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Patterns of growth and tract formation during the early development of secondary lineages in the *Drosophila* larval brain

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Abstract

The *Drosophila* brain consists of a relatively small number of invariant, genetically determined lineages which provide a model to study the relationship between gene function and neuronal architecture. In following this long-term goal we reconstruct the morphology (projection pattern and connectivity) and gene expression patterns of brain lineages throughout development. In the present paper, we focus on the secondary phase of lineage morphogenesis, from the reactivation of neuroblast proliferation in the first larval instar to the time when proliferation ends and secondary axon tracts have fully extended in the late third larval instar. We have reconstructed the location and projection of secondary lineages at close (4h) intervals and produced a detailed map in the form of confocal z-projections and digital 3D models of all lineages at successive larval stages. Based on these reconstructions we could compare the spatio-temporal pattern of axon formation and morphogenetic movements of different lineages in normal brain development. In addition to wild type, we reconstructed lineage morphology in two mutant conditions. (1) Expressing the construct UAS-p35 which rescues programmed cell death we could systematically determine which lineages normally lose hemilineages to apoptosis. (2) *so*-Gal4-driven expression of dominant-negative EGFR ablated the optic lobe, which allowed us to conclude that the global centrifugal movement normally affecting the cell bodies of lateral lineages in the late larva is causally related to the expansion of the optic lobe, and that the central pattern of axonal projections of these lineages is independent of the presence or absence of the optic lobe.

Keywords

Brain; Development; *Drosophila*; Larval; Lineage

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INTRODUCTION

Highly ordered connections of neurons represent the structural basis of the complex brain circuitry controlling behavior. Neuronal connectivity is contingent upon morphological and functional properties of individual neurons (“wiring properties”), which are shaped during development. A fundamental task of developmental neurobiology is to elucidate the genetic mechanisms that control neuronal wiring properties. A number of transcriptional regulators that exert a profound effect on neuronal shapes and target selection have come to light, many of them in genetic studies of *Drosophila* neurons (Jan and Jan, 2010). Despite this progress, our understanding of how genetic networks regulate neuronal wiring is still in its infancy.

Drosophila represents a favorable model system to address the relationship between gene function and neuronal architecture. Aside from the genetic and molecular tools that have been developed, *Drosophila* offers the advantage that its nervous system is built by a relatively small number of genetically and structurally defined modules, the neural lineages (Hartenstein et al., 2008; Ito et al., 2013; Wong et al., 2013; Yu et al., 2013). Most lineages (Type I) possess approximately 100–150 neurons that are produced by a single, stem cell-like neuroblast. A small number of lineages restricted to the dorsomedial brain (Type II lineages) are much larger (~500 neurons; Bello et al., 2008). Neuroblasts first appear in the early embryo and proliferate in a characteristic, stem cell-like mode. A neuroblast divides asymmetrically into a large daughter cell that continuously divides (self-renewal) and a second, smaller daughter cell (ganglion mother cell; GMC), which divides once more to produce two postmitotic cells that differentiate into two neurons or glial cells. In this manner, each embryonic neuroblast initially produces a lineage of 10–20 primary neurons that form the larval brain (Hartenstein et al., 1987; Larsen et al., 2009). Most neuroblasts become mitotically quiescent in the late embryo and reactivate proliferation during the larval stage (Truman and Bate, 1988; Prokop and Technau, 1991; Ito and Hotta, 1992; Prokop et al., 1998). Continuing to divide throughout the larval period, neuroblasts produce sets of secondary neurons which, together with remodeled primary neurons, give rise to the adult brain. Unlike Type I neuroblasts, those which give rise to Type II lineages exhibit a more complex pattern of proliferation; rather than generating GMCs, Type II neuroblasts divide into a series of intermediate progenitors, which themselves undergo several rounds of asymmetric divisions, much like Type I neuroblasts (Kang and Reichert, 2015).

During the embryonic phase, neuroblasts express specific and invariant combinations of transcription factors, which have been mapped in considerable detail (Urbach and Technau, 2003a). Several of the early expressed transcription factors remain active also during the secondary phase of neuroblast proliferation (Lichtneckert et al., 2007; Kumar et al., 2009; Sen et al., 2014). It is thought that a neuroblast, by the set of transcription factors it expresses, is provided with a specific “genetic address,” which plays a role in shaping the connectivity and function of the neurons it produces (Skeath and Thor, 2003; Urbach and Technau, 2003b). This notion bears well with the fact that neurons of the same lineage generally project together in one or two coherent fiber tracts, implying that they react in the same way to positional information controlling axonal pathfinding. In addition, upon differentiation, neurons of the same lineage typically target one or a few, spatially-restricted

brain compartments. This is particularly true for the secondary lineages, as documented in a detailed clonal analyses of the projection patterns of MARCM clones in the adult brain (Ito et al., 2013; Wong et al., 2013; Yu et al., 2013). By contrast, primary neurons of a given lineage, in particular those born early in the embryo, show a higher degree of diversity in terms of target selection.

Lineages are further subdivided into smaller units, called hemilineages and sublineages. Hemilineages are the result of the division of GMCs. In many, if not all lineages, the neurons resulting from one GMC are not equal, but form an “A” and a “B” neuron (Truman et al., 2010). Studies of secondary lineages showed that A-neurons and B-neurons systematically differ with respect to their axonal trajectory, whereby all A-neurons bundle into one tract and B-neurons into another. The branched secondary axon tracts (SATs) described for a number of lineages of both ventral cord (Truman et al., 2004; 2010; Kuert et al., 2012; 2014) and brain (Pereanu and Hartenstein, 2006; Cardona et al., 2010) in actuality represent the A-tract and B-tract combined. In many, if not all cases with an unbranched SAT, one hemilineage is eliminated by apoptotic cell death (Kumar et al., 2009b; Truman et al., 2010). Sublineages represent groups of neurons born sequentially during a certain time interval. In some cases, sublineages may differ with regard to their terminal arborization. They may all project along the same SAT, but choose different targets (e.g., layers; glomeruli) in the area of terminal arborization. This has been shown in detail for the four mushroom body lineages, where neurons born during different time intervals generate the γ -lobe, α/β -lobe, and α'/β' -lobe, respectively (Lee et al., 1999; Lin and Lee, 2012a).

