Published in final edited form as:

J Comp Neurol. 2010 August 1; 518(15): 2996–3023. doi:10.1002/cne.22376.

A development-based compartmentalization of the *Drosophila* central brain

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Abstract

The neuropile of the *Drosophila* brain is subdivided into anatomically discrete compartments. Compartments are rich in terminal neurite branching and synapses; they are the neuropile domains in which signal processing takes place. Compartment boundaries are defined by more or less dense layers of glial cells, as well as long neurite fascicles. These fascicles are formed during the larval period when the approximately 100 neuronal lineages that constitute the *Drosophila* central brain differentiate. Each lineage forms an axon tract with a characteristic trajectory in the neuropile; groups of spatially related tracts congregate into the brain fascicles that can be followed from the larva throughout metamorphosis into the adult stage. In this paper we provide a map of the adult brain compartments and the relevant fascicles defining compartmental boundaries. We have identified the neuronal lineages contributing to each fascicle, which allowed us to directly compare compartments of the larval and adult brain. Most adult compartments can be recognized already in the early larval brain where they form a “protomap” of the later adult compartments. Our analysis highlights the morphogenetic changes shaping the *Drosophila* brain; the data will be important for studies that link early acting genetic mechanisms to the adult neuronal structures and circuits controlled by these mechanisms.

Keywords

Drosophila; brain; lineages; connectivity; mapping

Introduction

The central nervous system of most animals consists of large numbers of neurons that are interconnected by highly branched processes. Nervous tissue is not homogenous: groups of neurons and/or their processes form morphologically distinct compartments. For example, in the vertebrate brain stem or spinal cord, one distinguishes discrete nuclei, such as the nucleus of the abducens or trochlear nerve (see, for a synopsis of compartments of the human brain, the synopsis by Nieuwenhuys et al., 1981). A nucleus is visible to the microscopist because of discontinuities in the packing of nerve fibers and nerve cell bodies. Cell bodies of a nucleus lie close together, and are typically separated from other, neighboring cells by a shell that contains fewer cell bodies, and that instead is filled with bundles of long axons that leave or enter, or simply pass by the nucleus. A nucleus is further defined by a characteristic input and output. Neurons of the trochlear nucleus, for example, receive sensory axons of the 8th nerve; neurons of the abducens nucleus are motor neurons innervating the abducens muscle. Because of their shared input/output, as well as other factors, including the elaboration of distinct short range connectivity within the nucleus,

expression of characteristic channels, transmitters, or other molecular markers, nuclei also may form distinct functional compartments.

The distinction of compartments in the brain is of great heuristic significance, because it allows one to break down the analysis of neuronal connectivity into two steps. On the one hand side, different compartments are interconnected by major axon tracts, which can be mapped and put in a map that displays the “macro-circuitry” of the brain. Such a map would show, for example, how a compartment like the trochlear or abducens nucleus is integrated into systems of nerve connection that include the brain as a whole. Secondly, one can focus on the analysis of neuronal connectivity at the level of individual neurons, within a given compartment. This approach looks at the “micro-circuitry”, asking how the activity of a single neuron “percolates” through the matrix of densely packed neuronal processes, thereby resulting in discrete neuronal patterns of activity or receptive fields. Aside from their importance for the study of neuronal function, compartments provide a system of landmarks used by neuroscientists to unambiguously localize relevant features, from lesions to gene expression.

Morphologically defined compartments have been also described for invertebrate brains, in particular insects and other arthropods (for a review of the classical literature, see Hanström, 1928; Bullock and Horridge, 1965). It should be emphasized that essentially all that is known about insect brain compartments is based on just a few, easily recognized and therefore widely studied cases, such as the mushroom body, the central complex, or the optic and antennal lobe. These structures share many features with compartments of the vertebrate brain defined above. Thus, the interior of an invertebrate compartment is rich in terminal neurite branching and synapses. On the outside, the compartment is surrounded by a frame of lesser synapse density, generated by the presence of long fibers and glial cells. Invertebrate compartments are also defined by a characteristic input or output, and (as becomes increasingly clear in more recent studies) by the expression of specific molecular markers.

For most compartments, boundaries are difficult to discern without the help of immunohistochemical methods. For this reason, the anatomy of the brain of invertebrates, including that of widely studied model organisms like *Drosophila*, is not at all well understood. Recently, given the increasing demand for brain subdivisions as landmarks for pinpointing functional or gene expression data, attempts have increased to define compartments for the entire insect brain. On the basis of confocal microscopy where whole-mounts of brains were labeled with markers for glia, fiber bundles, and synapses, we and others have recently introduced a 3D map of brain compartments for *Drosophila* (Rein et al., 2002; Jenett et al., 2006;). In this paper, we present a morphological and developmental analysis of brain compartmentalization in *Drosophila*.

Development plays a fundamental role in the definition of brain compartments. In vertebrates, the primordium of the nervous system arises in the dorsal ectoderm of the early embryo as the neural plate, which subsequently invaginates and becomes the neural tube. The neural tube is subdivided morphologically and molecularly into a system of orthogonally arranged domains and axon tracts (Kintner and Lumsden, 2007). Along the antero-posterior axis one distinguishes the neuromeres, each defined by the expression of a combinatorial code of transcription factors (Hox genes, Head gap genes). Medio-laterally the neural tube comprises longitudinal columns that are also characterized by the expression of specific combinations of regulatory genes. Much effort has been spent to backtrack the adult brain compartments to a stage when they form part of the neural tube. Such developmental analysis was always considered important to decipher connectivity: it is easier to analyze the pattern of nerve tracts between different parts of the nervous system at

an early stage, when fewer elements have differentiated, and the original simple topology of the neural tube has not yet been distorted by differential growth and morphogenetic movements. In other words, the early embryonic brain has been used as a “guide” to understanding the structure of the mature vertebrate brain (Martin, 1985). More recently, with the advent of molecular-genetic tools, developmental analyses provide the link between the determination of specific cells and compartments with domains of gene expression in the neural tube.

The genetic analysis of neuronal development has made rapid progress in invertebrate model systems like *Drosophila*. Most of the regulatory genes that are now known to be major players in specifying neuronal fate of all animals, including vertebrates, were first discovered in the fruit fly. However, with few exceptions, these studies were performed in the embryo, where individual neuronal lineages are easily accessible, and the fate of individual neurons can be assayed by molecular markers (Harris and Hartenstein, 2007). The *Drosophila* nervous system originates from an invariant set of stem cells, called neuroblasts, which delaminate from the ectoderm in the early embryo, and then start dividing to first produce the neurons of the larval nervous system (primary neurons), followed by adult specific (secondary neurons) which are born in the larva (Hartenstein et al., 2008). Detailed maps of embryonic neuroblasts, containing information about a large number of regulatory genes expressed in these cells, have been generated (Doe, 1992; Urbach and Technau, 2003); however, little is known about how embryonic neuroblasts and their lineages contribute to functional circuits, or morphological compartments, of the larval or adult brain. Only the four neuroblasts producing the mushroom body have been mapped at the embryonic stage (Noveen et al., 2000), in addition to those neuroblasts that give rise to primary motor neurons (Schmid et al., 1999). In recent years we have begun to follow the development of lineages and the compartments formed by them (Younossi-Hartenstein et al., 2003; 2006; Dumstreit et al., 2003; Pereanu and Hartenstein et al., 2006; Kumar et al., 2009; Larsen et al., 2009). As part of this effort, in this paper we analyze the axon tracts that form the basis of compartment boundaries in the adult brain, and follow them backward through metamorphosis into the larva. This approach allows us to generate a “protomap” of adult compartments at the larval stage, and thereby to put the definition of adult compartments into a developmental context.

Material and Methods

Markers

The nc82 antibody (Developmental Studies Hybridoma Bank, DSHB, nc82) labels synapses and served as a marker for neuropile. It is a mouse monoclonal antibody from a large library generated against *Drosophila* head homeogenates. The antibody recognizes the active zone protein Bruchpilot, which forms protein bands of 190 and 170 kDa in Western blots of homogenized *Drosophila* heads (Wagh et al., 2006). The immunoreactive signal disappears in immunohistochemical preparations as well as in Western blots if the bruchpilot gene is knocked down. Moreover, an additional band is detected specifically if GFP-tagged Bruchpilot is expressed in a pan-neuronal manner (Wagh et al., 2006; Kittel et al., 2006).

The DN-cadherin antibody (DSHB DN-EX #8), another marker for neuropile, is a mouse monoclonal antibody raised against a peptide encoded by exon 8, amino acid residues 1210–1272 of the *Drosophila* CadN gene. The antibody detected two major bands, 300 kDa and 200 kDa molecular weights on Western blot of S2 cells only after transfection with a cDNA encoding the DN-cadherin protein (Iwai et al., 1997). In addition, the specificity of this antibody was tested with immunostaining of *Drosophila* embryos. Signal was hardly detectable in homozygous mutant, *l(2)36Da^{M19}* with nonsense mutation causes premature

termination of protein translation (Iwai et al., 1997). In contrast, this antibody gave a signal in mutant embryos with N-cadherin transgene (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 (Patel et al., 1987). The antibody was used to screen a 9–12 hour embryonic *Drosophila* phage-gt11 cDNA library (Snow et al., 1988) that identified 2 phages containing a 435 bp EcoRI fragment that did not include the full open reading frame. A radio-labeled 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 aminoterminal sequence of this cDNA (11 amino acids) is identical to protein microsequence data from affinity-purified neurotactin protein (de la Escalera et al., 1990). A 1.9 kb KpnI-SalI fragment from the Neurotactin cDNA was subcloned into the protein A fusion protein vector (Hortsch et al., 1990). The Protein A-neurotactin fusion protein was expressed and isolated from pop2136 cells (Hortsch et al., 1990). The isolated fusion protein was used to immunize rats and generate a polyclonal antibody which detected the same *Drosophila* embryonic structures as that of the monoclonal antibody (Hortsch et al., 1990). The antiserum was used at 1:10 and incubated for 2 hours; PBS with 0.3% Triton-X was used throughout the procedure.

