BLA2 (#69), and BLA3/4 (#70/71). BLA1 arborizes in the LH and SIF (Fig. 14A); BLA2 is focused more on the SMP and SIF, but also an additional branch that follows the superficial t5s1 (t5s1s), and innervates the posterior SLP, IP, and IP/m (Figs. 14A, S5); BLA3/4 has restricted arborizations in the medial SLP (Figs. 14A, S5). BLA1 lineage (#72) has two separate hemineuromes. The dorsal hemineurome (#72d) projects along the t5s1 (Figs. 14 and 15B; D22) and arborizes in the posterior regions of the LH, SLP, and SMP compartments (Figs. 14B, S5). The medial hemineurome tract (#72m) follows the surface of the VLP, close to the anterior optic tract (green asterisk in Fig. 15A), and arborizes in the IP/m and PLP, respectively (Figs. 14B, S2).

The three ventral BLA lineages, BVAl (#73), BVLa2 (#74), and BLAvm (#75), have two hemineuromes each and interconnect ventro-lateral compartments of both hemispheres, also forming projections to the superior protocerebrum (Fig. 14G, F). The medial hemineurome of BLAv1 (#73m) projects over the anterior surface of
the VLPa compartment, following the anterior optic tract (Fig. 15A, D73); the tract then extends underneath the peduncle and crosses the midline in the superior arch commissure (SAC). Branches innervate (ipsi- and contra-laterally) the VLPa and the anterior SL/P/P compartments (Figs. 14C; S1). The medial hemisphere of BLAv2 (#74 m) projects medially through the VLPa compartment along the hVLpF tract (Fig. 15A, D74). Although the HSATp of the BLAv2 lineage arborizes in a similar ipsilateral territory as BLAv1m (IP/m, Fig. 14D), the hemisphere lacks a strong commissural component across the SAC, but forms a bundle which crosses posteriorly of the central complex towards the contralateral IP (Fig. 14D, blue arrowhead). The posterior hemineurones of BLAv1/2 (#73/74p) are directed through the ventral-lateral protocerebrum (Fig. 15A) and across the great commissure, arborizing bilaterally in the VLPa (Figs. 14C, D; 15D73, C74; S2). The posterior hemisphere of BLAv2 (#74p) has a strong dorsally-directed branch towards the LH and SL/P/P compartments (Figs. 14D; S1). The HSATp of BLAvm (#75 p) is located at the antero-dorsal surface of the VLP, where it projects dorsally, passing the anterior optic tract (green asterisk in Fig. 15A, D75). The HSATp of BLAvm has widespread terminal arborizations in the dorsal VLs and dorso-posterior adjacent compartments: the SLP, IP/m, and PLP (Figs. 14E; S1). The posterior hemisphere of BLAvm (#75 p) is located at the lateral surface of the ventro-lateral protocerebrum. Its tract, similar to that of BLA1, follows the trajectory of the anterior optic tract (Fig. 15B, D75). Anteriorly, it sends arborizations into the LAL compartment (Fig. 14E); posteriorly, it innervates the PLP (Figs. 14E; S2).

**BLD Lineages**

Six BLD lineages were defined in the larva. The anterior four of these BLD1-4 (#77-80), lie posteriorly adjacent to the dorsal BLAs and, like those, mostly innervate the superior protocerebrum with tracts forming the superficial component of the trS (Figs. 14F-I; 15B, D77-80; S5). The terminal arborizations of BLD1 (#77) are fairly restricted to the LH (Figs. 14F, S5); BLD3 (#79) and BLD4 (#80) also innervate the LH, but have more widespread arborizations in adjacent areas of the superior protocerebrum (SLP, LPM, SIP; Figs. 14H, I; S5). Ventrolaterally directed branches of BLD3/4 arborize in the VLP compartment (Figs. 14H, I; S4). BLD2 (#78) follows the t6S1 towards the posterior neuropil surface (Fig. 15B), arborizing in the posterior SLP (SMP, the IP/m, and PLP (Figs. 14G; S5). BLD1 and BLD2 each have an additional hemineurone. In the case of BLD1, these neurons (#77p) are located further posteriorly and project into the PLP (Fig. 15C, D77). Terminal arborizations are to be found in the PLP, IP and the lobula (Figs. 14F, S4). In the case of BLD3, one finds an anterior hemineurone (#79a) with projections to the ventral tip of the VLP compartment (Fig. 14I).

Two additional BLD lineages, BLD5 and BLD6 (#82 and 83), are located at the post-lateral neuropil surface and form connections with the lobula (LO) (Fig. 16A-B). BLD5, marked by the expression of the gene atonal (Hassan et al., 2000; Spindler and Hartenstein, 2010; Spindler and Hartenstein, 2011), has a characteristic straight commissural tract interconnecting the ipsi- and contra-lateral lobula (Figs. 15C, D82, 16A). BLD6 is located ventromost posteriorly (Fig. 15C, D83); it has widespread arborizations in the LO, but innervates a restricted "focus" located in the posterior VLP (Fig. 16B).

**BLP Lineages**

BLP lineages form two pairs in the posterolateral brain. BLP1/2 (#84/85) are located ventrally, projecting along the PLF fascicle that innervates ventral domains of the VLP compartments (Figs. 16C; 17C, D84, D85; S2). With the exception of a single clone (Fig. S7), all other clones assigned to the BLP1/2 pair have the same fairly restricted projection envelope (Fig. 16C). The clone belonging to the exception (Fig. S7) possesses much more widespread arborizations in the PLP and posterior domains of the IP. This clone suggests that BLP1 and 2 possess different envelopes. BLP3/4 (#86/87) lineages are located dorsally; the pair sends a short dorso-ventrally directed tract dorso-ventrally, innervating the LH and the adjacent SL/P/P compartment (Figs. 16D; 17C, D86, D87; S5).

**BLV Lineages**

BLV lineages, similar to the BIA lineages group, innervate the ventro- and dorso-lateral protocerebrum. BLV3/4 (#91/92) form a pair whose short SAT penetrates into the VLP from ventrally; the lineage pair forms restricted terminal arborizations in the VLP and the neighboring VLCi (Figs. 16F; 17A, D91, D92; S2). Even though the projection envelope of all clones recovered for this lineage is similar, it appears as if in some clones, the cell body cluster and SAT entry point are located more posteriorly than more other clones (Fig. 17D92/93, "posteror variant" of BLV3/4"). BLV4/2 (#89/90) form another pair with SATs that enter at the ventral side of the trSI fascicle (Fig. 17B) and terminates short time thereafter (at the junction of the IP/SIP; Figs. 16E; 17D90/96; S5). Terminal arborizations of the BLV4/2 pair are focused on the LH, SL/P/P, and adjacent IP/m compartments. Recovered clones of the BLV4/2 pair had very similar projection envelopes but differed with respect to the location of the cell body clusters. In four out of 13 clones, the cluster was located dorso-anteriorly of the VLP, at the level of the BIAd lineages (“dorsal variant” of BLV1/2; Fig. 17D). The BLV1/2 reporter, so-Gal4, is expressed in the larval lineages (Chang et al., 2003) and remains on in the “dorsal variant” in the adult (VH, unpublished). In the remaining eight BLV1/2 clones, the cell body clusters are spread out within an elongated volume of the cuticle in the deit between the optic lobe and VLP (Fig. 16E and left panel of Fig. 17D90/96). We speculate that these two variants (one with a dorsal position; the other with a ventral or spread-out dorsal-ventral position) represent the two lineages of the BLV1/2 pair.

BLVp1/2 (#93, 94) each have two hemineurones that migrate apart during metamorphosis. The posterior hemineurones of BLVp1/2 (#93/94p; HSATp) project along the PLF (Fig. 17C, D93, 94). The BLV1 HSATp innervates the VLPc and SL/P/P compartments and, via a commissural tract crossing as part of the great commissure, innervates the contralateral VLP (Fig. 16G; 17D94; S2). The HSATp of the BLV2p lineage has a different trajectory; two branches project dorsally to the superior protocerebrum (SLP, SIP, and SMP) and the anterior IP (IPa and IP/m; Figs. 16H; S1). The anterior hemineurones of BLVp1/2 (#93a/94a) are located at the ventro-anterior brain surface and laterally adjacent to BLV3/4 (Fig. 17A, D93, D94). Terminal arborizations branch in the VLP, SL/P/P, and LH (Figs. 16G, H; S1). BLVp2 has two strong commissural component which crosses the SAC and projects to the contralateral VLP (Figs. 16H; S1).

**Discussion**

Identification of MARCM clones with secondary lineages

We show in this paper that MARCM clones induced in the early larva, labeling post-embryonically derived (e.g. secondary) neurons, can be assigned to specific lineages based on the stereotyped trajectory of their axonal tracts. These tracts are formed in the larva and, as documented in the accompanying paper, remain
visible as coherent, BP104-positive fiber bundles throughout metamorphosis (Locke et al., 2013). Fifty-six lineages form tracts with unique properties; all but one of these 56 lineages (e.g., DALv3, marbled by en-Gal4; Kumar et al., 2008b) has been represented in our collection of MARCM clones. All other lineages were represented at least twice, with many of them occurring at high frequencies (n > 25). We currently have no explanation for the existence of such “hot” and “cold” spots of clone induction. Given that the lineages begin to proliferate at slightly different time points at the first-to-second larval instar transition (Ito and Hotta, 1992; J.K.I. and V.H., unpublished), we speculate that the exact timing of the heat pulse may play an important role for the large variations in the frequencies of clone generation.

Thirty lineages are paired (tracts of two adjacent clusters form one composite bundle) and eight form two “quartets” (four tracts coalesce into a single thick bundle). One of the “quartets” represents lineages of the mushroom body (MB), while the others are the four BLA4 lineages (BLA14–BLA4). Based on the individually labeled lineage MARCM clones, it is clear that within these composite bundles, axons of the different lineages do not intermingle. In brains labeled only with global markers (e.g., BP104), one cannot separate individual SATs within the pairs and quartets, and cannot predict how many clones with different projection envelopes to expect. All members of a pair/quartet could either form clones with identical projection envelopes or they could form two/four anatomically distinct clones. In nine cases, each member of the pair has a unique arborization pattern. For example, BAmas1/2, both of which project along the median bundle, appear to have distinct fields of arborization: proximal branches of BAmas1 project in the ventral PENMa (tricerebrum) and the SEG, projecting towards the V1/SMP, while BAmas2 form proximal arborizations dorsally in the PENPa and distally and bilaterally in the SMP. Importantly, although these projection envelopes are clearly different, they include adjacent brain territories. This generally holds true for most lineages: lineages with neurons (and, at an earlier stage, neuroblasts) located close to each other also typically innervate adjacent neuropil territories (see schematic representations of projection envelopes in Figs. 51–55).

Another case in point is the quartet BLA14–BLA4, for which we could identify three different types of clones whose projection envelopes were all confined to the superior protocerebrum where they targeted contiguous territories (BLA14: lateral horn; BLA4: lateral SIP, SIP, SMP, BLA3: medial SIP).

The fact that in the case of the BLA4 lineages we identified three, and not four types of clones could mean that “the fourth” BLA4 lineage has a projection envelope that is indistinguishable from one of the other three BLA4 members; alternatively, we might have missed the clone, since that particular lineage (like DALv3 mentioned above) represents a cold spot of inducibility. The same reasoning applies to six pairs of lineages (see Table 2) for which also a single clone type was noted. It is unlikely that for all of these pairs one of the members was missed, given their good overall representation (e.g., 10 clones for the BAla3/4 pair, 11 for DALcm1/2, 14 for DAmd2/3, and 13 for DAmv1/2). In these cases, we favor the interpretation that a lineage might have been “duplicated” to increase the overall number of neurons sharing the same projection envelope.

**Lineage-based analysis of brain macrocircuitry**

With respect to the overall shape of their projection envelopes, lineages fall into several classes. A more in-depth discussion of these different classes will have to await the detailed analysis of other projection envelopes relative to each other, and relative to the boundaries of neuropil compartments. To this end, clones are being registered to one “model brain,” in which their spatial relationships can be established. We anticipate that this work (D.C.C.W. and V.H., unpublished) promises to yield further insight regarding the validity of compartment boundaries (Do projection envelopes of multiple lineages respect the boundaries defined on the basis of antigen expression? Do projection envelopes respect additional subdivisions of compartments?) as well as regarding brain macrocircuitry (How strongly are compartments connected on the basis of sharing in a certain number of projection envelopes?).

Some of these questions have been already addressed in two recent papers where, employing a MARCM-based approach, Ito et al., (2013) and Yu et al. (2013) have published a comprehensive atlas of secondary lineages for the adult Drosophila brain. They chose a terminology in which the term for a lineage was based upon one of the neuropil compartments heavily innervated by that lineage. Both studies concur with our conclusion that the pattern of projection and arborization of secondary lineages is highly invariant. Based on their characteristic SAT projection and projection envelope, most lineages depicted in Ito et al. (2013) and Yu et al. (2013) can be identified with the secondary lineages described here, even on the basis of z-projections alone. Note for example the close correspondence between the projection envelopes shown for the well documented type II DPM, CM, and CP lineages (DPMm1–CM1; DPMp1–DM2; DPMp2–DM3; CM4–DM4; CM3–DM6; CM1–DM5; CP1–DI1; CP3–DI2), but also other new described lineages with long, characteristic SAT trajectories (e.g., BAmas1–RLAa2; BAmas2–FLAa3; BAmas2–Weda2; DALc11–AOTUV3; DALv1–VLPa2; BLAv1–VLPb1). However, given the large number of clones described in Ito et al. (2013) and Yu et al. (2013) are presented in the absence of labeled fasicles, the unambiguous matching of their nomenclature with ours should await the careful comparison of confocal stacks. Nonetheless, three aspects concerning the overall coverage of lineages applying different driver lines to visualize clones deserve mentioning.

First, “cold spots,” i.e., lineages, known to exist on the basis of independent data, which are not represented by MARCM clones are apparent in all three studies. Interestingly, DALv3 (independently documented by its expression of en-Gal4; Kumar et al., 2000) seems to be a “universal cold spot,” since it is not represented in our study, neither in that of Ito et al. (2013) or Yu et al. (2013). Other cold spots may depend on the driver line used; for example, the characteristic lineage of local antennal lobe interneurons, BALa2 (Das et al., 2013) is represented by multiple clones in our study, as well as in Yu et al. (2013), where it is called Alv2, but not in Ito et al. (2013). Similarly, BAmas1 (FLAa2) and BAmas2 (VESa2) are represented by clones in Yu et al. (2013), but not Ito et al. (2013).