In order to use lineages for genetic studies it is essential to provide detailed knowledge of the morphology of lineages throughout development. Quite extensive information exists for the lineages forming the mushroom body and the antennal lobe (e.g., Lee et al., 1999; Jefferis et al., 2002; 2005; Zhu et al., 2003; Yu et al., 2010; Lin et al., 2012b; 2013; Das et al., 2013). For other lineages, our knowledge is restricted to the above referenced maps that were generated for embryonic neuroblasts and for secondary lineages at the late larval stage and the adult stage. The developmental changes that shape the structure of lineages between the time when they first appear in the late embryo and the time of neuronal differentiation have not yet been elucidated for most lineages.

In the present paper, we have analyzed the early secondary phase of lineage morphogenesis, encompassing the time interval when neuroblasts resume proliferation (first larval instar) to the time point when secondary axon tracts have fully extended (third larval instar). The focus was on the larval brain (supraesophageal ganglion); a similar analysis of the ventral nerve cord (subesophageal ganglion, thoracic ganglia) is underway (Kuert et al., in preparation). Our analysis produced a detailed map, in the form of digital 3D models, of lineages at successive larval stages which allowed us to study axon growth and morphogenetic movements of lineage cell body clusters. Finally, we identified those brain lineages that lose hemilineages by cell death, using the apoptosis-inhibiting construct UAS-p35 (Yoo et al., 2002). Our results will assist future investigations addressing the mechanisms that control neuronal fate and differentiation in the *Drosophila* brain.

MATERIAL AND METHODS

Fly Lines

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

asense (ase)-Gal4 (Zhu et al., 2006), *engrailed (en)*-Gal4 (Tabata et al., 1995; #30564, Bloomington *Drosophila* Stock Center (BDSC), University of Indiana, IN, USA), *inscuteable (insc)*-Gal4 (Mz1407; Betschinger et al, 2006; #8751, BDSC), *sine oculis (so)*-Gal4 (Chang et al., 2003a; 2003b), UAS-chRFP-Tub (Rusan and Peifer, 2007; #25774, BDSC), UAS-mcd8::GFP (Lee and Luo, 1999; #5137, BDSC), UAS-p35 (on third; Yoo et al., 2002), UAS-dnEGFR (Chang et al., 2003a).

Clonal Analysis and Lineage Tracing Experiments

Primary lineage flip-out clones were generated using flies bearing the genotype: *hs-FLP¹*, *UAS-mCD8-GFP/+*; *UAS-mCD8-GFP/+*; *tub-FRT-GAL80-FRT-GAL4* (Zecca and Struhl, 2002). Briefly, embryos were heat-shocked at 38°C for 5 minutes and raised at 18°C until wandering third instar larva; third instar larval brains were dissected and processed for immunohistochemistry (see below).

Immunohistochemistry

Samples were fixed in 4% formaldehyde or 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 0.1–0.3% Triton X-100, pH = 7.4) and immunohistochemistry was performed using standard procedures (Ashburner 1989). The following primary antibodies were provided by the Developmental Studies Hybridoma Bank (Iowa City, IA): mouse anti-bruchpilot (nc82; 1:20); rat anti-DN-Cadherin (DN-EX #8, 1:20), and mouse anti-Neurotactin (BP106, 1:10). Additional primary antibodies: rabbit anti-Deadpan (Dpn; Bier et al., 1992). Secondary antibodies, IgG₁ (Jackson ImmunoResearch; Molecular Probes) were used at the following dilutions: Cy5-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), DynaLight 649-conjugated anti-rat (1:400), Alexa 568-conjugated anti-mouse (1:500).

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 or FIJI (National Institutes of Health, <http://rsbweb.nih.gov/ij/> and <http://fiji.sc/>) and Adobe Photoshop.

Digital removal of background fluorescence

For clarity in some panels, background fluorescence and/or fluorescence from other lineages (visualized with Gal4 drivers or clones) were digitally removed using FIJI (Schindelin et al., 2012; <http://fiji.sc/>) or Adobe Photoshop.

Electron Microscopy

For electron microscopy, larval brains were dissected and fixed in 2% glutaraldehyde in PBS for 20 min, followed by a postfixation for 30 min in a mixture of 1% osmium tetroxide and 2% glutaraldehyde in 0.15 M cacodylate buffer (on ice). Specimens were washed several times in PBS and dehydrated in graded ethanol and acetone (all steps on ice). Preparations were left overnight in a 1:1 mixture of Epon and acetone and then for 5–10h in unpolymerized Epon. They were transferred to molds, oriented and placed at 60°C for 24 h to permit polymerization of the Epon. Blocks were sectioned (0.1 μ m). Sections were mounted on net grids (Ted Pella) and treated with uranyl acetate and lead citrate. A JEOL 100CX transmission electron microscope was used for observation.

Morphological criteria for staging of larval brains

Animals were staged by placing larvae hatched from the egg within a 1h 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 considerable variability (in the order of 10%) in brain growth of larvae of the same age. We therefore defined specific morphogenetic parameters of the rapidly expanding optic lobe as structural hallmarks of the larval brain. These parameters include the ratio of optic lobe diameter (OOA) to neuropil diameter (OOA/NP), the ratio of neuroblasts versus epithelium within the outer optic anlage (NB/NB+E), and the thickness of the layer of medulla neurons (MN; Supplementary Figure S1).

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- μ m 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-DNCad 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 were created by manually labeling each lineage and its approximate cell body cluster location in TrakEM2.