The Neuroglial 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). The Nrg antibody was used to purify protein from whole embryo extracts by immunoaffinity chromatography. Protein microsequencing of the purified protein was performed to determine that the 18 N-terminal amino acids that is identical to the sequence determined for the N-terminus of the protein based on a full-length cDNA clone (Bieber et al., 1989).

We used *engrailed*-Gal4 (*enGal4*; Tabata et al., 1995) and *period*-Gal4 (*perGal4*; Kaneko and Hall, 2000) as driver lines for specific lineages.

Fly stocks and egg collections

As wild-type stock we used Oregon R. Flies were grown under standard conditions at room temperature (25°C). Egg collections were done on yeasted apple juice agar plates (Ashburner, 1989). Hatching larvae were transferred to Petri dishes filled with standard fly food at a density of three to five individuals per cc of food. Larvae were collected at desired time points and brains were dissected in PEMS (0.1M Pipes, 2mM MgSO₄, 1mM EGTA, pH7.0) buffered 4%Formaldehyde.

For the generation of selected GFP labeled clones, a stock containing a recombinant chromosome was constructed: *enGal4 UAS mCD8::GFPL*, *UAS-nls lacZ20b* on chromosomal arm 2R and FRT 82B *tubP-GAL80LL3* on chromosome 3. To generate MARCM clones (Lee and Luo, 1999) +; *UAS-mCD8::GFPL*, *UAS-nls lacZ20b*; *FRT82B* (Bello et al., 2003) males were crossed to females of the MARCM driver stock *hs-FLP*; *en-GAL4 UAS mCD8::GFPL*, *UAS-nls lacZ20b*; *FRT82B tubP-GAL80LL3* resulting in wild-type clones from within the lineages that express *en*. For MARCM experiments (Lee and Luo, 2001), embryos of the appropriate genotype were collected on standard cornmeal/yeast/agar medium supplemented with live yeast over a 4 hour time window and raised at 25°C for 21 to 25 hours before heat-shock treatment. Heat-shock induction of FLP was done at 37°C for 60 minutes.

Immunohistochemistry and histology

The Brp, Nrt, Nrg and DNaad antibodies were diluted 1:10. For antibody labeling standard procedures were followed (e.g., Ashburner, 1989). For fluorescent staining, the following

secondary antibody were used: Alexa Fluor 546 goat anti-Mouse IgG (H+L) at 1:500. This goat antiserum (Molecular Probes #A11039) was prepared against mouse immunoglobulin G (heavy and light chain). After washing in PBT (1XPBS with 0.1% Tween-20), embryos or brains were mounted in Vectashield mounting medium (Cat# H1000, Vector Laboratories).

Generation of Three-Dimensional Models

Staged *Drosophila* larval and adult brains labeled with suitable markers were viewed as whole-mounts by confocal microscopy [Biorad MRC 1024ES microscope using Biorad Lasersharp version 3.2 software; lenses: 40x oil (NA 1.0; WD 0.17)]. Complete series of optical sections were taken at 2- μ m intervals. Digitized images of confocal sections were imported into the Amira (www.amiravis.com) program. 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. Surface rendered digital atlas models were created by manually labeling each lineage and neuropile compartment within a series of confocal images. The Amira program also allows one to adjust virtual lighting, camera angle, transparency, reflection and other parameters in a straightforward manner.

Image Processing

Micrographs were processed using ImageJ and Adobe Photoshop by cropping and general digital adjustments to the brightness and contrast of the image. Schematics in Figure 1A–B, Figure 2A, Figure 8A, Figure 9A–C were generated using Adobe Illustrator. Three-dimensional models (in Figure 3A–C; Figure 5; Figure 6A–E, I; Figure 7; Figure 8D–E; Figure 11) were generated using Amira software, as described, and annotated using Adobe Photoshop.

Results

Structural elements of neuropile compartments and their boundaries: synapses, long fiber tracts, and glia

The fly central brain is formed by approximately 10,000–15,000 neurons per hemisphere whose cell bodies are arranged in an outer cortex that surrounds the neuropile, formed by the neurites (axons and dendrites) of the neurons (Fig. 1A, B). The neuropile is subdivided into anatomically discrete compartments which are rich in terminal neurite branching and synapses; they are the neuropile domains in which signal processing takes place. Compartments are surrounded by more or less dense layers of glial cells, as well as bundles of long nerve fibers that interconnect compartments (Fig. 1A, B). We will call these bundles “brain fascicles” in the following. Compartments can be easily visualized by global markers for neurites or synapses; the most widely used of these markers are antibodies against the synaptic protein Brp (nc82; Wagh et al., 2006) and N-cadherin (DNCad; Iwai et al., 1997). In brain preparations labeled with nc82 (Fig. 1C), compartments appear as domains of high expression levels, separated by bands of low expression which constitute layers of glia and brain fascicles. These inter-compartmental boundaries can be positively labeled by markers for glial cells, such as a GFP reporter driven by Nrv2-Gal4 (Sun et al., 1999; Fig. 1D).

As discussed in more detail below, the majority of brain fascicles visible in the adult brain are formed during the larval stage by the axon tracts of neural lineages (Pereanu and Hartenstein, 2006). As neuroblasts become mitotically active during the larval period, they produce tightly packed clusters of secondary neurons whose fibers fasciculate and form bundles called secondary axon tracts (SATs). SATs and the adult brain fascicles derived from them are entirely devoid of synapses and appear as signal-negative spaces in adult brain preparations labeled with nc82 or anti-DNCad (Fig. 2). In addition to these fascicles, a

second type of bundles is formed by less tightly packed fibers that are surrounded by terminal branches and synapses (Fig.2). These “sparse long fiber bundles” do not appear as signal-negative spaces on nc82-labeled brains and are therefore less easily detectable; however, on close inspection, they are usually visible, presenting as alternating lines of high and low density (Fig.2B’).

The majority of compartments to be discussed below are bounded by septa formed by glia and fascicles, as introduced in the preceding paragraphs. However, in some cases, septa are all but absent, and compartment boundaries are defined by abrupt changes in signal (i.e., synapse) density. Afferent fiber systems, be it sensory afferents (e.g., antennal axons) or interneurons (e.g., optic lobe derived axons) often form dense terminal arborizations contacted by equally densely packed postsynaptic fibers. These synapse-rich “glomeruli” or “foci” appear as strongly signal-positive domains in nc82-labeled brain preparations (Laisue et al., 1999; Rein et al., 2002; Otsuna and Ito, 2006). The presence of foci (or a globally increased higher synaptic density) help to define the boundaries of some compartments, such the antennal lobe (AL), posterior VLP (ventro-lateral protocerebrum), or lateral horn (LH).

Finally, it should be noted that at a number of locations, compartment boundaries represent virtual planes defined by easily detectable landmarks. For example, the peduncle of the mushroom body forms an elongated compartment that penetrates the center of each brain hemisphere in an anterior-posterior direction. Constructing a vertical plane through the length of the peduncle subdivides the neuropile domain surrounding the peduncle, called the inferior protocerebrum (IP; see below), into a medial and lateral half (IPm and IPl, respectively). Other than this virtual boundary, there are no morphological elements (e.g., glia, fiber tracts) that would demarcate the IPm from the IPl. The heuristic value of virtual boundaries for the analysis of brain connectivity and function remains to be demonstrated; at present, when boundaries are required for mapping patterns of neuronal projection and gene expression, such boundaries, when used sparsely, can be quite valuable.

Guide to the compartments of the central brain of the adult

Figure 3 introduces the major neuropile compartments of the central brain. Compartments that stand out most easily due to the presence of massive glial septa are the central complex and the mushroom body. With respect to these compartments, the central brain can be subdivided along the dorso-ventral axis (dorsal and ventral refers to the body axis) into three layers: a dorsal layer (above the level of central complex/mushroom body), middle layer (including and surrounding central complex/mushroom body), and ventral layer (below level of central complex/mushroom body; Fig.3A, B). The dorsal layer contains the medial, intermediate and lateral superior protocerebrum (SMP, SIP, SLP) and the lateral horn (LH). The middle layer is composed of the central complex, lateral accessory complex, mushroom body, and inferior protocerebrum, which contains a medial, lateral, anterior, and posterior subdivision (IPm, IPl, IPa, and IPP, respectively). The ventral layer includes, laterally, the ventro-lateral and postero-lateral protocerebrum (VLP, PLP); anteriorly the antennal lobe (AL); medially the ventro-medial cerebrum (VMC), the perioesophageal neuropils (PONP), and the suboesophageal ganglion (SOG). A detailed and systematic description of the adult neuropile compartments is currently being published by Ito and coworkers (Ito et al., 2010).

The central complex and some of the characteristically shaped surrounding compartments also serve as convenient landmarks to subdivide the neuropile of the central brain along the anterior-posterior (AP) axis into a series of frontal slices (Fig.3C, D). The virtual boundaries between these slices (“AP-levels”) do not coincide with many structurally compartment boundaries; nevertheless, the AP-levels are useful when comparing brains of different specimens, and of different developmental stages. In all, we distinguish between seven slices

of approximately 15–20µm thickness each. Fig.3 shows the AP levels and their hallmark structures. AP level 1 (AL) is characterized by the antennal lobe; level 2 (OTU) cuts through the optic tubercle, the horizontal lobes of the mushroom body, and the posterior half of the antennal lobe. Level 3 (EB) contains the ellipsoid body and lateral accessory lobe; level 4 (E/F) the posterior part of the ellipsoid and the flanking “wings” of the fan-shaped body; level 5 (FB) the main body of the fan-shaped body (note also the great commissure at this level). Level 6 (APT) shows the upward and laterally arching antenno-protocerebral tract, and level 7 (CX) the calyx and protocerebral bridge.