Secondly, the mapping of lineages in our study is restricted to clones that could be clearly assigned to the BP104-positive fiber bundles corresponding to lineage associated axon tracts traceable from the larva into the adult. Following this approach, we could assign a type of clone to each lineage originally defined in the larva based on possessing a neuroblast and a unique SAT; the only exceptions were the DALv3 “cold spot,” and the uncertainty concerning the six paired lineages for which we recovered only clones (see above). The study by Ito et al., (2013) lists a sizeable number of clones (e.g., the majority of their VPN clones), located in the lateral brain and including projections between optic lobe and central brain, that are most likely derived from neuroblasts that originate from the inner optic anlage. These lineages were not included in our larval catalog of central brain lineages (Pereanu and Hartenstein, 2006; Cardona et al., 2010), and are not considered in this paper. We have, however, observed frequent examples of VPN-type clones. The developmental definition of a boundary between central brain and optic lobe, in particular lobula, is complex, and will require further work.
Finally, the participation of secondary lineages in the production of glia also needs further clarification. All classes of glia increase in number during the larval period, and part of this increase is due to the proliferation of dedicated glial progenitors (or glial cells themselves, which continue to divide), whereas another part results from the generation of glial cells from secondary (neural) lineages (Pereanu et al., 2005). More recent studies have shown that several of the type II lineages are responsible for the generation of much of the ensheathing glia of the central complex (Viktorin et al., 2011), as well as some of the optic lobe associated glia (Viktorin et al., 2013). The use of elav-Gal4 as a driver precluded us from visualizing glial progeny among the dorsicles described in this study. However, Yu et al. (2013), utilizing flip-out techniques (actinSC-FRT-stop-FRT-GAL4 and actinSC-loxP-stop-loxP-GAL4) and twin spot MARCM (nSyb-GAL4), describe a small number of lineages that included glia among their progeny. One of these, DM5 (CM1 in our nomenclature), produced both ensheathing glia and astrocyte-like glia; another one, DL1 (CF2 in our nomenclature) generated a population of optic lobe-associated ensheathing glia that most likely corresponded to the set of glia described in Viktorin et al. (2013). However, none of the large number of central complex-associated ensheathing glia, derived from the type II DM lineages according to Viktorin et al. (2011), were labeled in Yu et al. (2013) or Ito et al. (2013). Aside from the possibility that this is due to a property of the driver line used, one may explain this discrepancy by the timing of clone induction. Thus, if the very first sublineages generated by the DM neuroblasts are dedicated glial progenitors, one might miss their progeny if dorsal induction occurs at a slightly later time point.

A lineage-based approach to study mechanisms controlling Drosophila brain development

In previous studies, a number of secondary lineages marked by Gal4-reporters had been identified in the larval brain and linked to their adult counterparts. The best characterized lineages are the four lineages of the mushroom body (Ito et al., 2007; Ito and Awasaki, 2008; Lee et al., 1999; Zhu et al., 2003) and the five which form the projection and local interneurons of the antennal lobe (Das et al., 2013; Das et al., 2008; Komiyama et al., 2003; Li et al., 2008; Yu et al., 2010). Additional lineages have been characterized based on the restricted expression pattern of transgenic reporters and protein markers (e.g., en-Gal4; ato-Gal4; per-Gal4; empty spiracles, ene; Hassan et al., 2000; Kumar et al., 2009b; Lichtneckert et al., 2008; Spindler and Hartenstein, 2010; Spindler and Hartenstein 2011; Srahna et al., 2006). The identification of projection envelopes of adult MARCM clones for all central brain lineages presented in this paper will aid in the identification of additional lineage-specific markers from among the numerous existing collections of Gal4 enhancer-trap lines (Payashi et al., 2002; Jenett et al., 2012; Pfeffer et al., 2008).

Taking advantage of the fact that lineages form structural units whose individual neurons share a common trajectory and terminal arborization, a selected number of genes (encoding for members ofdevelopmentally-relevant molecular pathways or important cell-cell interactions such as adhesion molecules) have been analyzed using a lineage-based approach. This type of approach was pioneered in a series of studies that revolve around the MB lineages. In these studies, the roles of many crucial players important for proliferation, cytoskeletal dynamics, and cell-to-cell adhesion were dissected through conditional loss- and gain-of-function experiments, using MB-specific drivers under Ga4/UAS control (Bilhurt et al., 2001; Lee et al., 2000a,b; Ng et al., 2002; Reuter et al., 2003; Scott et al., 2001). Additional lineage-specific (e.g. non-MB) Gal4 drivers have been identified and similar approaches (like the MB studies) have been taken to identify critical players for secondary lineage morphogenesis (through conditional knock-outs and gain-of-function, as described above; Bello et al., 2003; Kuert et al., 2012; Martin et al., 2012; Maurange et al., 2008; Spindler and Hartenstein, 2011; Zheng et al., 2006).

More recently, it has been demonstrated that neurons lineages, which may express a common set of molecular factors, react very differently to the loss of these factors. This has been made possible by the identification and characterization of lineage-specific Gal4 lines and highlights the importance of utilizing a multi-lineage approach when studying neural development. One example is the role of the Par-complex proteins, Bazooka (Baz)/Par-3/Par-6, in determining the shape of secondary neurons (Spindler and Hartenstein, 2011). Outside the nervous system, the Par complex plays an essential role for epithelial cell polarity and migration (Cong et al., 2010; Ellenbroek et al., 2012; Hurd et al., 2003; Ohno, 2001; Wang et al., 2006; Wang et al., 2012), in some vertebrate neurons, Par appears to be required for the differentiation of nascent neuronal processes into axons and dendrites (Chen et al., 2006; Nishimura et al., 2004; Shi et al., 2004). In the Drosophila post-embryonic brain, Baz is expressed by neuronal progenitors and postmitotic neurons. A Gal4-inducible Baz:GFP fusion reporter (under the control of UAS enhancer sequences; Sánchez-Soriano et al., 2005) driven by per-Gal4 (marking BAla1, BAlav1, DAlav2 and ato-Gal4 (marking BLD5) revealed that the Baz protein accumulates at positions along the SATs where terminal branches will appear (Spindler and Hartenstein, 2011). For example, in the PD-type lineages BAla1 (using per-Gal4) and BLD5 (using ato-Gal4), Baz:GFP is both concentrated at the cortex-neuropil boundary and the distal ending of the SAT. Loss of function of Baz by MARCM results in ectopic terminal branches, either along the SAT (DAlav2 and BAlav1) or at its distal tip (BLD5). In the case of BAla1, loss of baz results in aberrant pathway choices, forming additional SATs into the iALT (Spindler and Hartenstein, 2011). Interestingly, loss-of-function of another member of the Par-family, par6, phenocopies baz loss-of-function clones in case of the DALav2 and BLD5 lineage, but not BAla1 and BAlav1, further supporting the notion that the requirement of different Par-complex members varies from one lineage to the next. A previous study, demonstrating that the Par complex is not required for the development of mushroom body lineages (Rolls and Doe, 2004), also supports this idea. Although the mushroom body lineages have been traditionally used as “test lineages” to understand gene function, it is clear that such an approach may not be sufficient. Since gene function (in the case of baz and par6) may be lineage-dependent, a “multi-lineage approach” is more favorable and will provide a clearer picture of gene function in developing neurons.

Dissecting lineages: hemilineages, sub-lineages, neurons

Many type I lineages consist of two hemilineages (Truman et al., 2010). To generate hemilineages, a neuroblast divides asymmetrically to produce an intermediate cell (ganglion mother cell, GMC). The GMC divides symmetrically to produce two postmitotic sibling neurons. Typically, cell fate determinants (e.g., Numb, a repressor of Notch signaling) are asymmetrically segregated into the two neurons, such that one cell acquires an ‘A’ fate (inherits Numb and represses active Notch signaling) and the other acquires a ‘B’ fate (does not inherit Numb and has active Notch signaling; Lin et al., 2012; Truman et al., 2010). In some cases, one hemilineage is fated to undergo programmed cell death (Kumar et al., 2009a; Truman et al., 2010); in others, both hemilineages survive, but are morphologically and most likely, functionally unique. It was suggested that in cases where both hemilineages survive, two separate bundles or HSAIs, emerge
Table 1

List of abbreviations of neuropil fascicles (left), compartments (center), and entry portals of lineage-associated tracts (right)

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Anterior dorsal commissure</td>
<td>ADC</td>
<td>Antennal lobe</td>
<td>AL</td>
<td>Dorsal entry portal of the AL</td>
<td>ptAL d</td>
</tr>
<tr>
<td>Antennal lobe commissure</td>
<td>ALC</td>
<td>Antennal-ANO somosensory</td>
<td>AMMC</td>
<td>Lateral entry portal of the AL</td>
<td>ptAL l</td>
</tr>
<tr>
<td>Inner antennal-cerebral tract</td>
<td>iACT</td>
<td>and motor center</td>
<td>Ventral entry portal of the AL</td>
<td>ptAL v</td>
<td></td>
</tr>
<tr>
<td>Medial antennal-cerebral tract</td>
<td>mACT</td>
<td>Anterior periesophageal neuropile</td>
<td>PONPa</td>
<td>Ventrolateral entry portal of the AL</td>
<td>ptAL vl</td>
</tr>
<tr>
<td>Outer antennal-cerebral tract</td>
<td>oACT</td>
<td>Posterior periesophageal neuropile</td>
<td>PONPp</td>
<td>Ventromedial entry portal of the AL</td>
<td>ptAL vl</td>
</tr>
<tr>
<td>Cervical Connective</td>
<td>CCT</td>
<td>Fan-shaped body</td>
<td>FB</td>
<td>Lateral entry portal of the CA</td>
<td>ptCA l</td>
</tr>
<tr>
<td>Commissure of the lateral accessory lobe</td>
<td>LALC</td>
<td>Protocerebral bridge</td>
<td>PB</td>
<td>Medial entry portal of the CA</td>
<td>ptCA m</td>
</tr>
<tr>
<td>Dorsolateral root of the fan-shaped body</td>
<td>dirFB</td>
<td>Inferior protocerebrum</td>
<td>IP</td>
<td>Ventral entry portal of the CA</td>
<td>ptCA v</td>
</tr>
<tr>
<td>Horizontal VLP tract</td>
<td>hVLP</td>
<td>Anterior IP</td>
<td>IPa</td>
<td>Ventrolateral entry portal of the CA</td>
<td>ptCA vl</td>
</tr>
<tr>
<td>Frontomedial commissure</td>
<td>FrMC</td>
<td>Lateral IP</td>
<td>IPl</td>
<td>Anterior entry portal of the LH</td>
<td>ptLH a</td>
</tr>
<tr>
<td>Great commissure</td>
<td>GC</td>
<td>Medial IP</td>
<td>IPm</td>
<td>Posterior entry portal of the LH</td>
<td>ptLH p</td>
</tr>
<tr>
<td>Intermediate superior transverse fascicle</td>
<td>(superficial/deep)</td>
<td>trSis/d</td>
<td>Posterior IP</td>
<td>IPp</td>
<td>Anterior entry portal of the ML</td>
</tr>
<tr>
<td>Lateral ellipsoid fascicle</td>
<td>LE</td>
<td>Lateral accessory lobe</td>
<td>LAL</td>
<td>Dorso-lateral entry portal of the ML</td>
<td>ptML dl</td>
</tr>
<tr>
<td>Anterior LE</td>
<td>LEa</td>
<td>Lateral horn</td>
<td>LH</td>
<td>Dorso-medial entry portal of the ML</td>
<td>ptML dm</td>
</tr>
<tr>
<td>Posterior LE</td>
<td>LEp</td>
<td>Mushroom body</td>
<td>MB</td>
<td>Dorso-lateral entry portal of the PB</td>
<td>ptPB dl</td>
</tr>
<tr>
<td>Lateral equatorial fascicle</td>
<td>LEF</td>
<td>Calyx of MB</td>
<td>CA</td>
<td>Dorso-medial entry portal of the PB</td>
<td>ptPB dm</td>
</tr>
<tr>
<td>Anterior LEF</td>
<td>LEFa</td>
<td>Medial lobe of MB</td>
<td>ML</td>
<td>Ventral entry portal of the PB</td>
<td>ptPB v</td>
</tr>
<tr>
<td>Posterior LEF</td>
<td>LEFp</td>
<td>Peduncle of MB</td>
<td>P/PED</td>
<td>Lateral entry portal of the PLP</td>
<td>ptPLP l</td>
</tr>
<tr>
<td>Longitudinal superior lateral fascicle</td>
<td>IoSL</td>
<td>Spur of MB</td>
<td>SP</td>
<td>Postero-inferior entry portal of the PLP</td>
<td>ptPLP pi</td>
</tr>
<tr>
<td>Medial equatorial fascicle</td>
<td>MEF</td>
<td>Vertical lobe of MB</td>
<td>VL</td>
<td>Postero-superior entry portal of the PLP</td>
<td>ptPLP ps</td>
</tr>
<tr>
<td>Medial root of the fan-shaped body</td>
<td>mrFB</td>
<td>Anterior optic tubercle</td>
<td>OTU</td>
<td>Anterior entry portal of the SLP</td>
<td>ptSLP a</td>
</tr>
<tr>
<td>Longitudinal superior medial fascicle</td>
<td>IoSM</td>
<td>Posterior lateral protocerebrum</td>
<td>PLP</td>
<td>Lateral entry portal of the SLP</td>
<td>ptSLP l</td>
</tr>
<tr>
<td>Anterior IoSM</td>
<td>IoSma</td>
<td>Subesophageal ganglion</td>
<td>SOG</td>
<td>Posterior entry portal of the SLP</td>
<td>ptSLP p</td>
</tr>
<tr>
<td>Posterior IoSM</td>
<td>IoSmP</td>
<td>Superior protocerebrum</td>
<td>SP</td>
<td>Postero-lateral entry portal of the SLP</td>
<td>ptSLP pi</td>
</tr>
<tr>
<td>Median bundle</td>
<td>MBDL</td>
<td>Superior intermediate protocerebrum</td>
<td>SIP</td>
<td>Postero-medial entry portal of the SLP</td>
<td>ptSLP pm</td>
</tr>
<tr>
<td>Oblique posterior fascicle</td>
<td>obP</td>
<td>Superior lateral protocerebrum</td>
<td>SLP</td>
<td>Dorsal entry portal of the SP</td>
<td>ptSP d</td>
</tr>
<tr>
<td>Posterior commissure of the PLP</td>
<td>pPLPC</td>
<td>Anterior SLP</td>
<td>SLPa</td>
<td>Ventral entry portal of the SP</td>
<td>ptSP v</td>
</tr>
<tr>
<td>Posterolateral fascicle</td>
<td>PLF</td>
<td>Posterior SLP</td>
<td>SLPp</td>
<td>Dorso-medial entry portal of the VL</td>
<td>ptVL dm</td>
</tr>
<tr>
<td>Transverse superior posterior fascicle</td>
<td>trSP</td>
<td>Superior medial protocerebrum</td>
<td>SMP</td>
<td>Lateral entry portal of the VL</td>
<td>ptVL l</td>
</tr>
<tr>
<td>Lateral trSP</td>
<td>trSPI</td>
<td>Inferior Ventrolateral cerebrum</td>
<td>VLCi</td>
<td>Ventral entry portal of the VCLi</td>
<td>ptVLCi v</td>
</tr>
<tr>
<td>Medial trSP</td>
<td>trSPm</td>
<td>Ventrolateral protocerebrum</td>
<td>VLP</td>
<td>Antero-dorsal entry portal of the VLP</td>
<td>ptVLP acl</td>
</tr>
<tr>
<td>Superior commissure of the PLP</td>
<td>sPLPC</td>
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<td>VLPa</td>
<td>Inferior dorso-lateral entry portal of the VLP</td>
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<td>ptVLP dl</td>
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from the cell body cluster. Upon entering the neuropil, the two \( \text{HSATs} \) diverge and target different neuropil compartments. In this work and the accompanying paper (Lovick et al., 2013), we have identified 12 lineages possessing these properties (see Table 2). In the majority of cases, both the hemilineage cell body clusters and the neuropil entry points of their \( \text{HSATs} \) move apart to some extent during metamorphosis. This morphogenetic shift is extreme for several lineages (e.g. \( \text{DPL1/2} \), \( \text{DPLc5} \), \( \text{BLAV1} \), \( \text{BLAVm} \), \( \text{BLVp1/2} \)). For eight of these lineages, although there is a single cell body cluster and SAT; the tract splits into two components with different trajectories (Table 2). In these cases, it remains unclear whether the existence of more than one tract suggests that there are two separate hemilineages; an in-depth analysis of individual neurons forming parts of these lineages may help answer this question.