RESULTS

Reconstruction of secondary axon tracts in the early larval brain

Using an antibody against the adhesion protein Neurotactin (de la Escalera et al., 1990) and the Gal4 driver line *insec-Gal4* (Betschinger et al., 2006), which is expressed specifically in secondary lineages from the time of their appearance onward, we reconstructed the pattern of larval secondary lineages at short temporal intervals between hatching and 96h after hatching (AH). By analyzing the different aspects of the pattern of secondary lineages, including location of the tract entry points and characteristic trajectory and branching within the neuropil, at sequential, closely spaced time intervals, it was possible to identify

individual lineages, or lineage pairs, at all stages (Hartenstein et al., 2015; Suppl. Fig. S2, S3, Table 1). During the first larval instar, as described in previous works (Ito and Hotta, 1992), only five neuroblasts are mitotically active and are strongly labeled by *insc*-Gal4 and Deadpan (Dpn). These include the four neuroblasts of the mushroom body (MB) located in the posterior brain and the neuroblast generating the BALc lineage that flanks the antero-lateral neuropil (“3” in Fig. 1A). Neuroblasts of all other lineages are small cells, also marked by the expression of Deadpan (Dpn; Bier et al., 1992), which are scattered throughout the brain cortex, either close to the surface (“1” in Fig. 1A) or at deeper levels (“2” in Fig. 1A). Many neuroblasts already express low levels of *insc*-Gal4>UAS-chRFP-Tub, which allows one to recognize thin basal processes connecting the cell body to the neuropil surface (as in the case of “1” in Fig. 1A; BALp1/2 and DPLc neuroblasts in Fig. 1B). This peculiar phenomenon had already been described for the early larval neuroblasts of the ventral nerve cord (Truman and Bate, 1988; Chell and Brand, 2010); the connection between cell body and neuropil (green arrows in Fig. 1B) may serve as a structure that anchors those neuroblasts which are remote from the neuropil to the site where the primary axon tract enters the neuropil (magenta arrows in Fig. 1B).

All neuroblasts re-enter the cell cycle between 20 and 40h AH in a stereotyped temporal order (Ito and Hotta, 1992; Lovick and Hartenstein, 2015). This is accompanied by an increase in cell size, an increase in expression of *insc*-Gal4, and relocation to the brain surface (Fig. 1C). The appearance of these morphological and genetic changes defines the “time of birth” for a secondary lineage and closely corresponds to the birth date deduced from experiments where neuroblasts were systematically ablated, at short intervals, using the drug hydroxyurea (HU; Lovick et al., 2015; Table 1). The first lineages to appear around 20h AH are DALv1, BLAv2, BLD6, and BLVp1 (Table 1). Around 24–27h more than half of the secondary lineages can be detected; the last ones to be born are several lineages located in the antero-dorsal brain (DAMv, DAMd, DALcm).

Secondary axons follow the tracts laid down by primary lineages

Following their birth during the late first/early second larval instar, secondary lineages undergo a rather uniform phase of growth and axonogenesis. During the first 2–4 hours following their enlargement, neuroblasts perform one to two cell divisions. At this stage, lineages appear as small clusters of *insc*-positive cells, with one large superficial neuroblast accompanied by two to four smaller progeny (ganglion mother cells, neurons; see for example DAMd1 in Fig. 2K). Subsequently, with the onset of axonogenesis in the first-born neurons, secondary axon tracts (SATs) make their appearance as short stubs connecting the cell body cluster with the neuropil surface (Fig. 2A, I, L). SATs then extend into the neuropil, following in almost all cases the pattern that is prefigured by the primary axon tracts (PATs; arrows in Fig. 1D indicate representative examples). PATs are labeled by anti-Neuroglian (BP104) in the early larval brain, and a comprehensive map of PATs and their corresponding lineages has been generated (Hartenstein et al., 2015). The direct contact between SATs and PATs at the neuropil entry points can also be appreciated electron microscopically (Fig. 1E–G).

The only case where entry point and trajectory of PAT and SAT differed markedly is the lineage pair DALv2/3, labeled by the expression of *engrailed-Gal4* (Kumar et al., 2009a; Fig. 1H). Primary DALv2/3 neurons enter the neuropil at the ventro-lateral surface of the lateral accessory lobe (LAL) and fill this compartment with terminal arborizations (Fig. 1H; see also Hartenstein et al., 2015). Secondary DALv2/3 axons form a thick bundle that skirts the anterior surface of the LAL, and enters the neuropil close to the medial lobe of the mushroom body. These axons will differentiate into the large-field neurons of the ellipsoid body, a compartment that has no counterpart in the larval brain.

The sequence at which secondary axon tracts extend follows the birth order of lineages

The time of onset of axonogenesis and the time course of axon extension seems to correlate with the birth date of a lineage. In other words, neurons extend their axon at a fixed time interval following their birth; an early born lineage such as DALv1 has a well established SAT at 27h AH (Fig. 2A). The DALv1 SAT reaches across the midline around 64h AH (Fig. 2C). By contrast, a late born lineage such as DALcm1/2 produces short axons at 64h AH (Fig. 2H) and reaches the midline after 80h AH (Fig. 2J). The DAMd group of lineages consists of three members; one of them, DAMd1, has a slightly earlier time of birth than DAMd2/3 (Table 1; Fig. 2K, L). The DAMd1 tract extends towards the brain midline around 48h AH (Fig. 2L); the longitudinally directed DAMd2/3 tract follows suit several hours later (Fig. 2M). This temporally staggered extension of secondary axon tracts is in striking contrast to the near simultaneous sprouting of branches and formation of synaptic connections, events that do not occur for any secondary lineage (except the mushroom body) during the larval stage. Instead, secondary lineages, independent of birth date, appear to branch and form terminal arborizations in a temporally coordinated manner during the late pupal stage (Larsen et al., 2009).

The majority of Type I secondary brain lineages lose a hemilineage to programmed cell death

Previous work focusing mostly on the ventral nerve cord had shown that in many, if not all, secondary Type I lineages, the mitosis of each GMC is asymmetric, generating an “A” daughter neuron and a “B” daughter neuron (Truman et al., 2010). “A” neurons and “B” neurons each constitute their own A and B hemilineage and their axons gather in two separate bundles or tracts. When a lineage only has a single tract, the assumption is that one hemilineage is present and that the other has been removed via programmed cell death. This was confirmed for a number of secondary lineages of the VNC (Truman et al., 2010) and the brain (Kumar et al., 2009b), where the majority of Type I lineages (50 out of 87) possess only one tract (called “Type I 1H lineages” in the following; Table 2). To establish whether these lineages lose hemilineages due to programmed cell death we inhibited cell death in Type I lineages during the larval period by expressing a UAS-p35 construct (Yoo et al., 2002) under the control of the *ase-Gal4* driver line (Zhu et al., 2006). Following this treatment, none of the lineages which normally possess two tracts (“Type I 2H lineages”) showed structural abnormalities. By contrast, most of the Type I 1H lineages developed an additional tract (Table 2). In these lineages, the normal SAT was fully formed and followed the normal trajectory, but a second, often thinner bundle branched off the normal tract. In most cases, the branching occurred proximally, close to the point where the SAT entered the