The main systems of fiber bundles characterizing compartment boundaries

As mentioned above, compartment boundaries are largely defined by bundles of long fibers that are formed by secondary axon tracts born during the larval period. SATs were mapped in the larval brain (Pereanu and Hartenstein, 2006) and can be followed into the adult brain. We will describe in the following the main brain fascicles that contribute to compartment boundaries (Fig.4, Fig.5). A brief note regarding the nomenclature of fascicles: Only a few fascicles, including the antenno-protocerebral tract (also called: antenno-cerebral tract) or the ellipsoid fascicle (also called: lateral ellipsoid fascicle), have received names in the literature. Strausfeld (1976) introduces names for a large number of fiber connections visible in Bodian stains; however, most of these do not correspond to the fascicles considered here, namely the ones formed by lineage related axon bundles, but rather represent various “sparse long fiber bundles” as described above. For the lineage-related fascicles we try to choose names that provide topological information. In most cases, we include an adjective indicating the main direction (longitudinal, transverse, vertical) and location of the fascicle. Superior fascicles are related to the superior/dorsal protocerebral compartments; ventral fascicles are in contact with ventral compartments. For three longitudinal fascicles defining the lower boundary of the inferior protocerebrum (medial and lateral horizontal fascicle; posterior lateral fascicle) we took the names recently introduced by Ito et al. (2010).

Central complex and inferior protocerebrum—For didactic reasons, it is easiest to approach the brain fascicles as an assembly of longitudinal, transverse and vertical bundles that run along the surface of (and thereby define) the inferior protocerebrum (Fig.5A), which forms the center of each brain hemisphere. At central and posterior AP levels (E/F, FB, APT, see Fig.3D) the inferior protocerebrum appears as a roughly cylindrical domain surrounding the peduncle of the mushroom body. Medially, the inferior protocerebrum is separated from the adjacent central complex by a massive glial septum. Associated with this septum are several fascicles (Fig.4C, D, E; Fig.5A, B). First, there is the antenno-protocerebral tract (APT) that carries fibers from the antennal lobe to the calyx and lateral horn. A second thick, longitudinal fiber bundle, the medial equatorial fascicle (MEF) is formed mainly by neurons located in the posterior cortex and belonging to the CM lineages (Fig.5B, F). A third bundle closely associated with the MEF is the lateral root of the fan-shaped body (lrFB). The small-field neurons of the FB are mainly formed by four lineages that belong to the DPMm, DPMpm and CM4 groups, and are located in the posterior cortex (Ito and Awasaki, 2008). The fibers of these neurons extend antero-ventrally, passing the protocerebral bridge where they give off short dendrites, and then coalesce into three fiber masses (“roots”) that approach the FB from posteriorly.

The anterior domain of the inferior protocerebrum (IPa) surrounds the horizontal lobes of the mushroom body. A massive transverse fiber system, the ellipsoid fascicle (EF), separates the IPa from the IPm posteriorly, and the LAL ventrally (Figs.4A, B; 5B, F). The EF is formed by tracts of the DALv lineages whose cell bodies are located in the anterior cortex, around the spur of the mushroom body. Extending postero-medially, DALv axons form branches in the lateral accessory lobe, as well as the “bulbs” that flank the LEF (Fig.

3D, level EB; 4B). DALv2 fibers then approach the midline and form the ellipsoid body; DALv3 axons cross dorsal and ventral of the EB (Kumar et al., 2009). At its posterior pole, the inferior protocerebrum includes a small domain that extends as a crescent-shaped process (IPp) in between the fan-shaped body and the protocerebral bridge (Figs.3B; 5B, B').

Boundaries of the dorsal brain compartments—The dorsal compartments of the brain, namely superior protocerebrum and lateral horn, are shaped like an upside down shallow bowl which covers the inferior protocerebrum. Although less densely packed than the sheath around the central complex, glial cells are concentrated at the boundary between inferior and superior lateral protocerebrum (Fig.3D, levels E/F and FB). In addition, several longitudinal and transverse brain fascicles extend along this boundary (Figs.4A–D; 5A, C, E, G). The longitudinal superior-medial fascicle (loSM) is mainly formed by secondary axon tracts of DPmI lineages located in the postero-dorsal cortex, and of DAMd lineages in the anterior cortex (Fig.5G). The superior-lateral longitudinal fascicle (loSL) runs along the boundary between superior lateral and inferior lateral protocerebrum. It contains anteriorly projecting fibers emanating from postero-laterally located cell bodies (DPLp and DPLl lineages); in addition there are fibers that are formed by anteriorly located neurons (e.g., DPLd lineages) and extend in the opposite direction (Fig.5C, G).

Transverse fibers form several crescent-shaped bundles grouped around the boundary between superior and inferior protocerebrum. We distinguish a posterior, intermediate and anterior superior transverse fascicle (trSP, trSI and trSA, respectively). The posterior transverse fascicle is formed by DPLl lineages (lateral component; trSPl) and DPLc lineages (medial component; trSPm; Fig.4C, G; 5A, C, G). These bundles demarcate the boundary between LH and superior lateral protocerebrum (SLP), and between SLP and SMP, respectively (Fig.4C). At a more anterior level one finds the intermediate system of superior transverse fibers (trSI). It is formed by fibers of BLAd and BLD lineages, located in the dorso-anterior/dorso-lateral cortex, as well as more postero-ventrally situated BLV lineages (Figs.4B; 5A, E, C, G). The most anterior superior transverse system (trSA) has a conspicuous U-shape that demarcates the inner boundary of the SLP compartment (Fig.4A; Fig.5A, C) from the inferior lateral protocerebrum; the trSA is formed by fibers of the DPLal lineages (Fig.5G).

Boundaries of the ventral brain compartments—In the ventral half of the brain one can distinguish between two longitudinal systems of fascicles. One system extends posteriorly along the ventral boundary of the inferior protocerebrum (Fig.4C, D, E, and Fig. 5D). It is formed by the medial equatorial fascicle (MEF), already mentioned above; the lateral equatorial fascicle (LEF), and the posterior lateral fascicle (PLF). The lateral equatorial fascicle is formed by axons of certain CP lineages posteriorly, and of the DALv1 lineage (anteriorly (Fig.5D, H). Anteriorly, the lateral equatorial fascicle defines the boundary between three adjoining compartments, the inferior protocerebrum, lateral accessory lobe, and ventro-lateral protocerebrum (Fig.4A, B; 5D, H). The posterior lateral fascicle contains fibers of numerous lineages, among them BLVp and CP, both located in the posterior brain cortex (Fig.5D, H).

The ventral longitudinal fascicle (loV) extends ventrally of the great commissure and defines the boundary between VMC compartment (medially), and VLP/PLP compartment (laterally). Posteriorly the loV splits into numerous thin roots formed by different lineages, among them several CM lineages (Fig.4C; Fig.5G, H). Anteriorly, the loV fascicle comprises three large bundles, here called the medial, intermediate and lateral subdivisions of the loV (loVM, loVI, loVL, respectively). The loVM follows the ventral boundary of the VMC, defining the dividing line between this compartment and the ventro-medially adjacent

perioesophageal neuropile (Fig.3D, level EB; Fig.4A; Fig.5A, D). This bundle is formed by fibers of two BAMv lineages whose cells are located around the anterior surface of the antennal lobe (Fig.5H). The intermediate loV extends along the glial septum that separates VMC/antennal lobe from the ventro-laterally adjacent AMML (Fig.5D); loVI fibers are mainly formed by ventral BA lineages surrounding the anterior surface of the AMML (not shown). Finally, the lateral loV, located laterally and dorsally of the loVI, follows the glial septum in between lateral accessory lobe (which topologically represents a continuation of the VMC) and ventro-lateral protocerebrum (Fig.4A). Identified lineages that contribute to the loVL are the BALp lineages, whose somata are located in the groove between ventro-lateral protocerebrum and AMML (Fig.5H).

In addition to a large number of smaller fiber bundles which will not be mentioned here (their analysis has to await the detailed reconstruction of all of the lineages in the adult brain), several large, vertical long fiber systems are in contact with the longitudinal ventral systems, and serve as useful landmarks delineating neuropile compartment boundaries. These are the subesophageal-protocerebral fiber system (SPS; Strausfeld, 1976), the central descending protocerebral fascicle (deCP), and the ventro-anterior vertical fascicle (veVA). The subesophageal-protocerebral fiber system appears at central AP levels (EB, E/F; see Fig.4B'). where it extends, along the boundary between VMC and AMML upward into the ventro-lateral protocerebrum (Fig.4B'; 5D). It is not yet clear which, if any, secondary lineages form part of the SPS; labeling with anti-Neuroglial does not result in staining of the SPS (Fig.4B). The central descending protocerebral fascicle (deCP; Figs.4B; 5D) is formed by DALd and other DAL lineages whose somata are located in the anterior cortex, around the optic tubercle and spur (not shown). Fibers cross the proximal peduncle, and then turn ventrally along the glial septum between inferior protocerebrum and central complex towards the VMC compartment (Fig.4B). The deCP forms a convenient landmark of the boundary between VMC and LAL (arrow in Fig.5D, D'). Finally, the vertical ventro-anterior fascicle (veVA) forms a massive bundle traveling along the fundus of the glia-filled cleft that separates LAL from VLP (Figs.4A, 5A). The fibers are emitted by BALp lineages with cell bodies in the anterior-ventral cortex around the VLP and AMML (Fig.5E).

Boundaries and long fiber bundles defining compartmental subdivisions—

Several of the above described main compartments are large, in particular along the antero-posterior axis. Afferent tracts and intrinsic long fiber systems help to define smaller domains within the ventro-lateral and posterolateral protocerebrum, the ventro-medial cerebrum, and the superior protocerebrum.