Lineages and hemilineages are comprised of sequentially-born neurons, which may all share in the common projection envelope; however, they can be grouped into sub-classes which differ among each other in regards to their detailed phenotype (e.g. projection patterns). This has been investigated for the four lineages of the MB and for some of the lineages associated with the AL (Jeffries et al., 2001, 2004; Kunz et al., 2012; Lai et al., 2008; Lee et al., 1999; Yu et al., 2006; Zhu et al., 2003). The MB lineages undergo four sequential phases of proliferation, producing sub-lineages with different proportions (Lee et al., 1999; Zhu et al., 2003; Kunz et al., 2012). In the early embryo, MB neuroblasts give rise to a small set of non-intrinsic neurons (as opposed to the intrinsic neurons or Kenyon cells) that do not contribute to the neuropil of the mushroom body (Kunz et al., 2012). From mid-embryonic stages onward, the MB lineages switch to a mode that generates \( \gamma \)-neurons, followed by \( \alpha/\beta \) neurons (most of third instar larva), pioneer \( \alpha/\beta \) neurons (at the start of metamorphosis), and \( \alpha/\beta \) neurons (during mid-to-late phases of metamorphosis). Within these sub-lineages, neurons might form even smaller groupings, defined by the specific territory inside the calyx or MB lobes they innervate. For example, \( \alpha/\beta \) neurons born at different times in the pupa appear to send terminal arbors to different domains of the calyx (Zhu et al., 2003).

The correlation between birth order and neuronal projection has been elucidated in great detail for the adnB/BAmn3 lineage. The projection envelope of this lineage includes the antennal lobe, calyx, and lateral horn (Lai et al., 2008). The antennal lobe is formed by over 50 specific glomeruli, each glomerulus characterized by the endings of olfactory neurons sharing a common olfactory receptor (Jeffries et al., 2001; Vosshall et al., 1999). Single-cell clone analysis of the adnB/BAmn3 lineage (Yu et al., 2010) indicated that dendritic branches of neurons born at a certain time point always innervate a single, invariant glomerulus. In other words, dendrites innervate the antennal lobe in a largely non-overlapping, "tiled" manner. The same applies to axonal terminals which form an "odor map" in the calyx (Jeffries et al., 2001; Jeffries et al., 2004).

The projection envelopes shown for the central brain lineages will help to manage the large number of individual neurons that emerge in past and future studies of fly brain development and brain function. The shape of a large fraction of adult central brain neurons, imaged as single-cell clones, has recently become available (Chiang et al., 2011). Many of these neurons are readily identifiable as members of specific secondary lineages. A few examples are shown in Fig. 56. Panels A1–A5 show individual neurons that project along the median bundle, shared by lineages BAmn1 and BAmn2, and fall within the projection of BAmn2 (panel A; proximal arborization in dorsal PEnPa compartment, distal arbors bilaterally in antero-dorsal SMP). Aside from this shared envelope, neurons shown in A1–A5 clearly differ in regard to the details of their distal arborization. For example, A1 has
Table 2

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<td>Column A: Lineage names.</td>
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<td>Column B: Number identifying lineage-associated tracts (SATS). In lineages with multiple hemilinesage tracts or sublineage tracts, these are individually listed (e.g., dorsal hemilinesage tract of BAC is identified as “5d”, ventral hemilinesage tract as “5v”).</td>
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<td>Column C: Number of MARCM clones isolated for lineage.</td>
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<td>Column D: Main class of lineage based on contour projection envelope. “PO”: lineage with separate proximal and distal arborizations; “C”: lineage with terminal arborizations emerging at more or less regular intervals along the entire length of the SAT; “D”: lineages where arborizations are concentrated at distal tip of SAT. Lower case “p” (“local”) stands for small volume filled by arborization; “w” (“wide”) indicates large volume; “co” indicates commissural SAT; “de” signifies descending tract.</td>
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<td>Column E: Neuronal fascicle (join) by lineage-associated tract. For abbreviations of fascicle names, see Table 1. *“0” indicates that tract does not form part of any designated fascicle.</td>
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<td>Column F: Ipsilateral neuropile compartments receiving strong innervation by lineage. In cases of PD lineages where proximal and distal terminal arborizations can be distinguished based on labeled clone, a hyphen separates compartments receiving proximal arbor (left of hyphen) from those receiving distal arbor (right of hyphen). For abbreviations, see Table 1.</td>
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<td>Column G: Commissural join by lineage associated tract. For abbreviations, see Table 1.</td>
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<td>Column H: Contralateral neuropile compartments receiving strong innervation by lineage. For abbreviations, see Table 1.</td>
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Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.07.006.

References


Appendix 3.

Lineage-associated tracts defining the anatomy of the *Drosophila* first instar larval brain.
Lineage-associated tracts defining the anatomy of the Drosophila first instar larval brain

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ABSTRACT

Fixed lineages derived from unique, genetically specified neuroblasts form the anatomical building blocks of the Drosophila brain. Neurons belonging to the same lineage project their axons in a common tract, which is labeled by neuronal markers. In this paper, we present a detailed atlas of the lineage-associated tracts forming the brain of the early Drosophila larva based on the use of global markers (anti-Neuroligin, anti-Neuromatrix, inscutable-Ca14 > UAS-chRFP-Tub) and lineage-specific reporters. We describe 68 discrete fiber bundles that contain axons of one lineage or pair(s) of adjacent lineages. Bundles enter the neuropil at invariant locations, the tract exits in the ventral nerve cord. Correspondences and differences between early larval tract anatomy and the previously described late larval and adult lineage patterns are highlighted. Our 11 neuronal anatomical atlas of lineages constitutes an essential step towards following morphologically defined lineages to the neuroblasts of the early embryo, which will ultimately make it possible to link the structure and connectivity of a lineage to the expression of genes in the particular neuroblast that gives rise to that lineage. Furthermore, the L1 atlas will be important for a host of ongoing work that attempts to reconstruct neuronal connectivity at the level of resolution of single neurons and their synapses.

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1. Introduction

As a member of the holometabolan insects, Drosophila fashions two different bodies during its life cycle, living on or inside its food source, the larval body is designed for rapid ingestion of food and growth. The larva lacks segmental appendages for locomotion, and complicated sensory systems like compound eyes or the (auditory) Johnston’s organ, which, in the adult, are required for detecting food sources, mates and enemies. Corresponding to the lesser demands on controlling such complex behaviors, the early larval central nervous system is more than 1 order of magnitude smaller in size and neuronal number than its adult counterpart. However, in part because of its lower complexity, the larval brain has become a promising model system to address problems of neural structure and development, neural function, and behavior. Most of the individual larval sensory organs (sensilla), muscles, and motor neurons have been reconstructed at single cell resolution (Ghysen et al., 1993; Hartenstein, 1988; Kim et al., 2009; Landgraf et al., 2003a; Liu et al., 2003; Johansen et al., 1989; Kwon et al., 2011; Ramaekers et al., 2005; Schradt and Merritt, 2000; Sink and Whittington, 1991; Sprecher et al., 2011; Vactor et al., 1993) and their role in locomotory circuits is being established (Caldwell et al., 2003; Choi et al., 2004; Kohsaka et al., 2012). For some interneurons, including the projection neurons of the antennal lobe, the olfactory input and higher brain targets have also been mapped, and sophisticated learning paradigms are well established (Colomb et al., 2007; Gerber and Stocker, 2007; Masuda-Nakagawa et al., 2005, 2008; Python and Stocker, 2002; Schleyer et al., 2011; Sekito et al., 2009).
The Drosophila nervous system develops a population of asymmetrically dividing stem cells (neuroblasts) that are born in the neuroectodermal layer of the early embryo. Each of the segments of the ventral nervous system develops from 30 pairs of neuroblasts; the brain comprises approximately 100 pairs (Pelash and Tarchan, 2003; Younossi-Hartenstein et al., 1996b). Each neuroblast is characterized by the expression of a unique combination of transcriptional regulators, and produces a structurally functionally distinct lineage of neurons by an invariant sequence of asymmetric divisions (reviewed in Brody and Odenwald, 2005; Pearson and Doe, 2004; Urbach and Tarchan, 2004). A small number of 5–8 embryonic divisions generate the primary neurons that make up the larval brain (first wave of neurogenesis; Larsen et al., 2008). After a period of quiescence, these aforementioned neuroblasts reenter the cell cycle and generate the neuroblasts of the secondary lineages, which is classified during metamorphosis to form the adult brain (second wave of neurogenesis; Bibb and Hotta, 1992; Truman and Bate, 1988), reviewed in Hartenstein et al., 2000). Neural lineages constitute developing neuronal as well as neuron-anatomic lineages of the developing brain. This has been studied in most detail for the secondary lineages, that were mapped at the late larval stage (Cardona et al., 2010; Kuett et al., 2012, 2014; Pereana and Hartenstein, 2006). In the present work, we use two global neuronal metamorphosis into the adult stage (Lavick et al., 2013). The close ties between lineages and neuroanatomy can be easily appreciated at the late larval stage, where global neuronal markers, such as antibodies against the adhesion molecules Neurotactin (BTP106; de la Escala et al., 1990; Horwich et al., 1990), Neurabin (BPT104; Bieber et al., 1989), or DE-cactherin (Demstreri et al., 2001) show secondary lineages as cohesive clusters of immature neurons, located in the periphery of the brain (the rind or cortex; Fig. 1A–C). Neurons emit a single nerve fiber towards the brain center (the neuropil). Fibers of the same lineage from one or two tight bundles that follow an invariant trajectory by which the corresponding lineage can be recognized. These lineage-associated tracts (secondary axon tracts or SATs, for the secondary lineages) develop into fiber bundles that connect different neuronal components at the adult stage. For four lineages forming projection neurons connecting the antennal lobe with higher protocerebral centers, including the calyx and lateral horn (Oax et al., 2013; Lal et al., 2008), in the late larva, SATs of these lineages have extended all the way from the antennal lobe towards the brain center through the fiber bundle system. The projection pattern of the antennal lobe in the target neurons, respectively. Similar to the AL, the remainder of the fiber bundles of the adult brain is formed by other lineages during the larval stage (Lavick et al., 2013; Pereana et al., 2010).

In contrast to the now existing map of the late larval and adult brain neuropil (Pereana and Hartenstein, 2006; Wong et al., 2013), the pattern of axon tracts formed by differentiated primary neurons in the early larva has remained relatively obscure. The structure and development of larval neuropil compartments, as well as specific "pioneer tracts" that remain visible from embryonic to larval stages, has been documented in previous works (Nasif et al., 1998, 2003; Younossi-Hartenstein et al., 2003, 2006); however, the overall projection pattern of primary lineages is not known. Primary axon tracts (PATs) of all lineages emerge during the embryonic period; like SATs, they express Neurotactin and Neuroblastic, and can be visualized by antibodies against these epitopes (Truman et al., 2004) and by iontophoretic or with a global neuronal marker, insc-Gal4, expressed neuroblasts and their neuronal progeny, visualized by membrane-localized fluorescent reporter (Betscher et al., 2006), to follow the scaffold of secondary axon tracts backward from late to early larval stages, when it is utilized to identify the primary axon tracts. Previously, it has been shown for a few lineages (using enhancer or promoter-Gal4 driver lines), targeted by specific molecular markers, that the SATs forming in the larva follow pre-established pathways of primary axons (Larsen et al., 2009a). The findings presented here confirm this notion for lineages in general, which allowed us to generate an atlas of primary axon tracts for the larval brain. A number of Gal4 driver lines expressed in subsets of lineages from early to late larval stages augmented the resolution of the atlas. We here present the pertinent features of the atlas with the help of confocal sections and 3D models. Our work serves the following two main purposes.