neuropil (arrowhead in Fig. 3H); sometimes, the SAT was split already in the cortex and an additional cell cluster was visible (data not shown). In other cases, the supernumerary tract branched off the SAT at a more distal location within the neuropil. The trajectory of the supernumerary branch was relatively stereotyped for a given lineage; it either closely followed the path of a neighboring SAT or formed a novel pathway not normally followed by any lineage (Table 2). A representative example for each of these two behaviors is provided by Fig. 3A–H. In the first case, the lineage BAmas1 whose regular SAT turns dorsally towards the SMP compartment, forms a branch that follows the pathway of TRdm (a lineage of the subesophageal ganglion) into the anterior subesophageal ganglion (arrowhead in Fig. 3A–D). The second scenario is represented by DPLam that enters the neuropil from antero-dorsally and projects through the characteristic crescent formed by the tract of lineages DPLal1-3 towards the domain surrounding the peduncle of the mushroom body (Fig. 3E–F). In animals over-expressing p35 in secondary neurons, an additional branch emerged from DPLam that projected laterally, following a trajectory that does not exist in normal brains (arrowhead in Fig. 3G–H).

In some lineages, supernumerary tracts follow pathways that are prefigured by their corresponding primary neurons. The concept is schematically illustrated in Fig. 3O and exemplified in Fig. 3I–N. The example shown is lineage BAmD2 whose secondary neurons follow a single tract directed ventro-medially into the antennal lobe commissure, following the ventral (“v”) tract of BAmD1 (Fig. 3I–J). Following p35 over-expression, a thin supernumerary tract branches off the BAmD2 tract and projects dorso-posteriorly, following the axon bundle formed by the DAMD2/3 lineages (arrowhead in Fig. 3K–L). A GFP-labeled clone of primary neurons of BAmD2 showed axons that followed both the ventral pathway (arrow in Fig. 3M–N) and dorsal pathway (arrowhead in Fig. 3M–N); a clone of secondary neurons, as expected, has only neurons projecting ventrally (arrow in Fig. 3P). We surmise that the two pathways of primary neurons correspond to the two primary hemilineages of BAmD2, as suggested schematically in Fig. 3O (top). Taken together, these findings imply that during the primary phase of BAmD2 proliferation in the embryo, both hemilineages (A and B) survive and differentiate (Fig. 3O, top). During the secondary phase of proliferation, one hemilineage (arbitrarily called B in Fig. 3O, center) undergoes cell death, so that the SAT of BAmD2 has only an A-component. When p35 is over-expressed, the B component does not die and forms a supernumerary axon tract (Fig. 3O, bottom).

Hemilineages of a given lineage extend axon tracts at different time points

In most Type I 2H lineages, extension of the hemilineage-associated SATs occurs at different time points (Table 2, Fig. 4). The pair DPLal2/3 forms a dorsal tract, shared with DPLal1, that grows out between 48 and 74h AH (red arrowhead in Fig. 4B–C), and a ventral tract that appears later, between 74h and 96h AH (cyan arrowhead in Fig. 4D). Two similar examples are illustrated in Fig. 4E–H: The pair DALcl1/2 forms a ventral tract passing underneath the spur of the mushroom body (red arrowhead) and a dorsal tract passing above the peduncle (cyan arrowhead); the former forms between 20h and 48h AH, the latter between 64h and 96h AH. The lineages BLAv1 and BLAvm each possess a posterior tract extending posteriorly between 20 and 48h AH (yellow arrowheads) and a medial tract

passing medially over the anterior surface of the VLP between 74h and 96h AH (magenta arrowheads).

Morphogenetic movements of lineages during larval brain growth

Between hatching and the third larval instar, the brain undergoes a substantial increase in volume due to the proliferation of secondary lineages, the growth of the optic lobe, and continued branching of primary neurons. As a result of these events, the cell body clusters of many secondary lineages and, in some cases also the point of entry of SATs, shift in position (Fig. 5, Fig. S3). The most significant movements of lineage clusters can be observed in the lateral brain, where the optic lobe neuropils and the fiber systems connecting the optic lobe to the central brain emerge. Here, lineages that initially emerge at the lateral surface of the brain (BAIp, BLA, BLD, BLP, BLV; Fig. 5A) are displaced towards anteriorly (BAIp, BLA), dorsally (BLD), or posteriorly (BLP, BLV; Fig. 5B). Likewise, the antennal lobe and lineages grouped around it (BALa, BAmas, BAmv), start out at a fairly lateral position (Fig. S4D) and shift medially in the late larva (Fig. S4J). Growth of the lateral accessory lobe (LAL) and the lateral appendix of the medial lobe (LAML) are accompanied by a moving-apart of lineages of the BA and DAL groups (compare distance between DALcl, DALv, and BAmv in Fig. S4D vs J). At the dorsal brain surface, the expansion of the superior protocerebrum is accompanied by a shift towards posterior of the DPM lineages (note increasing gap between the DPM and the DAM group in Fig. S4F, I, L).

In order to test whether the movement of lineage clusters could be altered by blocking growth of certain parts of the brain, we ablated the optic lobe by expressing a dominant negative construct of EGFR in the embryonic anlage of the optic lobe, using the driver line *so-Gal4* (Chang et al., 2003a; 2003b; Fig. 5). Following this genetic manipulation, late larval brains lack the epithelial optic lobe anlagen (IOA, OOA) and the neurons produced by them (compare Fig. 5C and F). Lineages of the central brain develop their normal trajectories (Fig. 5F), even though lineage clusters, in particular those of the lateral half of the brain, have a location that is significantly different from that of the wild type (compare Fig. 5E). This finding implies that the growth of the optic lobe is causally related to the displacement of the cell bodies of central brain lineages that occurs during normal development; it also implies that the SAT trajectories do not react measurably if the normal displacement is blocked.