The posterior part of the ventro-lateral protocerebrum (VLPp) and posterior lateral protocerebrum (PLP) receive their main input from the optic lobe. Input fibers to both domains form distinctive, synapse-rich glomeruli, or “optic foci”. Optic lobe input to the VLPp comes from the lobula via several distinct bundles, the lobula optic nerves (Strausfeld 1976; Otsuna and Ito, 2006; Fig.6A, B). By contrast, input to the PLP is derived from the lobula plate (lobula plate optic nerves), as well as from the internal medulla (Fig.6A, B). All of these optic lobe-derived tracts are prefigured in the larval brain, where optic lobe neurons in the medulla and lobula complex start to differentiate and the first “pioneers” of the optic nerves reach the central brain (Fig.6C, D, H). Aside from their distinctive input, a vertical fiber bundle helps identifying the boundary between VLPp and PLP (Fig.6F). This bundle, termed the (vertical) posterior fascicle (veP) because it most likely corresponds to a structure with the same name defined by Strausfeld (1976) in *Musca*, represents an example of a “preterminal long fiber bundle” defined above. It connects the PLP with the lateral horn above it.

Anterior and ventral of the VLPP are the anterior VLP (VLPa) and inferior VLP (VLPI; Fig. 6G). These domains lack overt optic foci, although input from the optic lobe also exists. The boundary between VLPa and VLPP is demarcated by another preterminal long fiber tract, called the vertical lateral fascicle (veL; Fig. 6F), in accordance to a similarly positioned fiber system described by Strausfeld (1976) for *Musca*. The veL interconnects the VLPa with the SLP.

Transverse fascicles and commissural tracts serve as landmarks to subdivide the superior protocerebral compartments into an anterior and posterior subdomain (SLPa/p and SMPa/p, respectively (Ito et al., 2010); Fig. 7A). Commissural fibers belong to four systems which are visible already in the larval brain (Nassif et al., 2003): Dorso-anterior commissures and ventro-anterior commissures cross dorsal and ventral to the ellipsoid body, respectively; dorso-posterior and ventro-posterior commissures are found dorsally and ventrally attached to the posterior surface of the fan-shaped body (Fig. 7B–D). Each of these four systems consists of several discrete bundles, which will not be considered in detail here; representatives for each system are shown in Fig. 7B–H. The anterior commissures cross at the level where, further laterally, the vertical lateral fascicle and the anterior superior transverse fascicle are found (Fig. 7B). Taken together, the anterior transverse/commissural system defines a virtual plane separating SMPa/SLPa from SMPp/SLPp (Fig. 7A, B).

The great commissure, a massive fiber bundle measuring more than 10 μ m along the AP axis, is helpful as a landmark to further subdivide the large ventro-medial cerebrum (Fig. 7C, D). The region behind the commissure constitutes the postcommissural VMC (VMCp), or posterior slope (PS), which is further subdivided into a superior and inferior slope (sPS, iPS). The infracommissural VMC (VMCi) flanks the great commissure ventrally, the supracommissural VMC (VMCs) flanks it dorsally. Finally, the precommissural VMC (VMCpr) is located in front of the great commissure.

Development of the brain neuropile compartments

Neuropile compartments can be already recognized in the primordium of the central nervous system of the embryo (Younossi-Hartenstein et al., 2006). Compartments are formed around the axon bundles of contiguous primary neural lineages. Initially, a highly stereotyped subset of early differentiating neurons forms a scaffold of pioneer tracts. Soon thereafter, all lineages produce axon bundles (PATs) that orient themselves along the pre-existing pioneer scaffold. Primary axons then elaborate intertwined axonal and dendritic arbors which, together with sheath-like processes formed by glial cells, establish the neuropile compartments of the larval brain, as shown in Fig. 8D–E (for detail, see Nassif et al., 1998; 2003; Younossi-Hartenstein et al., 2006). In many cases, PATs form dense proximal arborizations at the point where they enter the neuropile. These proximal arborization typically outline compartments. We recently introduced the term “scaffolding lineage” for a lineage that, by itself or in conjunction with several other (neighboring) lineages, form a dense scaffold of terminal arborizations that define a discrete compartment (or subdomain thereof; Larsen et al., 2009; Fig. 8A). A typical example would be the four mushroom body lineages whose proximal (dendritic) arbors scaffold the calyx compartment. Shown here as an example is the primary DALv2 lineage whose proximal arbor fills the BC compartment (Fig. 8B, C). Several BA lineages have proximal (dendritic) arbors that scaffold the BA (antennal) compartment; other BA lineages project further posteriorly before forming dense arborizations in the BPM compartment. Most, if not all, BL lineages send their branched PATs radially towards the nascent BPL compartment (Fig. 8D, E).

All neuropile compartments recognizable in the embryo and early larva can be followed throughout larval and pupal development into the adult (Fig. 9). In the freshly hatched larva, one can easily recognize a dorsal, middle and ventral tier of neuropile (Fig. 9A, D), which

are comparable to the superior protocerebrum, inferior protocerebrum, and ventral cerebrum of the adult brain described above. Anteriorly and ventrally one finds the BA and BC compartments, which are the larval counterparts of the antennal lobe and lateral accessory lobe, respectively. Further posteriorly are the forerunners of the ventro-medial cerebrum and ventro-lateral/postero-lateral protocerebrum, as well as the perioesophageal neuropile, called BPM, BPL and BCv, respectively. The middle tier of the neuropile is divided by the peduncle and antenno-protocerebral tract into three domains, the CPL, CPI, and CPM. These will give rise to the lateral and medial/ventral inferior protocerebrum and the central complex, respectively. The CA compartment, surrounding the medial lobe of the mushroom body, prefigures the anterior domain of the inferior protocerebrum (IPa) of the adult brain. The calyx, formed by dendrites of the four mushroom body lineages, tips the posterior surface of the central neuropile, as in the adult. Least developed is the superior protocerebrum of the early larva. Medially, one can recognize a slender DA and DP compartment, foreshadowing the anterior and posterior SMP compartments of the adult. Lateral horn and SLP/SIP are essentially non-existent; a thin layer of neuropile on top of the CPL/CPI, called CPLd, represents the early precursor of these compartments, which (in terms of volume) dominate the central brain of the adult.

The larval neuropile compartments appear to receive a similar innervation, and therefore may carry out similar functions, as their adult counterparts. This is well established for compartments like the BA/antennal lobe that receives olfactory sensory input in both larva and adult (Stocker, 1994), or the calyx of the mushroom body that is formed by the dendritic arborizations of the four MB lineages and receives input from the antennal lobe in both larva and adult. Genetic markers expressed in specific lineages throughout development indicate that the larval-adult correspondence applies to other compartments as well. Several examples are illustrated in Fig.10. The *engrailed*-positive lineage DPLam innervates the anterior part of the VLP/BPL and SLP/CPLd and, further posteriorly, the ILP/CPL (Fig. 10A–D; Kumar et al., 2009). The *engrailed*-positive lineage DALv3 innervates the IPa/CA anteriorly, and adjacent parts of the IPm/CPI and LAL/BC further posteriorly (Fig.10E–H). BAla3 arborizes in the VMC/BPM (Fig.10I–L); in the adult, it branches out further into the VLP (Fig.10I). The *period*-positive BAmv branches in the dorsal LAL/BC and adjacent fan-shaped body/primordium of fan-shaped body (Fig.10M, N). The projection of the *period*-positive BAla1 terminates in the LH (Fig.9O); in the larva, the sparse terminal arborization of BAla1 in the lateral CPLd pin-points the location of the primordium of the LH (Fig.10P).

The ingrowth of secondary axon tracts during the late larval period follows pre-existing compartmental boundaries and establishes new ones (Fig.9C, F). Most important for the present context are the systems of secondary axon tracts that form in and around the middle layer of compartments. As mentioned previously, these SATs lay down the long fiber bundles still visible in the adult brain. As a result, the SATs provide reliable landmarks for compartments across all stages of development. SATs form dendritic and axonal arbors during the pupal period; these, along with ingrowth of sensory axons and axons from the optic lobe, account for the growth of neuropile compartments that occurs between the larval and adult stage. The transition from larval to adult neuropile architecture is shown in Fig.11, which depicts models of the neuropile at the late larval stage, followed by 12h, 24h and 48h after puparium formation, and finally the adult stage. Models are presented in anterior view (upper row), lateral view (middle column) and dorsal view (lower column); they are drawn at the same scale to allow for size comparisons. In the following, the most important events shaping the transition from larval to adult brain will be discussed.

Formation of the central and accessory complex—Medio-posterior SATs (DPMm, DPMpm, CM) penetrate the neuropile from posteriorly. Arborizations of these lineages form the fan-shaped body and protocerebral bridge of the central complex (Fig.4G, H; 5B, F; 9B;

see also Ito and Awasaki, 2008). Due to the intricate system of chiasms formed by the SATs of DPMm, DPMpm and CM lineages, the fan-shaped body develops as an unpaired compartment bridging the midline of the brain during the first day of pupal development (Fig.11B, C, L, M). No such unpaired structure exists in the larval brain (compare panels B and F of Fig.4, or D and F of Fig.9). The anterior part of the central complex, called ellipsoid body, is formed by the DALv lineages. Located in the anterior brain, SATs of these lineages grow in close proximity to the medial lobes of the mushroom body towards the midline (Fig.5B, F). During the pupal period, radially organized terminal arborizations of DALv lineages of both sides form the ellipsoid body (for detail, see Hartenstein et al., 2010). Highly focused dendritic arborizations of the DALv lineages, as well as other DAL lineages, form the dorsal and ventral bulb, the main input region of the ellipsoid body. Just like the ellipsoid body or fan-shaped body, the bulbs do not exist in the larval brain. By contrast, the lateral accessory lobe has a well defined larval forerunner, the BC compartment. Due to the arborization of several DAL lineages projecting into the BC, this compartment increases in volume during the pupal period (Fig.11A–E).