First, the L1 atlas of lineages constitutes another step towards the goal of following lineages backward in time towards the neuroblasts of the early embryo, with the underlying objective to link each lineage (with its specific structure and connectivity) to the gene expression pattern defining the parental neuroblast. This has been recently achieved for the lineages of the ventral nerve cord (Birkholz et al., 2015), and a few select lineages of the brain, including the mushroom body (Kunz et al., 2012). In Birkholz et al. (2015), the prior use of labeled embryonic clones was instrumental to identify larval lineages with specific neuroblasts, and we anticipate the same to be true for brain lineages. Our L1 lineage atlas, translated into the late embryonic brain, will provide an anatomical scaffold with discrete landmarks to which embryonic neuroblast clones, as well as lineage-specific markers expressed from the neuroblast stage towards the late embryo, can be related.

Second, the atlas will significantly aid ongoing work that attempts to reconstruct neuronal connectivity at single-cell and single-synapse resolution using electron microscopy. Along this line, projects are currently under way where complete series of contiguous ultrathin sections of early larval brains are recorded by transmission electron microscopy (TEM), assembled and registered using specialized software and digitally reconstructed (Cardona et al., 2010b, 2012). This reconstruction will be greatly aided by the anatomical landmarks provided by the lineage-associated tracts (PATs) chartered in the present work. PATs represent easily identifiable elements of the TEM images (Cardona et al., 2008), making it possible to identify the specific lineages they belong to by comparing the TEM dataset with appropriately oriented confocal stacks. Once identified in the TEM stack, the PATs define a dense grid of fixed "coordinates" to which ultrastructural, axonal geometry, placement of synapses, and specific interacting neurons (these properties can then be correlated with the parental neuroblast gene expression pattern. This will serve as a foundation for understanding whether and how specific transcriptional regulators define the various anatomical properties within a lineage or sublineage.

2. Materials and methods

2.1. Fly lines

Flies were grown at 25°C using standard fly media unless otherwise noted.

en-Gal4 (Tabatznik et al., 1995; #30564, Bloomington Drosophila Stock Center (BDSC), University of Indiana, IN, USA), FasII-Gal4 (Siebert et al., 2009), FasII-Gal4 (Hayashi et al., 2002; #169948, BDSC), CH146-Gal4 (a gift from R.F. Stocker, University of Fribourg, Switzerland).
Switzerland; Stocker et al., 1997; msc-Gal4 (Mz1407; Betschinger et al., 2006; #8751, BDSC, per-Gal4 (Kaneko and Hall, 2000; #7127, BDSC), psl-Gal4 (TH-Gal4; Frigg-Grein et al., 2003; #8848, BDSC), pnom-Gal4 (Boll and Noll, 2002), R16C11-Gal4, R82E10-Gal4, R13A1-Gal4, R76A11-Gal4, R67A11-Gal4 (Janelia Farm Gal4 Stock Collection, Jenett et al., 2012; #50262 #46525 #46540 #46557 #39400, BDSC), UAS-chRFP-Tub (Rusan and Peifer, 2007; #52774, BDSC), UAS-mcd8-GFP (Lee et al., 1999; #5187, BDSC).

2.2. Immunohistochemistry

Samples were fixed in 4% formaldehyde or 4% methanol-free formaldehyde in phosphate buffer saline (PBS, Fisher-Scientific, pH=7.4; Cat no. #B7999-4). Tissues were permeabilized in PBT (PBS with 0.1-0.3% Triton X-100, pH=7.4) and immunohistochemistry was performed using standard procedures (Ashburner, 1989). The following antibodies were provided by the Developmental Studies Hybridoma Bank (Iowa City, IA): mouse anti-bruchipithc (nC82, 1:20), rat anti-DN-Cadherin (DN-EX #8, 1:20), mouse anti-Fasciclin II (1D4, 1:20), mouse anti-Neuroglian (BP104, 1:30), and mouse anti-Neurotacin (BP106, 1:10). Secondary antibodies, IgG (Jackson Immunoresearch; Molecular Probes) were used at the following dilutions: Cy3-conjugated anti-rat Ig (1:100), Cy3-conjugated anti-mouse Ig (1:200), Cy5-conjugated anti-mouse Ig (1:250), Alexa 546-conjugated anti-mouse (1:500), Dylight 649-conjugated anti-rat (1:400), Alexa 648-conjugated anti-mouse (1:500).

2.3. Confocal microscopy

Staged Drosophila larval and adult brains labeled with suitable markers were viewed as whole-mounts by confocal microscopy (LSM700 Imager M2 using Zen 2009 (Carl Zeiss Inc.); lenses: 60 x oil (numerical aperture 1.3). Complete series of optical sections were taken at 2-μm intervals. Captured images were processed by ImageJ (National Institutes of Health, http://rsweb.nia.nih.gov/ij/) and Adobe Photoshop.

2.4. Morphologically defined stages in larval brain development

Animals were staged by placing larvae hatched from the egg within a 1 h period on food plates under non crowded conditions at 25°C. Since even when larvae are reared at low density to guarantee optimal food supply, there is a considerable variability (in the order of 10%) in brain growth of larvae of the same age. We therefore defined specific morphometric parameters of the rapidly expanding optic lobe as structural hallmarks of the larval brain. These parameters include the ratio of optic lobe diameter (OOG) to neuropile diameter (OOG/NP), the ratio of neuroblasts versus epithelium within the outer optic anlage (NP/BB), and the thickness of the layer of medulla neurone (MN; Supplementary Fig. S1A). Based on these parameters, presented in Supplementary Fig. 1A, larval brain development can be divided into 9 stages (1A-1E) of approximately 12 h length.

2.5. Generation of three-dimensional models

Digitized images of confocal sections were imported into FIJI (Schindelin et al., 2012; http://fiji.sc). Complete series of optical sections were taken at 2-μm intervals. Since sections were taken from focal planes of one and the same preparation, there was no need for alignment of different sections. Models were generated using the 3-dimensional viewer as part of the FIJI software package. Digitized images of confocal sections were imported using TrakEM2 plugin in FIJI software (Cardona et al., 2012). Surface renderings of larval brains stained with anti-Bruchipithc were generated as volumes in the 3-dimensional viewer in FIJI. Cell body clusters were indicated on surface renderings using TrakEM2. Digital atlas models of cell body clusters and SATs were created by manually labeling each lineage and its approximate cell body cluster location in TrakEM2.

3. Results

3.1. Dynamic expression of adhesion proteins Neuroglian and Neurtacin in developing brain lines

The adhesion proteins Neuroglian and Neurtacin serve as markers for the axon tracts of developing brain lines (Lovick et al., 2013; Perecans and Hartenstein, 2006; Truman et al., 2004). Both are expressed on neuronal somata and outgrowing neurites from mid-embryonic stages onward (Bieber et al., 1989; Hortsch et al., 1990; not shown). In the early larva, Neuroglian remains strongly expressed in primary neurons and their primary tracts (PATS; Fig. 1A, B), whereas Neurtacin is downregulated (Fig. 1A, C). Neurtacin appears again strongly in secondary neurons, which start to form at the second larval instar and produce secondary axon tracts (SATs; Fig. 1A, C). By contrast, Neuroglian expression reappears in secondary neurons at a later stage than that of Neurtacin (not shown). Similar to Neurtacin, we find that the instructive- (msc)-Gal4 reporter (Betschinger et al., 2006) is preferentially upregulated in secondary lines and SATs as soon as neuroblasts enter mitosis (Fig. 1A, C-C’). Double labelings of early larval brains, using Neuroglian (primary lines) and msc-Gal4 > UAS-chRFP-Tubulin (secondary lines), allowed us to correlate the trajectory of PATs and SATs (Fig. 1B). For several lines it had already been previously established that PATs follow PATs, formed by earlier born primary neurons of the same lines, into the neuropil (Larsen et al., 2013; Larsen et al., 2005).

Our present data demonstrate that the close association between PATs and SATs is true for almost all lines (Fig. 1G; Supplementary Figs. S2 and S3).
3.2. Reconstruction of primary axon tracts in the L1 larval brain

Drosophila brain lineages were initially identified and mapped for the late larval stage (L3), when each lineage forms a distinct SAT that can be visualized using global markers such as BP106 (Cardona et al., 2010a; Pereanu and Hartenstein, 2006). With only two exceptions of all these lineages were validated by MARCM clones in the adult brain (Kurr et al., 2014; Wong et al., 2013).

Using the above described markers for SATs and PATs, we traced lineages backward in time from the late larval stage into the late first-early second instar, when secondary lineages are born (Fig. 1D-I; Supplementary Figs. S2 and S3; see also Lovick et al., 2015). Given that SATs project along the tracts formed earlier by the corresponding primary neurons, we could establish a map of primary axon tracts for the L1 larval brain (“L1 PAT map”). In the absence of specific markers, the map is of less resolution than the map of lineages and tracts in the late larva, because fiber bundles formed by pairs or small groups (3–4) of lineages have collapsed into one tract. Thus, as previously described, most lineages are arranged in pairs (e.g., BAmv1/2) or small groups of 3–4 (e.g., DPla1–1–3; BlAd1–4) whose SATs enter and then extend through the neuropil in close apposition. In the late larva, when secondary neurons with their SATs have been added to each lineage, the SATs of these pairs or small groups can be separated followed from the cortex into the neuropil (see, for example, the two tracts formed by BAmv1/2, ISm shown by red arrows in Fig. 1E). In the L1 brain, at the level of primary lineages, the tracts have collapsed into one bundle (e.g., BAmv1/2 bundle indicated by red arrowhead in Fig. 1I). This decline in resolution aside, the primary axon tract map of the L1 brain reconstructed in this paper still represents a rich three-dimensional scaffold of structural landmarks around which neuromasts and their progeny are grouped.

3.3. Neuronal compartments and long axon fascicles form a neuroanatomical framework for the lineage map

The brain neuropil has been described in terms of distinct compartments, domains of high synaptic density surrounded by bundles of long axons and glial processes that form visible boundaries (Pereanu et al., 2010). Compartments and selected fiber bundles forming compartment boundaries constitute a framework of landmarks of the developing Drosophila brain. The points of entry of lineage tracts, defined as the “entry portals” of the corresponding lineages (Lovick et al., 2013, Wongs et al., 2013), as well as the fiber trajectories within the neuropil, can be described with respect to their invariant spatial relationship to compartment boundaries. We will therefore provide a brief review of the compartmental composition of the larval brain (see detail legend of Fig. 2).

The most conspicuous compartment is the mushroom body (MB), which is formed by four lineages located at the posterior surface of the brain (Figs. 2A and B), and comprises the peduncle (PED), calyx (CA), spur (SP), and medial lobe (ML; Fig. 2A). Four compartments, the antennal lobe (AL), anterior protopupal neuropil (PPNF), lateral appendix of the medial lobe (LAML), and anterior inferior protocerebrum (IPA), flank the MB lobes anteriorly (Figs. 2B, C, I, and L2). [Note that we will in the following use the nomenclature that reflects the correspondence between larval and adult compartments; see Ito et al. (2014) and Pereanu et al. (2010). For correspondences between these terms and the nomenclature originally introduced for the larval brain in Younesi-Hartenstein et al. (2003) see Table 1]. The lateral
accessory lobe (IAL), ventromedial cerebrum (VMC), and ventrolateral protocerebrum (VIP) represent the ventral compartments of the L1 brain (Figs. 3B–G and S4–C). The neuropil domains surrounding the peduncle and medial lobe of the mushroom body are termed “inferior protocerebrum” or “clasp” (Ito et al., 2014; Pererau et al., 2010; IFA, IM, M; Figs. 2, L3–L5 and S4B, C, F). The superior protocerebrum (SP), comprising a superior lateral (SLP) and superior medial (SMP) domain, forms the dorsal compartments of the brain (Figs. 2C–F, K3, K4, L2, L3 and S4G, H).

A system of longitudinal fascicles interconnects neuropil domains of the insect ventral nerve cord (VNC) at different anteroposterior levels (Power, 1948; Tyrer and Gregory, 1982). These fascicles, which in Drosophila are commonly marked by the expression of the adhesion protein Fasciclin II (FasII; Grenningloh et al., 1991), include a regularly spaced medial, intermediate, and lateral system (Fig. 3) Anti-Neuroligan, which more globally labels primary axons, also faintly visualizes these fiber systems (Fig. S3A–C). Medial and lateral tracts each have a dorsal (DMT, DLT) and ventral component (VMT, VLT), respectively. The intermediate fascicle has several components extending along the center of the VNC neuropil (CTI–3) (Nassi et al., 2003; Landgraf et al., 2003b). Anteriorly, the long axon tracts of the ventral nerve cord anastomose with each other and continue towards the brain (Nassi et al., 2003). They form three main bundles, termed medial cervical tract (MCT), lateral cervical tract (LCT), and posterior cervical tract (PCT). Each of these fiber systems, which carry ascending and descending axons connecting brain and VNC, splits up into smaller branches shown in Fig. 3B–F. A small number of these FasII-positive connectives associate with discrete primary lineages, which contain FasII-positive neurons (for specific detail, see below).

3.4. Synopsis of neuro-anatomical features of the early larval brain provided by lineage-associated tracts

We will in the following sections present detailed descriptions of all of the lineage-associated PATs labeled by the global marker Anti-Neuroligan, including their position of entry into the neuropil (entry portals) and trajectory within the neuropil. For didactic reasons, we will proceed by breaking down lineages into their topologically defined groups. Before going into this detail, we present first a summary of our findings in Fig. 4. Overall, we can distinguish 68 discrete fiber bundles that enter the brain. As indicated in the second column (B) of Table 2, these

Table 1: Abbreviations for fiber tracts and neuropil compartments of the Drosophila early larval brain.

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<th>Fiber tracts (cont’d)</th>
<th>Compartments</th>
<th>Abbrev.</th>
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Column A: List of fiber tract and associated abbreviations.
Column B: List of larval neuropil compartments and associated abbreviations.