DISCUSSION

Secondary neuroblasts at the onset of proliferation

Secondary lineages, visualized with global markers, can be traced from the time of their first appearance in the early larva throughout the remainder of the larval period. Analysis of larval brains labeled with global markers at close intervals enabled us to reconstruct the early “life history” of most secondary lineages. *insc-Gal4* appears in neuroblasts several hours prior to their first mitosis. Premitotic, spindle-shaped neuroblasts, labeled by *insc-Gal4* and the gene *deadpan* (Bier et al., 1992), are located at various depths within the cortex and emit a thin basal process towards the surface of the neuropil, much like early secondary neuroblasts of the larval ventral nerve cord (Truman and Bate, 1988; Chell and Brand,

2010). Embryonic (primary) neuroblasts do not possess a basal process, suggesting that this structure is not required for neuroblast proliferation, but may be important for the guidance of secondary neurons. Thus, basal processes attach to the neuropil at the entry point of primary axon tracts and serve to not only anchor the neuroblast in the cortex, but also allow secondary neurons, which are born in close contact with their older (primary) siblings, to extend their axons along the primary axon tract.

From an evolutionary/developmental perspective, it is tempting to speculate that the basal process hints at the “neuronal nature” of a neuroblast. In this view, a neuroblast might be seen as a neuron inhibited from differentiation and instead prompted to proliferate. After blocking cell division through mutations in the cell cycle regulator *string*, embryonic neuroblasts differentiate into neurons, expressing neuronal markers and producing axonal processes (Hartenstein and Posakony, 1990; Harris and Hartenstein, 1991). The basal process formed by neuroblasts during their time of mitotic quiescence could represent a rudimentary axon which then is retracted upon entry into the mitotic cycle.

In a previous study (Lovick et al., 2015), we determined the time-point at which individual secondary lineages are born, defined by the onset of proliferation of the corresponding neuroblast, by applying the mitosis inhibitor hydroxyurea (HU), which selectively ablates proliferating neuroblasts and their neuronal progeny. Birth dates determined by using the HU method closely match those found in this study. In the majority of cases, one can detect the neuroblast by its expression of *insc*-Gal4 several hours before the time at which it is ablated using HU (Table 1). The validated time table of the reactivation of neuroblast proliferation will assist us and others in developing strategies that target specific lineages for genetic manipulations.

Hemilineages of most brain lineages are lost through programmed cell death

Previous studies had indicated that axon tracts of hemilineages in the ventral nerve cord and brain typically enter the neuropil together, but then follow different pathways within the neuropil. Furthermore, in cases where a lineage, observed in the late larva or adult, emits only a single tract, this is the result of programmed cell death of one of the hemilineages (Kumar et al., 2009b; Truman et al., 2010). We provide evidence that, in most brain lineages with a single tract, one hemilineage undergoes cell death. Over expression of the caspase inhibitor p35 (Clem et al., 1991), which inhibits cell death in these lineages, results in the appearance of an additional secondary tract. Supernumerary tracts are typically smaller than “regular” tracts and could be due to a delay in the action of p35 in inhibiting programmed cell death. Alternatively, neurons of “rescued” hemilineages may suffer from intrinsic defects in specification that cause them to lag behind in differentiation. It is significant that in all cases, supernumerary axons bundle together and form a single, highly invariant tract supporting the idea that these are a cohesive group (hemilineage) of cells rescued from programmed cell death. Furthermore, our data suggests that this supernumerary tract may reflect the pathway taken by the corresponding primary hemilineage tract. We directly show this for the lineage BAm2. Primary clones demonstrate that BAm2 projects a ventral and a dorsal (hemilineage) primary tract, whereas the secondary BAm2 has only a ventral tract. When cell death is inhibited, the supernumerary secondary tract extends along a dorsal

trajectory, much like the dorsal primary tract. This implies that (1) BAm2 forms two intact hemilineages in the embryo; (2) initiates both hemilineages, with tracts identical to the primary ones, during the secondary phase of proliferation; (3) completely eliminates the dorsal hemilineage via programmed cell death. We anticipate that clonal analysis of primary brain lineages (J.O., J.L., and V.H., in preparation) will reveal that this phenomenon of “secondary hemilineage elimination” applies to many brain lineages.

Differential expression of Notch has been shown to be important for delineating hemilineages in the ventral nerve cord (Truman et al., 2010) as well as subsets of lineages in the brain (Lin et al., 2010; 2012b). Briefly, the Notch inhibitor Numb is segregated into one set of daughter cells, resulting in Notch^{ON} (Notch signaling active; ‘A’ fate) and Notch^{OFF} (Notch signaling inhibited; ‘B’ fate) sibling neurons. Most, if not all, lineages consist of A and B fated cells: in some lineages, Notch expression determines whether or not neurons undergo programmed cell death (Karcavich and Doe, 2005; Sánchez-Soriano et al., 2005; Kumar et al., 2009b; Lin et al., 2010; 2012a; Truman et al., 2010) or it determines what type of neural fate a cell will adopt (Notch^{ON} = local interneurons; Notch^{OFF} = projection neurons; Lin et al., 2010; 2012b). Notch has also been shown in numerous contexts to inhibit differentiation (e.g. high Notch activity in vertebrate neuroepithelial cells promotes proliferation and inhibits neuronal differentiation; Austin et al., 1995; Dorsky et al., 1995). We speculate that our finding that hemilineages of a given lineage (e.g., DALc11/2, DPLa12/3; see Fig. 4) emit tracts at slightly different time points may also be the result of differential Notch activity in the corresponding neurons. It is possible that GMC daughter cells receiving high Notch activity are temporarily inhibited to differentiate and, as a result, lag behind their siblings in regard to axon extension. Systematic studies that correlate high versus low Notch levels with hemilineages of all lineages are required to substantiate this idea.

Movement of secondary neuroblasts and lineages

In vertebrates, brain formation is accompanied by active migrations of undifferentiated neuronal precursors. Neurons are born apically within the pseudostratified neuroepithelium that forms the wall of the neural tube. Subsequently, neural precursors lose contact to the apical surface and migrate radially along the elongated neuroepithelial cells (at that point called radial glia) towards basally. In the brain, more widespread active migrations (or migratory streams) occur at certain locations, such as the rhombic lip (formation of the cerebellum; Wullimann et al., 2011) and the subpallium (inhibitory interneurons; Sultan et al., 2013). For a vertebrate neuron, the location of its cell body is of central importance for the structural properties of the network the neuron is engaged in. Thus, the cell body is the “center” from which dendritic and axonal processes sprout; if, for example, the cell body of a neuron is located in a deep cortical layer, dendrites of these cells are also typically focused in this region.