Superior medial protocerebrum—Aside from the fan-shaped body, SATs of the DPMm, DPMpm and CM lineages also form two massive fiber bundles, the loSM and MEF, that can be recognized in the larval and adult brain (Figs.4B–D; F–H; 5A, E, C, G; 9B, C, E, F). Other lineages, such as those of the CP and DAM group, also contribute to these bundles (Fig.5C, D, G, H). Arborizations developing along these SATs during the pupal period contribute to the significant growth of the superior medial protocerebrum (Fig.11A–E).

Superior lateral protocerebrum and lateral horn—Lateral lineages (DPL, BLD, BLA, BLV) form the systems of fiber bundles that extend in between lateral superior and inferior protocerebrum, i.e., the larval CPLd and CPL compartments. In the late larva, and to a lesser degree in the adult, these bundles, called the loSL and trS, impose as massive signal-negative “cavities” in brain sections labeled with antibodies against synaptic proteins (Figs. 4B, C, F, G; 9E, F). Arborizations of DPL and BL lineages are mainly responsible for the strong increase in volume of the superior lateral protocerebrum and lateral horn, from a thin “rind” in the late larva (Fig.9E; see also Fig.10P) to the large compartments of the adult (Fig.9F). The models shown in Fig.11 illustrate the growth of the superior protocerebrum and lateral horn that occurs in a gradual fashion throughout pupal development.

Inferior protocerebrum—This part of the neuropile, surrounding the peduncle of the mushroom body and bounded medially and dorsally by the systems of longitudinal and transverse SATs, changes relatively little in size during metamorphosis (Fig.9E, F).

Ventral neuropile compartments—The ventro-lateral protocerebrum evolves from its larval counterpart, the BPL. Growth occurs as a result of the axonal input that arrives through the optic nerves from the optic lobe, as well by the arborization of the BL lineages, most of which penetrate into the BPL. In the late larva, the optic nerves originating in the lobula, lobula plate and proximal medulla have reached the BPL compartment (Fig.6C, D), but dense terminal arbors are not yet formed. This can be most clearly deduced from the fact that the synapse-rich optic foci which dominate much of the adult ventro-lateral protocerebrum are absent from the larval BPL. Similar to the BPL, the larval forerunner of the antennal compartment (BA) grows through the combined addition of massive sensory input from the antenna and maxillary palp, as well as the elaboration of dendritic arbors of the BA lineages that form the olfactory projection neurons. These lineages are BA1c, BA1a1/2, and BAMv3 (Pereanu and Hartenstein, 2006; Lai et al., 2008). The ventro-medial cerebrum, which develops from the larval BPM, does not receive direct sensory input; its

growth during metamorphosis is due to arborization of several BA lineages and CM lineages which innervate this compartment.

Optic tubercle and AMMC—These two compartments have no larval counterpart and arise during early pupal development (Fig.11C, D, H, I). The optic tubercle, a well defined “optic focus” located dorsal of the spur and ventro-lateral protocerebrum, is formed by axons of optic lobe neurons projecting through the anterior optic tract, as well as arborizations of central brain lineages, among them DALcl (Hartenstein et al., 2010). The AMMC is the sensory compartment formed by mechanosensory afferents from the antenna and other parts of the head capsule. These sensory axons reach the brain during the pupal period and, along with arborizations of basal lineages (BALp) form the AMMC in the cleft in between the AL/BA and VLP/BPL (Fig.11D, I).

Discussion

The protomap of *Drosophila* neuropile compartments

This paper maps the structurally distinct neuropile compartments which were recently defined for the adult *Drosophila* brain (Ito). We emphasize here the brain fascicles that constitute part of the compartment boundaries, and follow these structures throughout development. A map of compartments with well defined boundaries is important for several reasons. First and foremost, there are numerous attempts ongoing to systematically screen for genetic markers (e.g., Gal4 lines), whose expression pattern needs to be established (Pfeiffer et al., 2008). To this end, a framework of internal boundaries is indispensable as landmarks. In addition, compartments will be important in the quest for functional centers and circuits. It is now possible to visualize neuronal activity in live brains (Wang et al., 2003; Marella et al., 2006; Wang et al., 2008; Liu and Davis, 2008). Just as in the case of mapping genetic markers, compartment boundaries provide the basis of localizing activity to specific regions of the brain.

Aside from providing a description of compartment boundaries in the adult brain, we have analyzed developmental stages, starting with the late embryo, and continuing through larval and pupal stages. In the vertebrate field it has proven to be of great value to map mature compartments onto the brain primordium (i.e., the neural tube) that is formed in the early embryo. To paraphrase the title of a chapter in one of the current standard text books on Neuroscience (Martin, 1985): “Development serves as a guide to an understanding of the regional anatomy of the brain”. Aside from this didactic aspect, the analysis of the developmental origin and morphogenesis of compartments is of even greater importance to link the function of genes (which are often expressed transiently in defined regions of the embryonic brain) to mature brain structures. To this end, a “protomap” of the brain, that indicates structurally and molecularly defined boundaries along the dorso-ventral axis and the antero-posterior axis, has become established for the mammalian brain (e.g., Rakic, 1988; Rubinstein and Beachy, 1998).

In the present paper, we have made a similar attempt to link the anatomy of the adult to larval stages. This was possible because the lineage-associated long axon tracts, which already appear in the late embryo, can be recognized by a number of antibody markers throughout development. We report that most of the compartments of the adult brain can be recognized already in the early larva. Subsequent changes that transform the larval into the adult brain involve (i) simple growth of a compartment; (ii) splitting (plus growth) of a compartment into several parts; (iii) addition of new compartments.

(i) The first mechanism, growth, is due to an increase in branch length and branch density (Larsen et al., 2009; Kumar et al., 2009). During the larval stage, many (if not all)

differentiated primary neurons grow by an increase in branching; as a result, all compartments grow rather evenly by a factor of about 10 (in volume) between early first and late third instar. With the exception of the mushroom body there is little or no addition of new neurons to the differentiated neuropile. Thus, the large number of secondary neurons that are born during the larval stage do not differentiate; their rudimentary axons remain unbranched, forming the SAT bundles that extend along compartment boundaries, or penetrate compartments, but without branching (Dumstrei et al., 2003; Pereanu and Hartenstein, 2006). During metamorphosis, secondary neurons differentiate and branch, which leads to a second phase of substantial neuropile growth. This growth spurts does not affect all compartments at the same rate. Thus, the central compartments grouped around the mushroom body (CA, CPM, CPI, CPL; giving rise to the inferior protocerebrum) grow only little between late third instar and adult; by contrast, the dorsal compartments (CPLd, DA, DP; giving rise to the superior protocerebrum) and some ventral compartments (BA/antennal compartment; BPL/ventro-lateral protocerebrum) grow substantially. When considering the changes in neuropile volume that occur during metamorphosis, one has to also factor in branch retraction and cell death of primary neurons. This has been shown to play a major role in the mushroom body and antennal lobe, but has not yet been analyzed for most other compartments. Both dorsal and medial lobes of the mushroom body shrink substantially during early pupal development due to the retraction of the axons of all γ -neurons, which make up the major fraction of larval mushroom body neurons (Watts et al., 2004). Likewise, the olfactory glomeruli of the larva disappear during early metamorphosis, presumably due to the disappearance of sensory input (which gets replaced by a new set of afferents), but also due to the withdrawal of dendrites of (primary) antennal projection neurons.

(ii) Several compartments for which no further intrinsic boundaries can be recognized in the larva, split into several distinct subcompartments during metamorphosis. This is most evident for the BPL, BPM and CPLd of the larva. The BPL gets invaded by a large number of high order visual interneurons originating in the medulla, lobula and lobula plate. Lobula plate afferents form distinct optic foci in the posterior part of the BPL, resulting in the formation of a distinct adult compartment, the PLP. Likewise, lobula afferents invade the center of the BPL, giving rise to the VLPp of the adult. The anterior and ventral “fringes” of the larval BPL stay devoid of optic foci, but grow substantially, forming the adult VLPa and VLPi. A similar splitting into multiple subcompartments (each further growing by branching of secondary neurons) occurs for the CPLd that forms the compartments of the superior protocerebrum (SLP, SIP, LH).

(iii) Compartments that arise *de novo* during metamorphosis are the central complex and part of the lateral accessory complex, the AMMC, and the optic tubercle. The latter two are mainly defined by a specific sensory input: the AMMC develops around the ingrowing mechanosensory afferents from the antenna and head capsule, and the optic tubercle forms around the anterior optic tract (from lobula and medulla). By contrast, the central complex is born “within” a larval compartment, the CPM. Like all other larval compartments, the CPM is a bilateral structure. Located posterior of the medial lobe of the mushroom body, left and right CPM hemicompartments are connected by the supraesophageal commissure, which in the early larva is a slender fascicle of axons. In the late larva, SATs of several lineages located in the anterior and posterior brain invade the CPM and commissure. These are the DALv2, DALv3, and possibly DALcl1/2 lineages from anterior, and the DPMm, DPMpm and CM4 lineages from posterior. The latter form the primordium of the fan-shaped body, the former the primordium of the ellipsoid body and the bulbs. Branching of these lineages during early pupal development (0–24h) results in the formation of dense layers of neuropile around the SATs, which give rise to the fan-shaped body (including noduli), protocerebral bridge and the ellipsoid body (including bulbs; Hartenstein et al., 2010). These structured compartments displace much of the former CPM, of which only small fringe regions remain.

One of these, located at the most posterior edge of the larval CPM, probably gives rise to part of the inferior protocerebrum (IPp). It is possible that many of the primary neurons forming the larval CPM retract their branches during metamorphosis and innervate other parts of the emerging adult neuropile, which would explain the virtual disappearance of the CPM.