* Indicates older version larval compartment nomenclature as described in Yuennui-Hartenstein et al. (2003).
bundles can correspond to: one lineage (e.g., BAm1) small sets of adjacent lineages (e.g., BAla1, BAla4); single hemi/sublineages (e.g., DPM2m1,2), or two hemilineages of neighboring lineages (e.g., DALc1m1,2m). Within the neuropil, these fiber bundles form larger fascicles that can be classified by their main orientation according to body axis, into longitudinal, transverse, and vertical (ascending/descending) fascicles. These fascicles could be identified with their counterparts described for the brain at later stages of development (late larva to adult; Pereira et al., 2016; Lovci et al., 2013). Table 2 and Fig. 4 represent these fascicles, color coded and assigned to the lineages that contribute to them. The basal-anterior (BA) lineages, according to previous studies (Kuent et al., 2012; Kumar et al., 2009), belong predominantly to the deuterocerebrum: two lineages, BAl and BAlp4, in addition to three other subesophageal lineages not considered here, are present for the Hox gene lobed, a marker of the tritocerebrum. BA lineages form a set of longitudinal fascicles (loVM, loVI, loVLo, dark blue), as well as two ascending fiber systems (red): the antennal lobe tract (ALT; formed by BAla1, BAlc, BAm1, BAlp4) connecting the antennal lobe and neighboring territories to the dorso-posterior protocerebrum, and (part of) the median bundle (MBDl, dark red; formed by BAm12) that leads from the PENPa to the dorso-anterior protocerebrum (Fig. 4A, top, R, F, D, K). Lineages of the DAL and DAM group form the anterior protocerebrum (note that “anterior,” relative to the body axis, corresponds to “ventral” relative to the neuraxis; see Nuo et al., 2014). DAL lineages, in addition to the dorsal most BA lineages (BAnd1/BAm1d), mainly form systems of transverse fiber bundles and commissures flanking the lobes of the mushroom body and the surrounding lipa compartment (ALC, FrMC, ScEC; green in Fig. 4A, top, B, F). The lineage DALd, and part of DALc1d, form the major descending bundle (decP; orange in Fig. 4A) projecting from the protocerebrum to the ventral brain and SEG (Fig. 4A, R, F, G). DAM lineages enter the anterior part of the SMP (superior medus protocerebrum) compartment and form commissural (ADC; green) as well as longitudinal fibers (loSMa; blue in Fig. 4A, R, F, H).
Fig. 4. Synopsis of lineages and neuropil tract. (A) List of lineages (first column), associated PIP tracts (second column), and neuropil entry portals (third column). Neuronal fiber tracts are represented by colored bars at the right of panels; longitudinal tracts are in blue, transverse tracts in green, and ascending and descending tracts in red and orange, respectively. Lines connect individual lineages (left) with the appropriate neuronal tracts (right). Lineages that project locally (according to specific labeling) are indicated by short gray lines and circles (third column); lineages for which no clear information exists are indicated by black ovals in second column. (B-I) Digital 3D models of lineages (B-D) and tracts (E-I). Mushroom body and ventral brain neuropil compartments are shown semi-transparently for reference. Anterior view (B, F, medial to the left); lateral view (C, G; anterior to the left); dorsal view (D, H; medial to the right); posterior view (I, I; medial to the right). Coloring of lineages reflects their projection along longitudinal fiber system (blue), transverse system (green), or ascending/descending system (red or orange, respectively). For abbreviations of fiber tracts and compartments see Table 1.
Table 2:
Lineages of the *Lemniscus* early larval brain.

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Column A: Lineage names based on topology (Peters and Hartenstein, 2006). Branching of CMS indicates that no primary lineage tract could be identified for this lineage.

Column B: Number identifying lineage-associated tracts (PATs) in Fig. 1. In lineages with multiple hemisegmental tracts or sublineage tracts, these are individually listed (e.g., dorsal hemisegmental tract of BAaMc identified as "5A", ventral hemisegmental tract as "5S"). Differential light and dark shading indicates lineage tracts that have merged into a single bundle; for example, a single PAT is formed by BAaMc and BAaMc2, or for the dorsal hemisegments 1M and 1M of DAaMc1 and DAaMc2, respectively.

Column C: Markers for lineages.

Column D: Entry portal of lineage-associated tract. For abbreviations see Table 1.

Column E: Numerical entrance portals in Figs. 2, 5-10, and 12.

Column F: Neurophil fascicle joined by lineage-associated tract. For abbreviations of fascicle names see Table 1.

^a Reviewed in Spindler and Hartenstein (2010).
^b Tan et al. (2013).
^c Jemt et al. (2012).
^d Lichtenecker et al. (2007).
Fig. 5. Tracts associated with baso-anterior (BA) lineages. (A–C) Digital 3D models of BA lineages and tracts in a single L1 brain hemisphere. Anterior view (A), later view (B), ventro-anterior view (C). Centers of cell body clusters of lineages are depicted as spheres; lineage-associated axon tracts are shown as lines. Mushroom body and antennal lobe (blue-gray) and fast-positive tracts (dark gray) are shown for reference. Fiber bundles of neuropil formed by BA lineage tracts are shown in light gray. Numbers are labeled circles on (A) and other panels represent entry points of lineage-associate tracts. "i" and "m" in (A) indicate dorsal and ventral homologous tracts of BAmd1 and BAmd2, respectively. Arrow in (B, C) points to entry of ventral hemilocus of BAmd into IoVM tract; arrowhead in (B, C) indicates convergence of tracts of BAmd, BALa1, BALa4, and BAmd2 into the antennal lobe tract (ALT); large arrowhead in (C) points at close parallel entry of antennal nerve (AN) and tracts of BALa1-4. Double-headed arrows in (A, C) and all other panels indicate brain midline. (D–M) Projections of frontal confocal sections of a single L1 brain hemisphere. Anterior-posterior levels shown by z-projections are indicated by letters (AL, SP, ML) at lower left corner (for definition of levels, see Fig. 2). Primary neurons and tracts are labeled by anti-Neurogranin (BIP104; magenta in panels D, F, H–L), white in panels E, I, M). BP104-positive antennal nerve (AN) and pharyngeal nerve (PN) is highlighted in green in panel (D). Lineages BAmd1 and BALa1 are labeled by pre-Gal4 > UAS-mcd8::GFP (green in F–I); BALa3 is labeled by m-Gal4 > UAS-mcd8::GFP (green in I–L). Panels (I) and (M) are high magnifications of central parts of (G) and (D), respectively. For abbreviations of compartiments and fiber tracts see Table 1; for numbering of entry portals see Table 2. Bar, 50 μm (D, F, I, M); 20 μm (F–H, J–L).

DPL, BLA, and BLD lineages are associated with the dorsolateral protocerebrum. Many of these lineages converge on three transverse fiber systems (trS A: DPLa1-3; trSIL: BIAL1-4, BLA1, BIL1-4; trSPL: DPLc1-5, DPLU) located in the superior lateral protocerebrum (SPL, light green in Fig. 4A center, B–I). BLA1/2, as well as the posterior-lateral lineages BILp1/2, form the main ventral commissural system, the great commissure (GC, dark green; Fig. 4A center, F, I). The GC is also joined anteriorly by fibers of the DLPc1 line and posteriorly by the CM group (Fig. 4A, F, I). The pair DPLc2/3 forms a longitudinal fiber system
(IoVM) in the L.P compartment.

DPM, CM, and CP lineages belong to the dorso-medial and posterior protocerebrum and mainly contribute to longitudinal fiber systems connecting the posterior protocerebrum with the anterior protocerebrum and deutocerebrum. These include dorsal bundles (IoSMp, formed by DPMp1/2 and part of DPMpm2; Fig. 4A bottom, D, E, H, I), as well as ventral bundles. Most prominent among these is the medial equatorial fascicle (MEF), which forms a thick fascicle running medially of and parallel to the peduncle of the mushroom body. It is formed by dorsal components of the CM lineages, as well as DPMp3 (Fig. 4A bottom, E, I). Further laterally are the lateral equatorial fascicles (LEFp, formed by CPl; Fig. 4A bottom, E, I), the posterior-lateral fascicle (PLFp, formed by ventral components of CM lineages and DPMp; PLFv, formed by BAl1 and 2; Fig. 4A bottom, E, I), and the posterior ventral longitudinal fascicle (kVp, associated with ventral parts of the CM lineages; Fig. 4A bottom, E, I). Dorsal components of the CP group and DPMp1/2 form the conspicuous oblique posterior fascicle (obF), which crosses over the peduncle where it emerges from the calyx (Fig. 4A bottom, H, I); the obF turns anteriorly and joins the IoSM bundle (Fig. 4A bottom, H). Two lineages, DPM1/2, form a descending tract towards the SEG (DPPT; Fig. 4A bottom, E, I).

Compartments missing from the larval brain are those of the central complex, a prominent structure of the adult brain. The main (secondary) lineages contributing to the central complex are the four posterior lineages: DPMm1, DPMp1/2, CM (fan-shaped body), and DALv2 (ellipsoid body). Several other lineages, including BAlv1 also contribute to the fan-shaped body (Hoffman et al., 2013). BAlv1 is the main output from the ellipsoid body, projecting to the LAL and medial lobes of the mushroom body (see below). Midline-crossing fibers of DALv2/3 form a thin commissure (FRMC) which demarcates the location where the ellipsoid body will form during early metamorphosis (Fig. 4A center).

3.5. Antero-ventral lineages: the BA group

The BA cluster contains 17 lineages which form 11 bundles entering the anterior neuropil in the vicinity of the antennal lobe (AL). BAl1–4 form an antero-lateral BA subgroup with PARs that pass over the AL surface and converge at an entry point at the anterior (secondary) boundary of the AL, close to the antennal nerve (entry portal AL v, #1; Figs. 2G, L, K1, K2 and 5A–E). The BAl1/2 tract turns medially along the posterior boundary of the AL (Fig. 5B). The BAla2 lineage includes local interneurons (Das et al., 2013) that terminate within the AL. BAla1, marked by several known Ga44 driver lines, including per-Ga44 (Larsen et al., 2010; Fig. 5F–H), represents one of the four antennal lobe projection lineages. Its tract turns dorsally, forming part of the antennal lobe tract (ALT) that leaves the antennal lobe at its posterior boundary (Fig. 5B, C). The BAl1 tract soon exits the ALT towards laterally, approaches the peduncle, and terminates in the inferior protocerebrum surrounding the peduncle (Das et al., 2013; Fig. 5G, H). This peculiar pathway, which matches the corresponding BAl1 secondary axon tract in the adult brain, represents the medio-lateral antennal lobe tract (mALT; Das et al., 2013; Lowick et al., 2013). BAL3 is marked by en-Ga44 (Kumar et al., 2009; Fig. 5J–M). The BAla4/3 tract projects postero-medially, passing the large IoVM bundle (see below) at its ventral surface, and branches in the ventromedial cerebrum (VMC; Fig. 5K, L).

BAlc, located posterior of the BAla1–4 cluster, enters the posterior AL at a position dorsal of BAla1/2 (entry portal AL l #2; Figs. 2G, L, K1 and 5B). Similar to the corresponding secondary tract in the late larva and adult, the BAlc tracts bifurcates with one branch projecting dorso-medially and the other one ventro-medially (Fig. 2K) and arrowhead in Fig. 5B). The dorso-medial branch joins BAla1 towards the antennal lobe tract (ALT; arrowhead in Fig. 5B, C). Primary BAlc neurons with this dorso-trajectory were described as local antennal interneurons, as well as atypical projection neurons (Das et al., 2013). In the adult brain, the dorsal BAlc neurons form a hemilineage of uni-glo-merular projection neurons (Lai et al., 2008). The ventral branch of BAlc, similar to its secondary counterpart at a later stage, converges upon the ventral longitudinal fiber system (sFLv, longitudinal ventral intermediate) fascicle, and projecting posteriorly towards the boundary between the ventromedial cerebrum and ventrolateral protocerebrum (VMC and VLP; arrow in Fig. 5B, C).

BAlp1–4 are located postero-ventrally of BAla1–4 (Figs. 2K, K3 and 5A–C). BAlp1–3 converge upon a single, short, posteriorly-directed bundle entering the neuropil in the cleft between the lateral accessory lobe (LAL) and VLP compartments (entry portal VLP vm, #3). This trajectory corresponds to the longitudinal ventral lateral fascicle (vLFL; Figs. 2K and 5A, C) and BAlp4, marked by the Ga44 driver UAS-CtC (Table 2), contains atypical antennal lobe projection neurons (Das et al., 2013). The BAlp4 tract projects dorso-medially along the posterior boundary of the antennal lobe (AL), to join BAlc at the root of the antennal lobe tract (ALT; entry portal AL v, #1; Figs. 2A–C, M). BAlc is located ventrally of the BAlp cluster and projects a short tract medially towards the boundary between VLP and subesophageal ganglion (SEG; entry portal VLP Ci v, #4; Figs. 2K3 and 5A, B, M). This entry point marks the position where a distinct compartment, the inferior ventrolateral cerebrum (IVC) which receives BAlp projection, will emerge (Lowick et al., 2013).

BAlm1/2 form a pair with a joined tract entering at the dorso-medial border of the anterior esophageal ganglion (PENLps), medially of the antennal lobe (entry portal AL vm, #6) and projecting dorsally towards the superior medial protocerebrum (SMP; Figs. 2K1 and 5A). Located dorsolaterally of BAlm1/2 and medially of the lateral appendix of the medial lobe (LAM1) are two lineages, BAlm1 and BAlm2. Both tracts project straight posteriorly towards the medial lobe of the mushroom body with the BAlm1 tracts entering slightly laterally of BAlm2 (entry portal Alv; #5; Figs. 2K1 and 5A). Similar to its secondary counterpart, BAlm1 bifurcates into a dorsal and ventral branch. The dorsal branch approaches the dorsal surface of the medial lobe (ML) and makes a sharp medial turn, joining a medio-dorsally directed tract of the DALm1/2 lineage (see below). The joined tracts of the medial DALm1/2 and dorsal BAlm1 cross the midline in the fronto-medial commissure (FRMC; Fig. 5A). The ventral branch of BAlm1 approaches the ventral surface of the ML, turns medially, and crosses the midline as the antennal lobe commissure (ALC; Fig. 5A, C). The BAlm2 tract, entering medially and ventrally of BAlm1, also turns ventrally and then medially as part of the ALC (Fig. 5A, C). The ventral components of both BAlm1 and BAlm2 and their commissural tract express Fasciclin II (Fig. 3B–D). Markers for BAlm2 (e.g., Ks40–Gal4; Table 2) and GFP-tubed clones (Lowick et al., 2015) reveal that BAlm2 also possesses a second, dorso-laterally directed branch (not visible with anti-Neurolase alone), similar to BAlm1 (Fig. 5A).