Circuit design is different in many invertebrates, including *Drosophila*, where the cell body of a neuron remains at the periphery of the neuropil, and dendritic or axonal branches occur at various distances from the cell body (Sánchez-Soriano et al., 2005). This implies that the location of the cell body isn’t as crucial for wiring as in vertebrates. Accordingly, active

migration of neuronal cell bodies to reach defined positions at a distance from the location where they were born has not yet been documented. In most cases described in the literature (e.g., embryonic neurons: Lekven et al., 1998; lineages in the larval or adult brain: Păreanu and Hartenstein, 2006; Ito et al., 2013; Wong et al., 2013; Yu et al., 2013; this study), neurons born by the same neuroblast remain relatively close to each other. However, in some cases, substantial displacement of cell bodies, typically of entire hemilineages, has been observed (for example, lineages DPL12/3 and BLVp1/2; Lovick et al., 2013; Wong et al., 2013). What remained constant is the location where axons entered (rather than merely touched) the neuropil. We speculate that at this location (as well as inside the neuropil) strong adhesive contacts are formed between the axons and its microenvironment (e.g., glia; other neurons). Once the neuropil grows during pupal development, the entry portals of the hemilineages shift further apart. Assuming that cell bodies are tethered to the portal by their proximal axon segments (i.e., the length of fiber between cell body and entry portal), one can imagine that the cell bodies are pulled along as the portals shift in position.

It is possible to interpret the large-scale displacement of lateral lineage clusters, which accompanies growth of the optic lobe, along similar lines. At an early stage of larval development (approximately 24–40h post hatching) secondary neurons of lineages that are located in the lateral brain cortex, surrounding the small primordium of the optic lobe, emit axons that, following a short, straight path, enter the neuropil (Fig. 5D). As the optic lobe grows, cell body clusters of these lineages are “pushed out of the way” towards more dorsal, ventral, anterior, and posterior positions. At the same time, the proximal segments of the axon tracts, bridging the distance between cell bodies and neuropil surface, lengthen (Fig. 5D). Following ablation of the optic lobe primordium, cell bodies of lateral lineages remain at their original position, supporting the conclusion that these cells move passively as a result of the growing optic lobe primordium. The fact that the central trajectories of the axon tracts of lateral lineages are not altered despite gross abnormalities in cell body position supports the idea that pathfinding of secondary axons heavily depends on the primary tracts, as already proposed in previous studies (e.g., Spindler et al., 2009). Once the first secondary axons have latched on to the primary tract at the neuropil entry portal, the secondary tract is stabilized; later outgrowing axons merely follow their younger siblings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Early development of secondary lineages in the larval brain. (A–D) Expression of *insc*-Gal4 (Insc; white in panels A, C; green in panels B, D) in secondary lineages. Panels are z-projections of confocal sections of larval brain hemispheres (midline at right margins) fixed at different stages of development [A: 12–24h after hatching (AH); morphologically stage L1A (see supplementary Fig. S1); B: 24h AH (stage L1B); C: 28–32h AH; D: 64h AH (stage L3A)]. (A): Insc is turned on in neuroblasts, co-labeled with anti-Deadpan (red), shortly before onset of secondary phase of proliferation. Arrow (1) points at neuroblast with superficially located cell body and basal process contacting neuropil surface; arrow (2) indicates neuroblast with deep cell body; neuroblast marked by arrow 3 (lineage BA1c) is large, strongly Insc-positive, and has commenced proliferation. Following onset of proliferation, neuroblasts upregulate Insc-expression, enlarge, and move to periphery of cortex (C). (B, D) Co-labeling of incipient secondary lineages (Insc, green) and primary axon tracts (BP104, magenta). Note close association of primary (magenta) and secondary (green) axon tracts (examples shown by white arrows in panel (D)). In panel (B), blue arrow points at neuropil entry point of PAT of lineages BA1p2/3; green arrow indicates fiber emitted by secondary BA1p2/3 neuroblasts towards this entry point. (E–G): Transmission electron micrographs of section of mid-third larval instar brain. (E): shows secondary axon tract (SAT) in deep layer of brain cortex, surrounded by cell bodies of primary neurons (pn). (F): Secondary axon tract entering the neuropil (np; upper right half of image). Group of primary neurons (pn lower left) form primary axon tract (PAT); secondary axon tract follows PAT. Thin lamella of neuropil glia (ng) surrounds primary axon tract. Profiles of primary axons are slightly thicker and more electron dense than secondary axons (G). (H) Z-projection of frontal confocal sections of late third larval instar brain, showing lineage pair DALv2/3 labeled by *engrailed*-Gal4>UAS-mcd8::GFP. Secondary lineage tracts are globally labeled by anti-Neurotactin (BP106; white). Primary neurons, distinguishable from undifferentiated secondary neurons by their large cell bodies and branched axons, were

rendered green; secondary neurons red. Note separate entries and trajectories of primary and secondary axon tracts (arrows). Scale bars: 10 μ m (A–D); 1 μ m (E, F); 0.5 μ m (G); 40 μ m (H)

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Figure 2.

Elongation of secondary axon tracts. Panels show parts of z-projections of frontal confocal sections of larval brain hemispheres imaged at sequential stages (panels of first row: 27h AH; second row: 48h AH; third row: 64h AH; fourth row: 74h AH; bottom row: 96h AH). Brains were labeled with *insc-Gal4>UAS-mcd8::GFP*, visualizing secondary lineages. For each column, one lineage and mushroom body (as reference; ML: medial lobe; SP/PED: spur/peduncle; VL: vertical lobe) were graphically isolated by using the FIJI paint tool to manually erase all labeled lineages except the one to be illustrated. Green asterisk indicates cell body cluster; red and blue arrowheads point at tip of advancing axon tracts. Left column (A–E) shows lineage DALv1 which has started to extend its axon tract already at 27h (A) and has reached midline by 64h (C). Center column (F–J) illustrates lineage pair DALcm1/2, which makes its first appearance at 48h (G) and starts to emit axons at 64h (H), reaching midline in the late third instar larva (J). Right column (K–O) shows lineages DAMd1-3. DAMd1 is represented by faintly *insc*-positive neuroblast at 27h (K) and emits a medially

directed tract around 48h (L). The pair DAMd2/3 produces a posteriorly directed tract at 64h (M, blue arrowhead). Scale bar: 20µm

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Figure 3.