Structure of compartment boundaries

Surprisingly, the nature and genesis of compartment boundaries are rarely considered in the literature that discusses neuroanatomy. It is usually taken for granted that the reader is able to perceive the artistically or photographically documented discontinuities that are used as dividing lines between neighboring compartments, without making it explicit what the structural basis of these discontinuities is, or how it comes about developmentally. At the most superficial level, outward shape is taken as a criterion for compartmentalization. We distinguish entities like the cerebral cortex, midbrain, or cerebellum because they have a characteristic shape and spatial arrangement, and are demarcated from each other (or other neighboring structures) by externally visible folds. In case of the mature structures, it becomes immediately evident that many other, intrinsic characteristics, like size and arrangement of neurons, distinguish structures like cerebral cortex and midbrain. Early in development this is not the case: the neural tube has the same intrinsic histological structure along its entire length, and it is merely the presence of creases and bulges in its outer relief that justifies us to distinguish a prosencephalon from mesencephalon or rhombencephalon (Jacobson, 1991).

More important for this discussion are compartment boundaries that exist below the surface of the brain, such as those (mentioned as examples in the Introduction) of the brainstem nuclei, cortical layers, or tangential cortical subdivisions such as the somatosensory barrels. These compartments are modules, consisting of a group of contiguous neurons/neuronal processes that among each other share certain structural/molecular/functional properties. Surrounding a given compartment is a boundary (“discontinuity”) that separates this compartment from other compartments formed by neurons with different structural and functional properties. In most cases, neurons belonging to a compartment are more tightly connected with each other than with neurons of neighboring compartments. As a consequence, compartment boundaries are defined visibly as layers (septa) with a reduced number of neuronal elements (somata, and/or terminal neurites/synapses). The barrels of the rodent somatosensory cortex are prototypical neuropile compartments (Van der Loos and Woolsey, 1973; Woolsey et al., 1975; Inan and Crair, 2007). Each barrel is associated with one whisker hair of the snout of the animal. A barrel is formed by a population of neurons that all receive input from that whisker, and that are preferentially connected with each other, rather than with neurons belonging to neighboring barrels. Thus, dendritic and axonal arbors of neurons of a given barrel are mostly restricted to the interior of that barrel, leading to a domain of high terminal fiber/synapse density separated from adjacent barrels by a septum of lower fiber/synapse density (Petersen and Sakmann, 2000). Other structural elements (e.g., glial cells) further accentuate barrel boundaries (Seo and Geisert, 1995). As a result, the neurons of a barrel function, to a certain extent, in isolation from neurons of neighboring barrels. The same idea applies to compartments in general, such as those defined here for the central brain of the fly. What we perceive as the boundary between a compartment like the calyx and its neighbors is a layer that contains fewer synapses, and is crossed by fewer terminal neurites (forming synapses!) than the interior of calyx or its neighboring compartments. It can be assumed that if one were to image neuronal activity in a living brain, a compartment stands out, at a given time point, as a focus of high or low activity, separated by a sharp discontinuity from neighboring domains.

What structural elements fill compartment boundaries? Shown in this and previous papers for the *Drosophila* brain (Younossi-Hartenstein et al., 2003; Pereanu et al., 2007) are glial processes and long fiber tracts, and both components define compartment boundaries in the brain of vertebrates and other animals as well. It has long been established that glial processes form the boundaries between olfactory glomeruli, well studied subcompartments of the insect antennal lobe (Tolbert and Oland, 1990). In the vertebrate CNS, glia and long fiber bundles are tantamount to the same thing, since axons (prior to splitting into terminal arborizations) are myelinated by oligodendrocytes. In the brainstem, the long fiber connections between different brain regions and the spinal cord form bundles or plates of fibers that form part of many compartment boundaries (e.g., Nieuwenhuys et al., 1981).

Astrocytes, the second major class of glial cells, also seem to play a major role in setting up and maintaining compartment boundaries. An important class of molecules expressed by astrocytes and implicated in a central role in brain compartmentalization are the chondroitin sulfate and keratan sulfate proteoglycans. Astrocyte processes expressing high levels of these ECM molecules are concentrated at the boundaries of somatosensory barrels (Seo and Geisert, 1995), olfactory glomeruli (Gonzalez et al., 1993), and many brainstem nuclei of the developing brain (Geisert and Bidanset, 1993). Keratan sulfate appears to form one of the ECM components that inhibits axon growth. Thus, scar tissue, formed by astrocytes, expresses high levels of keratan sulfate, which prohibits the growth of axons into the scar (Moon et al., 2002; Liu et al., 2006). The same mechanism may act during normal development, where glial cells expressing keratan sulfate and other ECM molecules set up compartment boundaries that act as (relative) barriers for axons.

Developmental origin of compartments

One can think of a number of different mechanisms that generate discontinuities within the developing brain tissue. One possibility is that discontinuities arising elsewhere in the body are superimposed upon the nervous system. A prime example of this is evident in specialized sensory organs, such as the eye or ear, consisting of receptors that are not evenly distributed over the body, but concentrated in restricted domains. These receptors form sensory nerves that invade the CNS and form terminal afferent arborizations in discrete regions. As a result of the specialized afferent input, the corresponding CNS region ("sensory compartment") acquires properties that set it apart from neighboring regions lacking that input. Given that the sensory afferents interact with their CNS target neurons, inducing certain biochemical, physiological and structural changes in these neurons (Kalb and Hockfield, 1990; de la Cruz et al., 1996), the properties that distinguish the sensory compartment from neighboring CNS domains will be further accentuated. A similar mechanism might act in the reverse direction: Efferent neurons (motor neurons) might encounter a specialized, spatially restricted peripheral target (muscle, gland) which interacts with the innervating efferent neurons and induces in them a set of changes, resulting in a specialized CNS compartment associated with that particular peripheral target. One might call this mechanism of compartment formation "peripherally induced compartmentalization".

A well studied example of peripherally induced compartmentalization is the trigeminal sensory system in many mammals, including rodents (van der Loos and Woolsey, 1973) and marsupials (Weller, 1993). Here, as already alluded to in the previous section, individual whiskers of the snout of the animal are represented by morphologically distinct "barreloids" in the thalamus, and "barrels" in the primary somatosensory cortex. Barreloids and barrels form a pattern that topologically corresponds to the pattern of the whiskers themselves; furthermore, if whiskers are experimentally deleted or increased in number, corresponding changes are induced in the pattern of both barreloids and barrels (van der Loos and Woolsey, 1973; Ohsaki and Nakamura, 2006). What this shows is that the induction of

compartments acts across synapses: the periphery (trigeminal afferents) induces barreloids in the thalamus, and these in turn induce barrels in the cortex. One might distinguish these two forms of induced compartmentalization as peripherally versus centrally induced compartmentalization.

Induced compartmentalization also plays a role in the insect brain. Numerous experimental studies have shown that by manipulating sensory input, one can change the shape of neurites of central neurons (Murphey and Levine, 1980). It has been directly shown that olfactory sensory (antennal) afferents play a very similar role in setting up olfactory glomeruli in the insect antennal lobe than olfactory afferents in vertebrates (Oland et al., 1990; Oland and Tolbert, 1996). In the absence of antennal sensory neurons, the rearrangements of glial and central dendritic processes that normally result in the formation of olfactory glomeruli does not take place.

Opposed to the above described mechanism by which brain compartments are induced by afferent or efferent connections is the control of compartmentalization by intrinsic factors (“intrinsic compartmentalization”). For vertebrates, an idea which in recent years has been bolstered by a rich literature (Kintner et al., 2008; O’Leary et al., 2007; Rubenstein et al., 1998), is that at an early stage, the expression pattern of transcription factors, including Hox- and other Homeobox proteins (Otx, Emx, Tlx, Pax proteins, Nkx proteins) subdivides the neural tube into distinct domains (“proto-compartments”) which are already fated to become structural compartments of the mature brain. An essential prediction of this hypothesis is that the pool of neurons generated within a “proto-compartment” remains together, once boundaries are set up at which prohibit the mixing of neurons. The mechanisms that account for the setting up of boundaries are poorly understood in vertebrates, but models involving a multistep process (prepattern set up by morphogens; inhibitory interactions leading to a “fuzzy boundary; adhesion molecule-based sorting, generating the definitive boundary) are discussed (Kiecker and Lumsden, 2005).

We assume that the major neuropile compartments of the *Drosophila* brain analyzed in this paper (e.g., mushroom body, antennal lobe, VLP, etc), are set up by the central neurons themselves: they are intrinsic compartments. It is true that, as discussed above, afferent input contributes to further subdivide the major compartments into smaller units, such as olfactory afferents shaping the individual glomeruli of the antennal lobe. However, the antennal lobe as a whole, or the other major compartments, are still present as distinct compartments despite deafferentation. What appears to be peculiar to *Drosophila* and other insects (as opposed to vertebrates) is the prominent role of defined lineages in shaping individual compartments. This has been directly shown for compartments like the calyx of the mushroom body, which is absent as a morphologically detectable unit if the four MB lineages are ablated (Ito et al., 1997; Malun, 1998). We are currently trying to identify the scaffolding lineages for the remainder of the brain compartments, following a similar approach. (Lovick et al., in prep). To distinguish the two types of intrinsic compartmentalization, in insects and vertebrates, we propose the terms “closed lineage” and “open lineage” mode of intrinsic compartmentalization. “Closed lineage”, as in *Drosophila*: Neurons of a lineage form a closed unit, where somata, axons and major parts of their arborizations stay together and define discrete modules of the brain neuropile (Larsen et al., 2009). Experimental removal of a lineage leads to the absence of the corresponding module; there is little or no regulation (i.e., neighboring neuroblasts do not “sense” the absence of a given lineage and produce extra-neurons that may close the gap). By contrast, vertebrate neural lineages, at least up to a certain point, appear “open”. Thus, within a given volume of the brain neuropile, one finds somata of numerous neighboring lineages intermingled; projections of members of a given lineage do not form distinct bundles or compartments (Tan et al., 1998). Of course, at some point in development, boundaries between

compartments are fixed, and from that time onward, descendants of a cell (dividing afterwards) do not cross compartment boundaries (Kiecker and Lumsden, 2005; see above). It will be interesting to establish when and how compartment boundaries are formed, and draw comparisons with the lineage-related mechanism that operates in insects. Moreover, it will be fascinating to unravel the phylogenetic history behind the “open lineage” and “closed lineage” mechanism. With the exception of the nematode *C. elegans*, detailed lineage-focused analyses of neural development in invertebrate taxa have not been carried out; anecdotal evidence suggest that clusters of neurons with discrete bundles, possibly representing individual lineages, exist in many protostomes (e.g., Morris et al., 2004). Important insight will be garnered by investigating neurogenesis in “basal deuterostomes”, such as hemichordates or echinoderms. Much of the nervous system of these organisms consists of a dense basiepithelial nerve plexus (“nerve net”), in which structural modules (clusters of somata, bundles of neurites) are at least not obvious (Bullock and Horridge, 1965). This could imply that the dichotomy of open versus closed lineage mode of CNS compartmentalization occurred deep, around the split of deuterostomes and protostomes. However, many more details regarding neural lineages and their role in CNS development in different invertebrate taxa need to come forward in order to be certain.