The last group of BA lineages, BAlm3/4, is located dorsally of the antennal lobe (AL; Fig. 5A–C). Together, the BAlm1/2 lineages form a common, thick tract that projects postero-ventrally and enters medially of the AL (entry portal AL v, #5; Figs. 2G, K1, K2
Fig. 6. Tracts associated with dorso-anterior lateral (DAL) and dorso-anterior medial (DAM) lineages. (A–C) Digital models of DAL and DAM lineages and tracts in a single L1 brain hemisphere. Anterior view (A), lateral view (B), dorsal view (C). Aside from mushroom body and antennal lobe (blue-gray), and fast-spiking tract (dark gray), the lateral accessory lobe (LAL) and lateral appendix of the medial lobe (LAML) (both in magenta-gray) are shown for reference. Fiber bundles of neuropil formed by DAL lineage tracts are shown in light gray. Numbered hatched circles in (A) and other panels represent entry portals of lineage-associated tracts. Double-headed arrow in (A, C) and all other panels indicates brain midline. D–O: J-projections of frontal confocal sections of a single L1 brain hemisphere. Antero-posterior levels shown by t-projections are indicated by letters (AL, SP, ML) at lower left corner (for definition of levels, see Fig. 2). Primary neurons and tracts are labeled by anti-Neuropil (BP104; magenta in panels D, F–H, J–O, N, O); white in panels E, I, M(M’); lineage DALch is labeled by R22410-Ga4 > UAS-modF > GFP (green in D, F, G); DALv1 is labeled by per-Gal4 > UAS-modF > GFP (green in H, J, K, L); DALc3 and DPLam are labeled by en-Gal4 > UAS-modF > GFP (green in L, N, O). Panels (D), (I), (M), and (M’) are high magnifications of central parts of (D), (J) and (N), respectively. For abbreviations of compartments and fiber tracts see Table 1; for numbering of entry portals see Table 2. Bars: 20 μm (A, F–O, H–J, L, O); 10 μm (E, I, M).
and 5A). The tract, defining the medial longitudinal ventral fascicle (LVM), continues posteriorly, first along the boundary between the lateral accessory lobe (LAL) and anterior perireceptive ganglion (PEND), then towards the boundary between the ventromedial ventral commissure (VVC) and ventral lateral protocerebrum (VLP; Figs. 2K, K3 and 5A–C, G). BMV1 is marked by per-Ga4 (Larsen et al., 2009; Fig. 5F–I; Table 2), which reveals additional detail about the trajectory of this lineage. As described for the secondary BMV1 lineage, primary BMV1 gives off a crescent-shaped branch projecting dorsally along the lateral boundary of the LAL (Fig. 5H, arrowhead). BMV2 constitutes the fourth antennal lobe projection neuron lineage; it contains all of the 20 or so projection neurons connecting the larval AL to the calyx and lateral horn (Das et al., 2013; Ramaswamy et al., 2005). BMV3 can be marked by several reporters (Table 2; Stokoe, 1997; Table 2). The BMV3 tract (which is difficult to discern solely by anti-Neuroglian) enters the AL from a dorso-medial position, projecting medially right in front of the downward path of the ventral BAM1 tract, and then turning posteriorly to join BAA1/ BAM1A/BAM1 by the root of the antennal lobe tract (AAL; entry portal AL d. #7; Fig. 5A–C).

3.6. Antero-dorsal linesages: DAL and Dam

The DAL group possesses 10 lineages located anterior and lateral of the spur (SP) and vertical lobe (VL) of the mushroom body (Fig. 5A–C). DALCl1/2 form a paired cluster which flanks the SP and emit a dorsal and a ventral tract (Figs. 2K, K2 and 6A, B, D–G). DALCl1 is marked by the expression of en-Ga4 (Table 2; Fig. 6D–G). The ventral tracts of DALCl1/2 project medially, passing the lateral appendix of the medial lobes (AML) and entering medially of this compartment via the portal SP (6A, B, E, M). As shown by marker NRZ1 (Fig. 6E, F, G), the ventral tract of DALCl1 continues medially and crosses the midline in a commissure that we interpret as the forerunner of the adult subellipsoid commissure (Seck, 1982; Fig. 6A, F, G), as defined by the secondary DALCl1 tract (Lindvall et al., 2013). The dorsolateral DALCl2 tract extends posteriorly and medially, crosses the peduncle at its dorsal surface, then turns ventrally (entry portal SP d. #5; Fig. 6A, E, M). These trajectories of DALCl2/3 primary axons resemble the pattern of secondary DALCl2/3 tracts (Lindvall et al., 2013). Terminal arborizations of the dorsal DALCl1 tract (labeled by NRZ1-Ga4) fill the anterior and medial inferior protocerebrum (Pa, fhm), posterior to the elbow formed by the root of the mushroom body (Fig. 6G, G); branching of the ventral tract occurs in the LAL (Fig. 6F, G).

DALClm1/2 and DALd are located medially of DALCl1/2, flanking the antero-lateral surface of the vertical lobe (VL; Figs. 2K, K2 and 6A–C). The DALClm1/2 lineages form a cluster that produces a medial tract and a ventral tract. The medially-directed tract passes in front of the VL and is directed towards the midline; its crossing defines the forerunner of the frontal commissure (PFC; entry portal VL vm. #11; Fig. 6A, B, E, I). The posterior tract curves around the lateral and posterior surface of the VL and then turns ventrally, joining the single tract of DALd which forms the descending decTrP tract (entry portal VL vl. #10; Figs. 2K2 and 6A, B, E, I).

Three DALv lineages are located ventrally of DALCl1/2 (Figs. 2K and 6A–C). The DALVl tract projects posterolaterally into the space between the lateral accessory lobe (LAL), ventral lateral protocerebrum (VLP), and spur (SP; entry portal VLP dm. #12; Figs. 2K, L2 and 6A, B, E, I). It is closely attached to the ventromedial surface of the peduncle and continues posteriorly towards the great commissure, defining the anterior LEf fascicle (LEf; Figs. 2K, K3 and 6B, C). DALVl2 forms a cluster ventral of DALVl (arrowhead in Figs. 2Q and 6A, H–K); DALVl2 is marked by per-Ga4 (Spindler and Hirthenstei, 2010, 2011) and pixon-Ga4 (Boll and Noll, 2002; Minson et al., 2009; PNLv by en-Ga4 (Kumar et al., 2006; Larsen et al., 2009; Fig. 6L, N, O). The DALVl2/3 tracts, which express Neuroglian only faintly, approach the lateral surface of the LAL, where they form terminal arborizations (entry portal LAL v. #13; Figs. 6A, B, E, I, J). The DALVl3 tract (labeled by specific markers) then turns dorso-medially and forms dense arborizations in the LAL and surrounding the medial lobe of the mushroom body (Fig. 6J, K). Some axons cross the midline with the FMc commissure and terminate in the medial lobe of the contralateral hemisphere (not shown).

Two lineages, DAL1U1/2, form the DALU group among the secondary lineages, and are located laterally of DALV1–3 (Cardona et al., 2010a; Lindvall et al., 2013). Secondary DALU2 axons enter the antero-medial surface of the ventral lateral protocerebrum (VLP) in a very short tract. A cluster of neurons, that we interpret as DALU2, with axons converging onto the medial VIP close to entry portal VLPdm (12) is also apparent in the L1 brain (Fig. 6A–C). DALU1, whose secondary component has a highly characteristic trajectory along the lateral surface of the peduncle and then backward to the anterior superior protocerebrum (Lindvall et al., 2013) was difficult to follow backward to the L1 stage. A primary lineage closely associated with DALVl, its tract running parallel to the DALVl tract, is the only candidate for the DALU1 (entry portal VIP dm. #12; Figs. 2Q and 6A, B).

A group of five DAM lineages is located medially of the mushroom body vertical lobe (Figs. 2K1–3 and 6A–C). The most ventral component, DAMV1/2, projects two adjacent, thin tracts posteriorly into the superior medial protocerebrum (SMP; entry portal VL dm. #14; Figs. 2K1 and 6A, C). The DAMl1 lineages are located dorso-posteriorly of DAMV and enter the neuropil through the VL dm entry portal (14 in Figs. 2 and 6). A medial group of neurons, interpreted as DAMd1, has medially-directed axons which reach the dorsal midline, defining the anterior-dorsal commissure (ADc, Fig. 6A, C). Secondary neurons of DAMd2 project posteriorly, forming the anterior longitudinal superior medial fascicle (IsotM1; Lindvall et al., 2013). Fibers emitted from the primary DAMd2/3 cluster which follow a similar posterior route are only faintly visible in some preparations (indicated as “IoM1a” in Fig. 6A–C).

3.7. Dorsal-lateral lineages: the DPLu group

DPLu lineages are widely dispersed over the dorso-lateral surface of the superior protocerebrum (SProtC), with the following subgroups with characteristic tract entry points: an antero-lateral DPLu with an adjacent DPLum and a DPLd cluster; a postero-lateral DPLu cluster; a posterior DPLp cluster; a dorsal DPlc cluster; and a dorso-posterior DPLm cluster. The DPLu cluster, presumably formed by three lineages, DPLu1–3, enters the superior lateral protocerebrum laterally (SIP; entry portal SIP l. #15a; Figs. 2G–I, K3 and 7A) and projects a thick bundle, the transverse superior anterior fascicle (tSfA), ventro-medially towards the peduncle (PED; Figs. 2K and 7A, D, H, I). The tSfA tract demarcates the boundary between the SLP compartment (above) and the ventrolateral protocerebrum (VIP; below). DPLm is marked by the expression of en-Ga4 (Kumar et al., 2009; Fig. 6L, N). The short DPLm tract enters the SIP compartment medially of the tSfA (entry portal SIP a. #16; Figs. 2G–I, K3 and 7A, B) and forms terminal arborizations in the SIP and the lateral inferior protocerebrum (LIP; Fig. 6N, arrowhead).

The DPLu group, which consists of three uniquely identifiable secondary lineages (DPLu1–3), is located posterior of DPLm. It forms a short tract entering the superior lateral protocerebrum (SLP) compartment latero-posteriorly (entry portal SLP pl. #19; Figs. 2H, I, K4 and 7B, C) and projects anteriorly, forming the longitudinal superior lateral fascicle (IoSL; Figs. 2K4 and 7B, C, G, K, Q). Even
Fig. 7. Tracts associated with dorsoro-posterior lateral (DPL) lineages. (A–C) Digital 3D models of DPL lineages and tracts in a single L1 brain hemisphere. Anterior view (A), lateral view (B), dorsal view (C). Mushroom body and antennal lobe (blue-gray) and tail-positive tract (dark gray) are shown for reference. Numbered hatched circles in (A) and other panels represent entry portals of lineage-associated tracts. Double-headed arrow in (A, C) and all other panels indicates brain midline. (D–O) Z-Projections of frontal confocal sections of a single brain hemisphere of early L1 (64 h; D–G), late L1 (48 h; H–K), and L2 (12 h; L–O). Antero-posterior levels shown by a-projections are indicated by letters (ML, anterior GC level (GCan), intermediate GC level (GCin), posterior GC level (GCpost)) at lower left corner (for definition of levels, see Fig. 2). Secondary lineages are labeled by anti-Neurotactin (BP194), white in D–G), or Anti-Gal4 > UAS-eYFP-Fu (green in H–K). Primary neurons and tracts are labeled by anti-Neuropilin (BP194; white in H–K and L–O, magenta in H–K). For abbreviations of compartments and fiber tracts see Table 1; for numbering of entry portals see Table 2. Bar: 20 μm (D–O).
further posteriorly and ventrally one finds the DPLp group, which, because of their close association with the CP lineages, is discussed along with these (see below).

DPLp includes five lineages (DPLp1-5) at the secondary stage (second to third larval instar; Fig. 7D-G). The cell body clusters are spread out over a fairly wide area topping the superior lateral protocerebrum (SLP); tracts converge on a thick bundle (called the medial transverse superior posterior fascicle; tsPm) that forms a conspicuous entry portal at the boundary between the SLP and superior medial protocerebrum (SMP; Penauna and Hartenstein, 2006; Lovick et al., 2013; Fig. 7F). Within the neuropil, DPLp tracts have a medially directed trajectory that passes towards and then underneath the longitudinal superior medial fascicle (lsSM), DPLc2 and 4 reach the neuropil from a more lateral position, and form a posterior tract that runs parallel to the DPLp. The DPLp5 tract is followed backward from late L3 to approximately 48 h posthatching, when secondary tracts start to elongate (Fig. 7H-K). Prior to this stage, primary DPLp tracts form one thick bundle that passes superficially from laterally over the SLP (tsPm in Figs. 2K4 and 7A, C, N). This bundle, fed by a larger and more medial cluster, corresponds to the DPLc2/4 tract. Expression of FasII in DPLc2/4 throughout the larval period (Supplementary Fig. S3) helps identifying these DPLp clusters in the early larval brain. Further posteriorly and ventrally the DPLp5 tract is directed towards the DPLc2/4 tract; these clusters (DPLp anterior in Fig. 7L M) are interpreted as DPLc1/3. For DPLp, a substantial ventrally directed tract, which projects parallel to the descending DPM1 tract (Fig. 7A, G, C, K, G, O; see also below) can be distinguished.

DPM1/2 form a pair located posterior of the DPLp group, laterally adjacent to the calyx (Figs. 2K4, K5 and 7B, C, G, K, O). A short tract enters at SLP p (#20 in Figs. 2H, I and 7C, G, K) and projects anteriorly at the boundary between the superior lateral and superior medial protocerebrum (SMP, SLP). One remaining DPL lineage, DPLp, is difficult to identify in the L1 brain. The secondary DPLp lineage enters laterally adjacent to the tip of the vertical lobe (entry portal VL dl, #17 in Fig. 7D, H), and has a characteristic branched tract, with one branch projecting medially anteriorly and a second branch extending ventrally toward the midline (Fig. 7A, H, H). The other branch is directed posterolaterally towards the intermediate superior transverse fascicle (tsR). In the L1 brain, we can only identify a small cell cluster located laterally to the VL tip that corresponds in position to DPLp (Fig. 7A, H, H).