Identification of hemilineages that undergo programmed cell death during normal development. (A–L) Z-projections of frontal confocal sections of late third instar brain hemispheres (medial to the left) in which secondary lineages are labeled by *ase-Gal4>UAS-mcd8::GFP* (green) and anti-Neurotactin (BP106; magenta). Panels are organized in sets of four (A–D, E–H, I–L) focusing on a specific lineage (A–D: BAmas1/2; E–H: DPLam; I–L: BAm2). In lower pair of each quartet (B/D, F/H, J/L) the lineage or lineage pair was graphically isolated and is shown in white; isolated mushroom body (MB; CA calyx; ML medial lobe; PED peduncle; VL vertical lobe) is shown in red for reference. Left two panels of each quartet (A/B; E/F; I/J) show wild-type, right ones (C/D, G/H, K/L) illustrate phenotype following *ase-Gal4*-driven expression of UAS-p35. (A–D) Trajectory of the paired tract of BAmas1/2 is indicated by letters “a” (proximal segment), “b” (turn), and “c” (distal, ascending segment). In brain expressing p35 (C–D) an ectopic tract branches off the turn “b” (arrowhead in C–D; see inset in C for higher magnification) and joins the SAT of

lineage TRdm (shown in cyan). (E–H) Proximal and distal segment of tract of DPLam is marked by letters “a” and “b”, respectively. Ectopic branch (arrowhead in G–H) projects laterally in p35 brain. (I–L) Lineages BAmD1 and BAmD2 enter the anterior neuropil at a location right underneath the medial lobe. BAmD1 branches into a dorsal (d) and ventral (v) tract; BAmD2, in wild type, has only a ventral tract (a). In p35 brain, BAmD2 forms ectopic dorsal branch (arrowhead in K–L) which joins the SAT of DAMd2/3 (shown in cyan). (M–P) Ectopic secondary branch of BAmD2 follows a trajectory established by primary BAmD2 lineage. (M–N) Z-projection of frontal confocal sections of third larval instar brain in which the primary BAmD2 lineage is visualized as a GFP-labeled flp-out clone (green; see Material and Methods). Secondary lineages are globally labeled by BP106 (magenta). Note that primary BAmD2 emits a ventral branch (arrow) and a dorsal branch (arrowhead). The dorsal branch follows the same, posteriorly-directed pathway as DAMd2/3 (cyan in panel N). (O) Schematic presenting development of BAmD2 (and, possibly other) lineages: Primary neurons include two hemilineages, called (arbitrarily) A and B; following secondary phase of proliferation, only one secondary hemilineage (“Asec”) differentiates and follows the pathway of the corresponding primary hemilineage, whereas the other one (“Bsec”) undergoes programmed cell death. “Bsec” is rescued by expression of p35. (P) MARCM clone of differentiated secondary BAmD2 lineage in adult brain (white; neuropil labeled by anti-DNcad (blue); from Wong et al., 2013). BAmD2 possesses only a ventral tract (arrow) that gives off a posterior contralateral branch (p) and an ipsilateral branch (i). Scale bar: 40µm (for all panels)

Figure 4.

Hemilineage tracts of a given lineage extend at different time points. Panels show parts of z-projections of frontal confocal sections of larval brain hemispheres imaged at sequential stages (panels of first row: 27h AH; second row: 48h AH; third row: 74h AH; bottom row: 96h AH). Brains were labeled with *insc-Gal4>UAS-mcd8::GFP*, visualizing secondary lineages. For each column, one or two lineage(s) and mushroom body (as reference; green) were graphically isolated (see Material and Methods). Red and blue arrowheads point at tip of advancing hemilineage tracts. Left column (A–D) shows lineages DPLa1-3. In late larva (D), all three of these have a hemilineage extending a crescent-shaped, dorsally-directed hemilineage tract (red arrowhead; see also Lovick et al., 2013; Wong et al., 2013). In

addition, DPLal2/3 form a ventral hemilineage tract (cyan arrowhead). Between 27h and 74h after hatching (A–C), only the dorsal tract is present. Right column (E–H) illustrates lineage pairs DALcl1/2 and BLAv1/vm. In late larva (H), DALcl1/2 possess two hemilineage tracts, one passing underneath (red arrowhead), the other over (cyan arrowhead) the mushroom body peduncle. The ventral tract extends at an early stage (27h; E), the dorsal one later (74h; G). Similarly, BLAv1/vm form posteriorly directed tracts (yellow arrowheads) before medially directed ones (magenta arrowheads). Scale bar: 20µm

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medulla neuropil; MEco medulla cortex) has grown to cover the entire lateral surface of the brain. Lineages located in the lateral brain hemisphere at an early stage (A) shift centrifugally to more anterior (BLA lineages), dorsal (BLD and DPLl/DPLc lineages), posterior (BLP and DPLp lineages), or ventral (BLV) positions. This morphogenetic shift is shown schematically in panel (D). (E–F): Topology of secondary lineages in 96h larval brain following ablation of optic lobe primordium by *so-Gal4>UAS-dnEGFR*. Panels show digital 3D model of lineages (E) and z-projection of BP106-labeled brain (F) at same orientation and magnification as those in adjacent (B) and (C), respectively. Notice absents of optic lobe primordium in (F); compare to (C). Scale bar: 40µm (for all panels) Additional abbreviations: loSM longitudinal superior medial fascicle; trSI intermediate superior transverse fascicle

Table 1

Steps in the development of secondary lineages. All lineages are listed at the left.