Acknowledgments

This work was supported by NIH Grant RO1 NS054814 to VH.

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Fig. 1. Definition of neuropile compartment boundaries. A, B: schematic cross section of brain depicting exterior cortex (cx), central neuropile (np), and glial cells (gl; green). The neuropile is subdivided by glial processes and long axon tracts into discrete compartments. C: confocal section of one hemisphere of adult central brain labeled with synaptic marker nc82. The interior of compartments shows strong label due to the high density of terminal neurite arborizations containing synapses. Compartment boundaries (cb) appear as elongated areas showing low signal. D: confocal section of brain hemisphere taken at the same level as in C. In this preparation, glial cells are labeled by Nrv2-Gal4 driving UAS-GFP.

Compartment boundaries are rich in glial processes and appear as strongly signal-positive areas.

Bar: 25 μ m

Fig. 2.

Two types of long fiber bundles. A: Schematic depiction of a neuronal lineage. B: Confocal cross section of adult brain hemisphere labeled with synapse marker nc82. C: Same section as in B; part of axon tract and neurite arborization of one lineage is labeled by *engrailed-Gal4* driven UAS-GFP (green). Axons of all neurons belonging to one lineage fasciculate and form one coherent long fiber bundle (purple lines in A; represented by purple arrow in C). Typically, several lineage associated axon tracts come together in the neuropile and form a brain fascicle. Lacking synapses, this fascicle appears as a sharply delineated, signal-negative space in sections labeled against synaptic markers (purple arrow in B). A second type of long fiber bundle is formed by pre-terminal neurites (red lines in A; red arrow in C).

Compared to the brain fascicles formed by lineage associated tracts, preterminal fibers form loose bundles because they intermingle with short terminal branches and synapses. In sections labeled with synaptic markers, pre-terminal long fiber bundles appear as signal-poor, striated domains (red arrow in B; B').
Bar: 25 μ m

Fig. 3.

Neuropile compartments of the adult *Drosophila* brain. A: Anterior view 3D digital model of the brain. Compartments of one brain hemisphere are depicted in different colors. The color code for compartments used here will be applied in all other panels of this and the following figures. Grey lines subdivide the central brain and the compartments contained within it along the dorso-ventral axons into three major regions, the dorsal cerebrum, middle cerebrum, and ventral cerebrum. B: 3D digital models of one brain hemisphere. Panels in left column represent latero-dorsal view, those of the right column antero-dorsal views. First row: Surface view of all major brain compartments. Second through fourth row: “exploded” model, showing the compartments of the dorsal cerebrum, middle cerebrum and ventral

cerebrum, respectively. C: Dorsal view of brain model. Grey lines subdivide the neuropile along antero-posterior axis into seven 15–20µm thick slices. These slices are represented as Z-projections of confocal sections in D (left column: compartments visualized by labeling with synaptic marker nc82; right column: labeling of compartment boundaries with Nrv2-Gal4>UAS-GFP). Each brain slice is characterized by a conspicuous hallmark compartment or tract. From anterior to posterior, the slices and their hallmark structures are (1) antennal lobe (AL); (2) optic tubercle (OTU); (3) ellipsoid body (EB); (4) posterior ellipsoid/anterior fan-shaped body (E/F); (5) fan-shaped body (FB); (6) antenno-protocerebral tract (APT); (7) calyx (CX).

Abbreviations of compartments: AL antennal lobe; α -L alpha lobe of mushroom body; AMMC antenno-mechanosensory and motor center; β/γ -L beta and gamma lobes of mushroom body; CCX central complex; CX calyx; EB ellipsoid body; FB fan-shaped body; IP inferior protocerebrum; IPa anterior subdivision of IP; IPl lateral subdivision of IP; IPm medial subdivision of IP; IPP posterior subdivision of IP; IPv ventral subdivision of IP; LAL lateral accessory lobe; LO lobula; LP lobula plate; LH lateral horn; MB mushroom body; MD distal medulla; MP proximal medulla; Nd noduli; OTU optic tubercle; PB protocerebral bridge; PLP postero-lateral protocerebrum; PONP periesophageal neuropile; SIP superior intermediate protocerebrum; SLP superior-lateral protocerebrum; SLPa anterior subdivision of SLP; SLPP posterior subdivision of SLP; SMP superior-medial protocerebrum; SMPa anterior subdivision of SMP; SMPp posterior subdivision of SMP; sp spur of mushroom body; SOG subesophageal ganglion; VLP ventro-lateral protocerebrum; VMC ventro-medial cerebrum; VMCpr precommissural VMC

Bar: 50µm

Fig. 4.

Larval-to-adult persistent long axon tracts associated with compartment boundaries. All panels show Z-projections of frontal confocal sections of adult brain (two columns on the left) and late larval brain (two columns on the right) labeled with antibodies nc82 (synapses) and BP104 (secondary axon tracts; SATs). Z-projections represent brain slices at the antero-posterior levels defined in Figure 3. BP104-positive SATs of groups of lineages converge in the neuropile and form a stereotyped array of fascicles that persist through metamorphosis into the adult brain. Fascicles were grouped into longitudinal (lo), transverse (tr) and vertical (ve) ones. Many fascicles extend along compartment boundaries, and therefore allow one to follow compartment boundaries from larva to adult. For example, as shown in panels B–C' and F–G', the intermediate and posterior transverse superior fascicles (trSI, trSP), along with the medial and lateral longitudinal superior fascicles (loSM, loSL) demarcate the boundary between the superior compartments [SMP (larval primordium: DP), SLP, LH (larval primordium: CPLd)] and inferior protocerebrum [IPm, IPI (larval primordia: CPI, CPL)]. For more detail, see Results.

Abbreviations: APT antenno-protocerebral tract; coLAL inferior commissure of lateral accessory lobe; deCP descending central protocerebral; dlrFB dorso-lateral root of fan-shaped body; GC great commissure; EF ellipsoid fascicle; MEF medial equatorial fascicle; PLF posterior lateral fascicle; LEF lateral equatorial fascicle; loSL longitudinal superior lateral fascicle; loSM longitudinal superior medial fascicle; loV ventral longitudinal fascicle; loVI intermediate ventral longitudinal fascicle; loVL longitudinal ventro-lateral fascicle; loVM longitudinal ventro-medial fascicle; MeB median bundle; mrFB medial root of fan-shaped body; obP oblique posterior fascicle; P peduncle; PP peripeduncular fiber system; SPS suboesophageal-protocerebral fiber system; trSA transverse superior anterior fascicle; trSI transverse superior intermediate fascicle; trSP posterior transverse superior fascicle; trSPI transverse superior postero-lateral fascicle; trSPm transverse superior postero-medial fascicle; veVA vertical ventro-anterior fascicle

Bar: 25μm

Fig. 5.

Brain fascicles associated with compartment boundaries. All panels show 3D digital models of one brain hemisphere. Compartments are rendered transparent. Left column (A–D') represents adult brain, right column (E–H') late larval brain. First row shows anterior view, second and third row dorsal view, fourth row ventral view. In models shown in large panels (A–D, E–H) BP104-positive fascicles that are useful to delineate compartment boundaries are rendered in different colors. Small panels (A'–D', E'–H') present models in which compartments are color coded and annotated. In panels E–H, identified secondary lineages whose SATs contribute to the long axon fascicles of the adult are identified by name framed by colored ovals. Models shown in anterior view (A, A', E, E') emphasize the notion that longitudinal and transverse fascicles and their larval precursors are grouped around the inferior protocerebrum (IPm/IPv, IPI; larval precursors: CPI, CPL). Panels of the second row (B, B', F, F') show fascicles that extend along the boundary between central complex (CCX; larval precursor: CPM centro-posterior medial compartment) and inferior protocerebrum. These fascicles are the antenno-protocerebral tract (APT) and the longitudinal central fascicle (MEF). Shown are also the medial and dorso-lateral roots of the fan-shaped body (mrFB, dlrFB) which invade the central complex from posteriorly. Anteriorly, tracts of the DALv and DALcl lineages extend towards the central complex, forming the ellipsoid fascicle (EF). The third row of panels singles out longitudinal and transverse fascicles extending along the boundary between superior protocerebrum and inferior protocerebrum. Panels of the bottom row (D, D', H, H') show inferior fascicles that extend along the ventral boundary of the inferior protocerebrum, and the ventral longitudinal fascicle that delineates the ventro/postero-lateral protocerebrum (VLP, PLP, laterally) from the lateral accessory lobe/ventro-medial cerebrum (LAL, VMC, medially). Secondary lineages whose SATs contribute to the inferior and ventral fascicles are depicted in H.

For abbreviations of adult neuropile compartments see legend of Fig.3; of adult fascicles, see legend of Fig.4. For nomenclature of secondary lineages, see Pereanu and Hartenstein, 2006.