3.8. Posterior-medial lineages: DPM and CM

The DPM and CM lineages are clustered along the dorso-medial-posterior edge of the superior medial protocerebrum (SMP). Among the DPMs, one can further distinguish, based on distinct projection pattern, a medial group (DPMm1/2, DPMm1/2) from two lateral groups (DPM1/2, DPM1-3). The medial DPM lineages (except for DPMm2) are marked by the expression of several known driver lines (BDI1-Gal4, Bayur et al., 2013 and B3A10-Gal4 (see Table 2)) and represent Type II lineages which, at the secondary stage, produce much larger progeny by means of intermediate progenitors (Bello et al., 2008; Yang et al., 2013). DPMm1 and DPMm2 also express Fasciculin III throughout larval development (Supplementary Fig. S5B, C, E, F). These lineages, together with CMd (see below), generate the columnar neurons of the central complex; following the nomenclature of Bello et al. (2008) they were called DML1, respectively. In the L1 brain, DPMm1, DPMm1/2, and CMd form already larger clusters than other (Type I) lineages. DPMm1/DM1 enter close to the dorsal midline at the PB m entry portal (#25 in Figs. 2J and 8A-C). Towards posterior-laterally it is followed by DPMm1/2 and DPMm2/DM3 which form the PB d entry portal (#27 in Figs. 2J and 8A-D). These two lineages are also positive for the adhesion molecule Fasciculin III, which is expressed in a discrete subset of lineages throughout larval development (Fig. 5B, C, E, F).

Each one of the three medial DPM Type II lineages has a tract that follows an anterolateral trajectory into the posterior inferior protocerebrum (IIP), before turning mediolaterally towards the midline (Figs. 2K4, K5 and 8A, B). The convergence of medially-directed fibers of DPMm1, DPMm1/2, and CMd (see below) represents the primordium of the fan-shaped body (FtFB), as recently defined by Reubi et al. (2013) who used a Gal4 driver line specifically expressed in primary neurons of these four lineages. Aside from the tract destined for the primordium of the fan-shaped body, DPMm1/2 produce a second axon bundle that has a projection identical to that described for the corresponding secondary lineages (Lovick et al., 2013; Figs. 8A-D). This tract is followed backward from late L3 to approximately 48 h posthatching, when secondary tracts start to elongate (Fig. 7H-K). The signature of DPLc2/4 tract. Expression of FasII in DPLc2/4 throughout the larval period (Supplementary Fig. S3) helps identifying these DPLp clusters in the early larval brain. Further posteriorly and ventrally the DPLp5 tract is directed towards the DPLc2/4 tract; these clusters (DPLp anterior in Fig. 7L M) are interpreted as DPLc1/3. For DPLp, a substantial ventrally directed tract, which projects parallel to the descending DPM1 tract (Fig. 7A, G, C, K, G, O; see also below) can be distinguished.

The second lateral DPM group, DPM2, is represented by one lineage (DPM11) with a thick, highly visible tract. DPM11 is located at the level of DPM1/2, and projects a main branch directed posterolaterally towards the intermediate superior transverse fascicle (tsR). In the L1 brain, we can only identify a small cell cluster located laterally to the VL tip that corresponds in position to DPLp (Fig. 7A, H, H).

The CM lineages CM1, CM3, and CMd are Type II lineages located at the posterior-medial surface of the brain. Their short axon bundles converge upon two entry portals, a dorsal one (PB v; #28 in Figs. 2J, K, 8A and 8B), and another one (PB v; #29 in Figs. 2J, K, 8A, B, D, E). The dorsal convergence (CMd; Fig. 8A, B) is mostly formed by fibers of CMd, the fourth Type II lineage that, during the secondary stage, generates the columnar neurons of the central complex; CMd forms a thick bundle extending into the inferior protocerebrum; this bundle defines the medial equatorial fascicle (MEF; Figs. 2K4 and 8A-H). A branch of these forward-directed axons tums medially towards
Fig. 8. Tracts associated with dorso-posterior medial (DPM) and centro-medial (CM) images. (A, B) Digital 3D models of DPM and CM images and tracts in a single 11 brain hemisphere. Anterior view (A), medial view (B). At top of each panel, lineages are shown in relationship to mushroom body (gray) for spatial orientation; bottom of panels shows higher magnification of lineages and neuropil tracts (light blue). Numbered hatched circles in (A) and other panels represent entry portals of lineage-associated tracts. Arrows in (A) and (B) point to convergence of tracts of DPMm1, DPMm1/2, and CM to form a commissural tract that represents the primordium of the fan-shaped body (pFB). Letters "x"-"y" indicate additional tracts formed by these lineages (see text). Double-headed arrow in (A, B) and all other panels indicates brain midline. (C-H) z-Projections of frontal cortical sections of medial half of a 11 brain hemisphere (24 h; C-E) and 12 lateral (48 h; F-H). Antero-posterior levels shown by z-projections are indicated by letters (Ca int, Cc post, CA) at lower left corner (for definition of levels, see Fig. 2). Primary neurons and tracts are labeled by anti-Neurolin (BP1DA; white in panels C-E; magenta in C-E). Primary neurons representing the Type II lineages DPMm1, DPMm1/2, CM1/3/4 are labeled by DsRed-Gal4 > UAS-EGFP (green in C-E); from 12 onward the same markers label secondary neurons of these lineages (green in panels F-H). For abbreviations of compartments and fiber tracts see Table 1; for numbering of entry portals see Table 2. Bar: 20 μm (C-H).
Fig. 9. Tracts associated with posterior lineages (CP, BII, DPLp), (A, B) Digital 3D models of posterior lineages and tracts of a single L1 brain hemisphere. Lateral view (A), posterior view (B). At the top of each panel, lineages are shown in relationship to mushroom body (blue-gray) for spatial orientation; bottom of panel shows higher magnification of lineages and neurite tracts (light blue). Numbered hatched circles in (A) and other panels represent entry portals of lineage-associated tracts. Double-headed arrow in (B) indicates brain midline. (C-L) Projections of frontal confocal sections of lateral half of brain hemisphere of L1 larva (24 h: (C-I), L2 larva (48 h: (J-L)), and early L3 larva (56 h: (K, L)). Lateral in all panels is to the left. Antero-posterior levels shown by z-projections are indicated by letters (CApost, CAant) at lower left corner (for definition of levels, see Fig. 2). Primary neurons and tracts are labeled by anti-Neuroligin (BII4; white in panels C, D, I, J; magenta in all other panels). Primary neurons representing the Type II lineages CP2 and CP1 are labeled by Shh-Gal4 > UAS-mcd8::GFP (green in I, J); from L2 onward, the same marker labels secondary neurons of these lineages (green in panels I, J). Primary neurons of CP1 are labeled by Dpp-Gal4 > UAS-mcd8::GFP (green in G, H). Secondary neurons are globally labeled by imac-Gal4 > UAS-cherry::YFP (green in K, L). For abbreviations of compartments and fiber tracts see Table 1; for numbering of entry portals see Table 2. Bar: 26 μm (C-L).
the primordium of the fan-shaped body (prFB; Fig. 8A, B). A second lineage projecting into the MEF is DIPi/2, located dorsally adjacent to CM4 (see above; Fig. 8A, B, E,E,’H). Extending anteriorly, the MEF splits up into branches that turn ventrally towards the great commissure (GC; Fig. 2K4), and antero-laterally towards the lateral accessory lobe (LAL1, not shown).

Located ventrally and laterally of the origin of the median equatorial fascicle (MEF) two clusters, interpreted as CM1 and CM3, have axons that contribute to the MEF, but mainly contribute on the VMC po portal (#28) they project forward and ventrally, forming the longitudinal ventral posterior fascicle (LPF; Figs. 2I, K5 and 8A, B, D, F, J, L) and projects dorsomedially, forming the oblique posterior tract (obpt; Fig. 9A, B, J, L). The ventral branch projects anteriorly, parallel to the peduncle. This tract enters at the PLP po portal (#23 in Figs. 2I, J, K5 and 8A, B, D, F, J, L) and forms the dorsal component of the posterior lateral fascicle (PLF; Figs. 8A, J, K5). Marker expression by the expression of R79A11-Gal4 (Fig. 9C, H) enters medially of DPLi/2 and also forms a branched tract (entry portal CM1, #22; Figs. 2I, J, K5 and 8D–G, F, L). The dorsal CPli tract joins the obpt, forming the ventral component of this thick bundle (Fig. 9A, B, J, L). The ventral CPli branch, which projects laterally of the calyx (entry portal CM1, #22; Figs. 2I, J, K5 and 8A–D, F, J, L) and projects dorsolaterally, forming the lateral equatorial fascicle (LEF; Fig. 2L4 and 9A, B, G, H, J). The fourth CP lineage, CP4 (as defined at the secondary stage in the late larva), also projects along the obpt, close to CP1; CP4 neurons and their primary tract are located dorsally of CP1 (entry portal CM1, #22; Figs. 2I, J, K5 and 9A–C, I, J, K).

Three lineages of the DPL group, DPLi/2 and DPLi/2, are known to the clusters in location and projection. The DPLi/2 cluster is located dorsally of CP1/2, laterally of the calyx (entry portal CM1, #22; Figs. 2I, J, K5 and 9A–D, F, J, L). The lateral DPLi/2 branch converges onto the dorsal CP2/2 tract as it enters the oblique posterior tract (obpt). DPLi/2 is located ventro-laterally of DPLi/2, bordering its outer optic anlage of the optic lobe primordium (entry portal PLP, #23; Figs. 2J, I and 9B, F, J, L). At the secondary stage, DPLi/2 has a characteristic, bifurcated tract, with one short lateral branch towards the optic neuropil and a medial branch that projects anteriorly along the posterior-lateral fascicle (PLFd), together with the CP2/2 axons (Fig. 9K, arrowhead). A primary cluster with a short, simple tract, projecting ventrally of the DPLi/2 tract, can be followed back towards the L1 stage and has been tentatively defined as DPLi/2 (Fig. 9A–C).

BPL lineages are located at the posteoro-lateral brain surface, posterior to the optic lobe primordium. Their axon tracts approach the lateral neuropil surface (more precisely: the point where the lateral surface of the ventrolateral protocerebrum (VLP) is joined by the larval optic neuropil) from posteriorly (Pereau and Martenstein, 2006). At the secondary stage, BPL lineages form two lineages with similar projection: BPLi/2, whose cell bodies clusters located dorsally extend their axons antero-ventrally towards the lateral surface of the VLP compartment. BPLi/4, located further ventrally, project upward towards the lateral superior lateral protocerebrum (SLP; Lowick et al., 2013). Two BLP clusters with similar location and axonal trajectory can be followed backward towards the L1 stage: one cluster (termed BLPi/2) approaches the junction between the larval optic neuropil (LON) and VLP from postero-dorsally, the other one (BLPi/4) from postero-ventrally (Fig. 2I, J, K5 and 9A–D, F, J, L). Tracts cannot be followed any further anteriorly, and their entry portals into the brain neuropil cannot clearly be defined.

BLV lineages are located ventrally of the optic lobe primordium. The two posterior-most member of this group, BLVi/2, are located ventrally adjacent to CP2/3, and have closely apposed axon tracts that converge on the posterior-lateral fascicle carrying the ventral CP2/3 axons (PLF; Figs. 2K5 and 8A–D, F, J, L). BLVi/2 tracts form a separate entry point (PLP pi; #24 in Figs. 2I, J, K5 and 8A–D, F, J, L) and continue as the ventral component of the PLF (PLV) that projects anteriorly towards the lateral inferior protocerebrum.

3.10. Lateral lineages: BLA, BLD, BLV

BLA lineages are located along the anterior edge of the optic lobe primordium. At the secondary stage, they comprise a ventral group of 3 lineages (BLA/v1/2, BLA/m) and a dorsal group of five lineages (BLAD1–4; BLA). The BLAV lineages can be individually followed backward towards the L1 stage: primary BLAV lineages from three separate tracts that project posteriorly towards the lateral surface of the ventrolateral protocerebrum (VLP). Only BLA1 is labeled by R79A11-Gal4 (Fig. 10D,D”). The BLAV lineages terminate medially and enter the VLP neuropil (entry portal VLP d, #32; Figs. 2I, J and 10A, B, D, E, G, H, I, K); BLAV1/2 fibers continue medially as part of the great commissure (GC; Figs. 2K3 and 10A, B, D, E, G, H). BLAD lineages form a cluster dorsally of the BLAV lineages. Three to four short, posteriorly-directed tracts converge on a thick bundle which enter the superior lateral protocerebrum (SLP) as a tract directed towards the posterior lateral superior anterior fascicle (SLA) formed by DPLa (entry portal SLIP d1/15b; Figs. 2G–I, K1 and 10A–D, G, J). Right posterior of the trSLA, the BLAD bundle turns dorsal-medially, forming the transverse superior intermediate fascicle (tsrIF; Fig. 10A–D). The BLAI lineage, located at the medial edge of the BLAD cluster, has a characteristic bifurcated tract that sends one branch posteriorly, the other one antero-medially. The posterior branch follows the neuropil surface to join the axon bundle formed by BLAV; the antero-medial branch extends around the anterior neuropil surface along the boundary between the superior lateral protocerebrum (SLP) and ventrolateral protocerebrum (VLP), and approaches the spur of the mushroom body (Figs. 2K2, J3 and 10A, C).