	20	24	28	32	36	40	44
BAla1			b				c
BAla2			b				c
BAla3							b
BAla4			a				b
Balc	c						
BAlp1							c
BAlp2		a	b				c
BAlp3		a	b				c
BAlp4			b				c
Balv			b				c
BAnas1			a				c
BAnas2							a
BAnd1			a				b
BAnd2			a				b
BAmv1	a		c				c
BAmv2			b				c
BAmv3			b				c
DALc11	a		b				c
DALc12			a				c
DALcm1							b
DALcm2							b
DALd			a				c
DALi1	a		b				c
DALj2	a		b				c
DALv1	b						c
DALv2			b				c
DALv3	a		b				c
DAMd1			a				b
DAMd2			a				b

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	20	24	28	32	36	40	44
MB1-4	c						

The horizontal axis represents the time axis, subdivided into 4h intervals. Numbers at the top indicate hours after hatching. Onset of blue shading indicates time interval at which the corresponding secondary lineage is ablated by HU pulse (see Lovick et al., 2015a; dark blue = ablation in >90% of cases; medium blue = ablation in 50%–90%; light blue = ablation in 10–50%). Grey shading in left column points out lineage pairs with highly similar or identical axonal trajectory in larval brain. Lower case letters (a–c) signify stages in secondary lineage development, based on their appearance in preparations of brains expressing the secondary lineage marker *inisc-Gal4>UAS-mcd8-GFP* (see Fig. 1). Letter “a” indicates presence of neuroblast at/prior to entering mitosis; “b” signifies stage shortly after onset of proliferation when lineage consists of neuroblast and small cluster of progeny; at stage marked “c”, lineage has formed axon tract entering neuropil.

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Table 2

Secondary lineage tracts.

A	B	C	D	E	F
Lineage	#	type	tracts	p35	p35
BAla1	1	IH1		+	BAla3/4
BAla2	2	IH1			
BAla3	3	IH1		+	n
BAla4	4	IH1			
Balc	5	IH2	dv	-	
BAlp1	6	IH1		+	p *
BAlp2	7	IH1		+	p *
BAlp3	8	IH1		+	n *
BAlp4	9	IH1		-	
Balv	10	IH1		-	
BAnas1	11	IH1		+	TRdm
BAnas2	12	IH1		+	p *
BAnd1	13	IH2	dv	-	
BAnd2	14	IH1		+	DAMd2/3
BAmv1	15	IH2	Pd	-	
BAmv2	16	IH1		-	
BAmv3	17	IH1		-	
DALc11	18	IH2	dV	-	
DALc12	19	IH2		-	
DALcm1	20	IH2	Mv	-	
DALcm2	21	IH2		-	
DALd	22	IH1		-	
DALj1	23	IH2	u	-	
DALj2	24	IH1		+	DALj1
DALv1	25	IH1		+	p *
DALv2	26	IH1		+	DALc11/2
DALv3	27	IH2		-	
DAMd1	28	IH1		+	p

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A	B	C	D	E	F
Lineage	#	type	tracts	p35	p35
DAMd2	29	I		+	n *
DAMd3	30	I			
DAMv1	31	IH2	u	-	
DAMv2	32	IH2		-	
DPLal1	33	IH1		+	DPLal2/3
DPLal2	34	IH2	Dv	-	
DPLal3	35	IH2		-	
DPLam	36	IH1		+	n
DPLc1	37	IH1		-	
DPLc2	38	IH1		v	p
DPLc4	40	IH2	u	-	
DPLc3	39	IH1		-	
DPLc5	41	IH2	aP	-	
DPLd	42	IH2	mP	-	
DPLl1	43	IH1		-	
DPLl2	44	IH2	u	-	
DPLl3	45	IH2		-	
DPLm1	46	IH1		+	n *
DPLm2	47	IH2	u	-	
DPLp1	48	IH2	Ma	-	
DPLp2	49	IH1		+	CP2/3
DPLpv	50	IH2	u	-	
DPMl1	51	IH2	u	-	
DPMl34		I 2	u	-	
DPMm1	53	II			
DPMm2	54	IH1		+	p
DPMpl1	55	IH1		-	
DPMpl2	56	IH1		+	n
DPMpl3	57	IH1		+	p *
DPMpm1	58	II			

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A	B	C	D	E	F
Lineage	#	type	tracts	p35	p35
DPMpm2	59	II			
CM1	60	II			
CM3	61	II			
CM4	62	II			
CM5	63	IH1		-	
CPI	64	IH2	Dv	-	
CP2	65	II			
CP3	66	II			
CP4	67	IH1		-	
BLAd1	68	IH1		+	p
BLAd2	69	IH1			
BLAd3	70	IH1			
BLAd4	71	IH1			
BLAl	72	IH2	u	-	
BLAv1	73	IH2	mP	-	
BLAv2	74	IH2	mP	-	
BLAvm	75	IH2	mP	-	
BLD1	77	IH2	u	-	
BLD4	80	IH2	u	-	
BLD2	78	IH1		n	
BLD3	79	IH2	u	-	
BLD5	82	IH1		+	BLAl
BLD6	83	IH1		+	p
BLP1	84	IH1		-	
BLP2	85	IH1		+	p
BLP3	86	IH1		-	
BLP4	87	IH1		-	
BLVa1	89	IH1		u	
BLVa2	90	IH1		u	
BLVa3	91	IH1		+	BLVa1/2

A	B	C	D	E	F
Lineage	#	type	tracts	p35	p35
BLVa4	92	I H1			
BLVp1	93	I H2	Dv	-	
BLVp2	94	I H2	u	-	

Column (A): lineage name; column (B) number used for lineage in supplementary figures S2 and S3. Column (C): classification of lineage (I H1: type I lineage with one axon tract; I H2: type I lineage with two tracts; II: type II lineage). Column (D): designation of lineage tracts (see Lovick et al., 2013, Tab.2; a or A: anterior; d or D dorsal; m or M: medial; p or P posterior; v or V ventral). If capital letter is used, corresponding tract extended earlier than its partner written in lower case (e.g., “Dv” in lineage pair DPLaI2/3 indicates that the dorsal tract of DPLaI2/3 appeared earlier than the ventral tract). Letter “u” indicates that order of appearance of tracts remains undetermined. Column (E) reports effect of expression of UAS-p35 in corresponding lineage (+ supernumerary tract present; - no supernumerary tract). Orange shading marks lineages where no supernumerary tract was observed despite the fact that only a single tract exists in wild type (see column C). Gray shading labels members of lineage pairs/groups where presence or absence of supernumerary tracts could not be ascertained. Column (F): trajectories of supernumerary tracts caused by UAS-p35. If tract followed another, established lineage tract, the corresponding lineage name is given. Letter “p” stands for cases where supernumerary tract extended parallel to the regular tract of corresponding lineage; “n” indicates novel trajectories (not encountered in wild type brains). Asterisks marks cases where supernumerary tracts appeared in only a fraction of preparations analyzed.