BLD lineages are located dorsally of the optic lobe primordium and project tracts ventrally towards the lateral surface of the neuropil. At the secondary stage, six lineages, further subdivided into three pairs with similar trajectories, were identified. The posterior pair, BLD5/6, has long, ventrally-oriented tracts that are directed towards the junction between the larval optic neuropil (LON) and the ventrolateral protocerebrum (VLP). Here, tracts turn medially or in near the great commissure (GC). The BLD5/6 pair, located furthest posteriorly and marked by R79A11-Gal4, can be followed backward towards the L1 stage (Figs. 2K4 and 10B, C, F, I, J, L). The PAT of this pair projects straight ventrally and enters the lateral neuropil immediately dorsolateral of the larval optic neuropil (LON; PLP1 entry portal, #30; Figs. 2I and 10B, F, I, J, L). The four anterior BLD lineages, BLD1–4, have tracts that approach the lateral surface and make a characteristic sharp turn towards dorso-medially, joining the transverse superior intermediate fascicle (tsrIF) formed by BLAd (see above). At the secondary stage, the BLD1–4 lineages have characteristic branching patterns, which do
Fig. 10. Tracts associated with lateral lineages (BL, BLD, BLV) (A–C). Digital 3D models of BL lineages and tracts in a single L1 brain hemisphere. Anterior view (A), posterior-lateral view (B), dorsal view (C). Aside from mushroom body and antennal lobe (blue-gray) and fascicle-positive tracts (dark gray), the larval optic neuropil (LON) is shown for reference. Fiber bundles of neuropil in which BL lineages contribute are shown in light gray. Numbered hatched circles in (A) and other panels represent entry portals of lineage-associated tracts. (D–L) Projections of frontal confocal sections of lateral half of L1 brain hemisphere (24 h; D–F) and early L3 larva (64 h; G–I). Lateral in all panels is to the left. Antero-posterior levels shown by projections are indicated by letters (ML, GCan, GPost) at lower left corner (for definition of levels, see Fig. 2). Primary neurons and tracts are labeled by anti-Neuramin (BP104; white in panels D–F and G–I; magenta in D–F and G–I). Primary neurons of BLDs, BLD5, BLD2, and BLF5 are labeled by GFP/Gal4-UAS-mCherry::GFP (green in D–F). Secondary neurons are globally labeled by syn-Gal4::UAS-shibireUAS::GFP (green in G–I) and anti-Neurotactin (BP106; white in J–L). On all panels, parts of larval optic neuropil (LON) are shown close to left marginIOA inner optic anlage; OOA outer optic anlage). For abbreviations of compartments and fiber tracts see Table 1; for numbering of entry portals see Table 2. Bar: 20 μm (D–F); 50 μm (G–I).
not exist at the primary stage in the L1 brain. Here, BD1-4 form a lateral and a medial pair, directly adjacent to the dorsal edge of the optic lobe primordium (inner optic anlage, IOA; Fig. 10B, C, E, H/W, K); axons of both clusters converge and enter the superior lateral protocerebrum (SLP) next right to the DPLa1 and BLAd tracts (SLP 1
entry portal, #15c; Figs. 2G-1 and 10R, C, E, H/H, K). BD1-4 tracts continue along the trSI fascicle. BLV lineages are grouped around the ventral edge of the optic lobe primordium and project their tracts dorso-laterally, into the field formed between the inner optic anlage (IOA) and the ventral lateral protocerebrum neuropil (VIP; Figs. 2K4 and 10A, B, F/F', I/F', I/F, I). The two posterior BLVs (BLVp1/2) were discussed in the previous section (see above). The remaining, anterior BLV lineages form two pairs, BLV a1/2 and BLV a3/4. The BLV a3/4 pair is located posteriorly and medially of the BLV a1/2 pair. As described for the corresponding secondary lineages, the primary BLVa3-4 tract is shorter, ending below the LON-VLP junction at the lateral surface of the VIP (entry portal VIP vl #31; Figs. 1J, K4 and Fig. 10A, B, F/F', I/F', I/F, I). It funnel dorso-laterally, running in front of the LON-VLP junction towards the superior lateral protocerebrum (SLP; entry portal IJ/a, #15c; Figs. 2G-1, K4 and 10A, B, F/F', I/F', I, I).}

4. Discussion

4.1. The use of pan-neuronal markers in reconstructing brain architecture

The brain neuropil of insects and most other invertebrates is composed of the thin processes of neurons and glial cells. One can distinguish neuropil domains where terminal axonal and dendritic branches form synaptic connections (synaptic neuropil) from bundles of long processes (tracts or fascicles) that interconnect different domains of synaptic neuropil. Globally expressed neuronal membrane proteins, such as Neuroglian or Neurotactin, are concentrated in long axon bundles and, when labeled by proten-specific localization, they stand out against the surrounding synaptic neuropil. It is important to note that many known pan-neuronal proteins are expressed dynamically (Fung et al., 2008). Both Neurotactin and Neuroglian appear at high levels in young neurons that send out their initial axon during embryonic development (Fung et al., 2008). Neurotactin and Neuroglian are important markers for the early larval brain, but there are already differences in expression level which are most likely correlated with the birthdate of neurons. Thus, the intensity of labeling of neuronal cell bodies in the cortex is not identical for all cells; clusters of strongly labeled cell bodies, always closely associated with the beginning of the lineage axon tract (PAT), are surrounded by more weakly labeled cell bodies (see, for example, clusters indicated by arrows in Fig. 2L). We suspect that the cells with higher expression levels are the late born neurons and that their strongly labeled axon tracts form the visible “backbone” of the PATs visible in the L1 brain. In the late larval brain, primary neuron expression of Neurotactin and (to a lesser extent), Neuroglian, wanes, while expression of these markers in secondary axon tracts is very robust. Secondary tracts maintain expression of Neurotactin and Neuroglian throughout early metamorphosis; at late pupal stages, Neuroglian remains strong all the way into adulthood, which makes it possible to relate the long axon tracts of the larva to those of the adult (Lovick et al., 2013).

One needs to point out that, aside from the lineage-associated axon bundles, there exists a second type of tract or fascicle which consists of less tightly packed parallel fibers with interspersed short terminal branches and synapses. A prominent example are the longitudinal, fasti-positive axon tracts of the ventral nerve cord and the lobes of the mushroom body. The long nerve fibers that scaffold these domains, also called “tract neuropil” (Virtual Fly Brain; Miljaver et al., 2012), are not necessarily related to lineage. This is very clear in case of the longitudinal tracts of the VNC, where lineage-associated tracts form predominantly transverse (commissural) or vertical bundles (Stuart et al., 2014; Truman et al., 2004), but do not become part of the fasti-positive longitudinal fascicles. Instead, these fascicles are most likely formed by single or small subsets ("sublineages") of axons belonging to several different lineages. In the embryo, fasti is expressed in groups of neurons that are born and differentiate early and thereby establish neuropil “pioneer” or “founder” tracts (Goodman and Doe, 1995; Nassif et al., 1998). The exact role of each of these pioneer neuron clusters to lineages has not yet been established; however, it seems clear that these clusters are derived from multiple lineages.

4.2. Factors controlling the spatial pattern of lineages and lineage-associated tracts

The pattern of PATs reflects in part the pattern of neuroblasts that had generated the lineages giving rise to the PATs. Based on clonal analysis, neuronal cell bodies belonging to one lineage cluster around their mother neuroblast and the PAT begins at the base of each cluster (Bassing et al., 1996; Lassen et al., 2009; Schmidt et al., 1997). There is no large scale migration of cell bodies away from the location in which they were placed at birth. The differences that one observes between the position of lineages in early and late embryos are brought about by a general movement of the brain primordium as a whole, whereby the neuraxis shifts posteriorly. For example, neuroblasts of lineages that are located dorso-posteriorly in the larval brain (e.g., mushroom body) start out quite anteriorly in the neuroblast map of the stage 11 embryo (Chang et al., 2003; Kunz et al., 2012; Nowe et al., 2000). It is not known what kind of morphogenetic mechanisms cause this shift; most likely, forces act upon the brain primordium itself, such as the moving foregut and heartbeat. However, in terms of local neighborhood relationships between individual neuroblasts and the clusters of neurons they give rise to, there do not appear to be major changes between early and late embryonic stages (Chang et al., 2003; Sprecher et al., 2007).

Aside from neuroblast location, another determining factor of the pattern of PATs appears to be affinities of certain lineages to each other. Thus, PATs of most lineages do not enter the neuropil as individual bundles, but travel together in groups of 2-4 members. Such groups of entering PATs form “entry portals” that represent distinct landmarks at the neuropil surface. They appear as funnel-shaped depressions, or as sheaths, in volume renderings of confocal stacks of preparations where the neuropil is labeled by global markers such as NCd-cadherin (Figs. 2 and 11). Many portals form part of the boundaries between neuropil compartments; examples are the portals that surround the antennal lobe at the anterior neuropil surface. Importantly, the combinations of lineages that group together during the primary phase of neurogenesis remain in contact during secondary neurogenesis in the late larva. In other words, the entry portals defined by PATs in the L1 larva correspond to those formed by SXTs in the adult brain (Lovick et al., 2013; Wong et al., 2013), which makes the entry portals a useful structural feature to follow neuropil morphogenesis throughout development (see below).

What do lineages that adhere together and enter the neuropil at the same portal have in common? Part of the answer probably lies in similarities in projection and connectivity mediated by the joined lineages. In the majority of cases, joined lineages project along the same fascicle within the neuropil, and therefore connect
overlapping or closely adjacent neuropil domains. Examples are Bamvl/2, BAbp/2/3, BL23/4, DALd1/2 (hemilineages), DALc1/2, DALd (hemilineages), DFLd1-3, DFLd1-3, BLAd1-4, BIF3/4, and BIV3/4. The secondary components of all of these lineages have been visualized as GP-labeled clones, and show not only common tracts, but also similar, largely overlapping domains of terminal arborization (Joo et al., 2013; Wong et al., 2013; Ye et al., 2013). It will be interesting to establish for these groups of lineages
the corresponding neuroblasts and their genetic identities; one
would expect that the commonalities in anatomical properties of a
group are reflected in the expression of similar genes during early
development.

4.3. Structural elements of the L1 and adult brain: a comparison

We had documented in previous studies (Nassif et al., 1998,
2003; Pereira et al., 2010; Younessi-Hartenstein et al., 2006) that
many structural elements of the adult brain can be already

Fig. 12. Entry portals of lineage-associated tracts (numbered hatched circles in the L1 larval brain (A–C), L3 larval brain (D–F), and adult brain (G–I)). Each panel shows a view: a rostral view (A, D, G), posterior view (B, E, H), and left lateral view (C, F, I) of the brain. The panels indicate brain midline. Rendering of entry portals follows color scheme used to differentiate between groups of lineages in Figs. 1 and 5–10.
recognized at the early larval stage. The identification of lineages and their associated tracts add many new details to these findings. Thus, the change in distances between fiber tracts (Fig. 11) and lineage entry portals (Fig. 12) that occur during development reflect the changes in neuropil growth that occur during larval and pupal development. This growth is due to the enlargement of primary neuronal arbor (from early to late larva) and the addition of secondary neurons (from early larva to adult). Neuropil growth is highly anisotropic: some compartments grow much more than others. Compartments that grow most are related to the highly increased number of sensory afferents that characterizes the transition from larva to adult (32 photoreceptors in the larva; 5500 photoreceptors in the adult; 11000 in the adult (Laisvys and Vosshall, 2008)), and to the control of complex motor acts the adult fly is capable of. 

Neuropil growth is most pronounced in four regions of the central brain: (1) the lateral protocerebrum and anterior protocerebral optic tubercle which receives higher order input from the optic lobe (visual system); (2) the antennal lobe, a primary sensory center for olfaction; (3) the superior lateral protocerebrum and lateral horn, which presumably serve as a multimodal association center; (4) the central complex which controls locomotion behavior.

(1) The growth of the ventral lateral protocerebrum can be best appreciated in the anterior and lateral/medial views of the neuropil presented in Figs. 11A–C and 12A–F respectively. Note the increase in distance between the vertically oriented tracts deCP and DPPT, and in length of the longitudinally oriented ventral fascicles (IoV, IoVM, kVp, Fig. 11 D–F). The portals of the laterally entering lines BSa3/4 (#31) and BSbP4/2 (#32) to each other in the L1 brain (Fig. 12D), are pushed away (Fig. 12E, F), the same change occurs for the entries of BLAv/12 (#32) and BLDS/#30). The anterior optic tubercle (AOTU) bulges out of the anterior surface of the larval SLP compartment (Fig. 12D–F).

(2) The adult antennal lobe develops at the dorsal margin of its larval counterpart, as described in previous works (ref). The enormous growth of this compartment can be appreciated by the increasing vertical and horizontal distance between the entry portals of dorsal projection neuron lineages BAlm3/#7 and the ventrolateral lineages BAlb1/#1 (Fig. 12A–C). The portal of BAlm1/#5, and the location of the ventrolateral medially fascicle (kVfM) formed by these lineages, shifts to a ventral position (Figs. 11A–C and 12A–C). The BAlm31 portal (#40) is also pushed ventrally and the median bundle (MBdX) formed by the BAlm31/2 lineages lengthens (Figs. 11A–C).

(3) The neuropil domain located dorso-laterally of the peduncle is relatively underdeveloped in the larval brain, compared to the adult. Here, the neuropil forms prominent bulges, the superior lateral protocerebrum (SLP) and lateral horn (LH). By contrast, the corresponding domain (SLP/LH) in Fig. 12C, F, I) is represented by a thin layer of neuropil. The growth of these compartments is reflected in the increasing distances between the entry portals of dorsal projection neuron lineages DPMl (#15), DPMl (#16) and posterior DPL lineages [DPlc (#18), DPlm (#19), DPlm (#20); see Fig. 12D, E, F]. The DPl and DPlm portals, occupying a lateral position in the larva (Figs. 11G, H and 12D, E), have been pushed towards the posterior brain surface by the outgrowing SLP and LH (Figs. 11A and 12A).

(4) The central complex develops during metamorphosis, primarily through the differentiation of the massive number of columnar/small field neurons generated by the type II lines DPMm1/D1, DPMm1/D2, DPMm2/D3, and CM/D4 (Riebl et al., 2013; Yang et al., 2015). In the early larval brain, primary neurons of these lineages form a commissural bundle that crosses the midline right behind the medial lobe of the mushroom body (Fig. 11D, G). This commissure, which grows into a sizeable primordium of the fan-shaped body of the third larval instar (Riebl et al., 2013; Fig. 11E, H), foreshadows the position of the central complex as a set of compartments formed during pupal development by elaboration of secondary commissural fibers that “squeeze” in between the commissures formed by previously established primary neurons (the SuEG/LALC antero-ventrally, SEC and SAC antero-dorsally, GC ventrally, and PLPC postero-dorsally: Fig. 11D–F).

An anteriorly located lineage, BAL43, contributes the (large field) ring neurons of the ellipsoid body that represents the anterior part of the central complex (Fig. 11F).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jneurosci.2014.05.063.

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