Abbreviations of larval neuropile compartments: BA baso-anterior (larval antennal lobe); BC baso-central (primordium of LAL); BPL baso-posterior lateral (primordium of VLP and PLP); BPM baso-posterior medial (primordium of VCM); CA centro-anterior (primordium of IPa); CPI centro-posterior intermediate (primordium of IPm); CPL centro-posterior lateral (primordium of IPI); CPLd dorsal CPL (primordium of SLP and LH); CPM centro-posterior medial (primordium of CCX); DA dorso-anterior (primordium of SMPa); vl vertical lobe of mushroom body; DP dorso-posterior (primordium of SMPp); hl horizontal lobe of mushroom body; prEB primordium of ellipsoid body; prFB primordium of fan-shaped body

Fig. 6.

Sub-compartmentalization of the ventro-lateral protocerebrum is defined by input from optic lobe. A, B: Digital 3D model of adult brain hemisphere in dorsal view (A) and posterior view (B). The neuropiles of the optic lobe (LO lobula; LP lobula plate; MD distal medulla; MP proximal medulla) along with their major output tracts are rendered in different colors. C, D: Dorsal and posterior view of late larval brain hemisphere, with primordia of optic lobe neuropiles and output tracts rendered in the same colors as in A and B. E–G: lateral view of brain hemisphere, with optic lobes removed but optic lobe output tracts (rendered in same color as in A, B) left in place, to show their spatial relationship with central brain compartments. H–J: Lateral view of models of late larval brain, rendered in the same

manner as adult models shown in E–G above. The terminals of the lobula optic nerve (lon) form discrete glomeruli, called “optic foci” which define the posterior VLP (VLPP; enclosed by red hatched line in G). Domains devoid of optic foci are the anterior and inferior VLP (VLPa and VLPI, respectively; panel G). Two vertical fascicles of central brain axons, the vertical lateral (veL) and vertical posterior fascicle (veP) are additional landmarks demarcating the boundary between VLPa and VLPP, and VLPP and PLP, respectively (panel F). Output from the lobula (and proximal medulla; not shown) also contributes the major source of afferents to the optic tubercle (OTU) via the anterior optic tract (aot; A, E, F). Axons originating in the lobula plate project via the lobula plate optic nerves (lpon) to the postero-lateral protocerebrum (PLP; A, B, E, F). Output from all three optic neuropiles (MP, LO, LP) is already present in the late larva (C, D, H, I). However, these optic lobe efferents have not formed terminal arbors. The larval primordium of the VLP/PLP, called baso-posterior lateral compartment (BPL), is small and lacks optic foci J). For abbreviations of brain compartments (G, J) see legends of Figs. 3 and 5. All models are drawn to the same scale. Other abbreviations: CCX central complex; GC great commissure

Fig. 7.

Transverse tracts and commissures define subcompartments in the superior protocerebrum and ventro-medial cerebrum. Panels of the left column (A–D) show digital 3D models of adult brain hemisphere in dorsal view (A, B), ventral view (C) and medial view (D). The right column presents models of late larval brain hemisphere viewed and rendered in the same manner as the adjoining adult models to their left. In panels of upper row, compartments of the superior cerebrum (A) and their larval primordia (E) are rendered in their respective colors. In second row, neuropile compartments are rendered transparent to allow for viewing selected transverse tracts and commissures (green, blue, purple). Likewise, panels of third row show ventral compartments rendered transparent, and selected

transverse tracts and commissures opaque (green, blue, purple). Models of bottom row present surface view of the medial neuropile compartments. The supraesophageal commissures ("brain commissures") of the adult form four systems that flank the central complex dorso-anteriorly (DAC dorso-anterior commissures; B, D), ventro-anteriorly (VAC ventro-anterior commissures; C, D), dorso-posteriorly (DPC dorso-posterior commissures; B, D) and ventro-posteriorly (VPC ventro-posterior commissures; C, D). Forerunners of these commissures can be recognized in the larva (F, G, H). Note the close proximity of commissures in the larva compared to the adult, where the central complex has pushed commissures away from each other (compare D and H). Each of the four commissural systems contains several tracts of which only a few representatives are shown (panels B and D: sec supra-ellipsoid commissure for the DAC system; plpc commissure of postero-lateral protocerebrum for the DPC system; panels C and D: aco antennal commissure for the VAC system; GC great commissure for the VPC system). Dorsally, the DAC system along with the laterally adjoining transverse superior anterior tract (trSA) defines a convenient boundary between anterior and posterior domains of the superior protocerebral compartments (SLPa/SMPa and SLPp/SMPp, respectively; hatched red line in panels A and B). Ventrally, the great commissure subdivides the ventro-medial cerebrum (VMC) into precommissural, supracommissural, infracommissural, and postcommissural domains (VMCpr, VMCs, VMCi, VMCpo, respectively; red hatched lines in panel C and D). Panels F and G specify the secondary lineages whose tracts produce the commissures and transverse fascicles shown. For abbreviations of adult and larval brain compartments, see legends of Figs.3 and 5; for long axon tracts, see Figure 4; for nomenclature of secondary lineages, see Pereanu and Hartenstein (2006).

Fig. 8.

Origin of neuropile compartments in the late embryo. A: Schematic cross section of late embryonic brain. Neurons belonging to one lineage (e.g., L1, L2) form a cohesive cluster. Axons fasciculate and form the primary axon tract (PAT). In many cases, proximal arborizations sprout from the SAT at the position where axons enter the neuropile; these arborizations, formed by one or several neighboring lineages, “scaffold” a compartment. In this manner, lineage L1 would represent the scaffolding lineage for compartment C1; L2 for a subdomain of compartment C2 (sC2). Glial cells (green) form processes around compartments. B, C: Z-projections of confocal sections of late embryonic brain (lateral view). Neuropile is labeled with anti-DNcadherin (white in B, magenta in C). Note that compartments (BA baso-anterior; BC basocentral; BPL baso-posterior lateral; SOG suboesophageal) are clearly delineated by DNcadherin-negative septa. In C, somata (so) and proximal arborizations (pa) of lineage DALv2 are shown (per-Gal4>UAS-cd8 GFP). Proximal arborizations of DALv2 form a dense scaffold of fibers restricted to the BC compartment; no neurites are found in neighboring compartments. D, E: 3D digital models of a late embryonic brain hemisphere; dorsal view (D; anterior to the bottom) and lateral

view (E; anterior to the left). Brain is outlined in gray. Primary axon tracts of groups of primary lineages are rendered in different colors and identified by acronyms in circles (for nomenclature of lineages, see Younossi-Hartenstein et al., 2006; Pereanu and Hartenstein, 2006). Emerging compartments are rendered in the same colors as the groups of lineages that most strongly contribute (scaffold) the corresponding compartment. For abbreviations of compartments see legend of Fig.5.

Bar: 10 μ m

Fig. 9.

Morphogenesis of neuropile compartments from larva to adult. A–C: Schematic cross sections of brain hemisphere at early larval stage (A; L1/2), late larval stage (B; L3) and adult stage (C). D–F: Z-projections of frontal confocal sections of brain hemisphere labeled with synaptic marker nc82. Stages as in adjoining schematic sections above. In all panels, neuropile compartments are annotated in black letters (A–C) or white letters (D–F; for abbreviations, see legends of Figs.3–5). Colored circle in neuropile center indicates peduncle of the mushroom body (P). During late larval stage, secondary axon tracts (gray lines in B; nc82-negative spaces in E) penetrate neuropile preferentially along the septa surrounding compartments of middle cerebrum (CPI, CPL) and form the fascicles depicted

in Fig.5 (subset of fascicles annotated in blue lettering; for abbreviations of fascicles see legend of Fig.5). Arborization of secondary lineages and ingrowth of sensory afferents from the antenna/head capsule and the optic lobe, both occurring during the pupal stage, results in growth of compartments, particularly those of the dorsal cerebrum and the BLP, and in the addition of several new compartments (e.g., FB fan-shaped body; AMMC antenno-mechanosensory and motor center).

Bar: 25 μ m

Fig. 10.

Arborization of identified lineages in corresponding larval and adult neuropile compartments. All panels show Z-projections of ten contiguous 2µm confocal sections labeled with synaptic marker nc82 (magenta). Individual lineages, identified at the top of panels, are labeled by specific Gal4 drivers (*per*Gal4; *en*Gal4) driving UAS-cd8;GFP. Pairs of adjoining panels (e.g., A, B; E, F) show brain slices at antero-posterior levels indicated at lower left corner (EB level of ellipsoid body; E/F level at transition ellipsoid/fan-shaped body; FB level of fan-shaped body; OTU level of optic tubercle; see Fig.3). The left panel of each pair shows slice of adult brain, right panel corresponding slice of larval brain. Clusters of cell bodies of lineages are indicated by yellow arrowheads. For abbreviations of adult and larval compartments see legends of Figs. 3 and 5, respectively; for long axon tract abbreviations, see legend of Fig.4. A, B: The *engrailed*-positive DPLam lineage at level EB forms arborizations in anterior SLP and VLP (A) and in their larval forerunners, CPLd and BPL (B). C, D: Same lineage at level FB, arborizing in adult IPI and larval CPL (D). E, F: Arborization of *engrailed*-positive DALv3 lineage in IPa and LAL, and the corresponding larval compartments, CA and BC. Further posteriorly (G, H), this lineage has arborization in IPm/SMP or CPI/DP, respectively. I-L: The deutocerebral, *engrailed*-positive lineage BAla3 has dense arborizations in the precommissural, infracommissural and supracommissural VMC (VMCpr, VMCi and VMCs, respectively) and its larval forerunner, the BPM. In the adult, but not the larva, some distal branches of this lineage reach the VLP (I). M, N: The *period*-positive BAMv1 lineage branches in the adult LAL and fan-shaped body. In the larva, arborizations are found in the BC and the CPM, which contains the primordium of the fan-shaped body (prFB). O, P: The *period*-positive BAla1 lineage contains neurons projecting from the antennal lobe (anterior to the slice shown) to the lateral horn (LH). In the larva, this lineage has proximal arborizations in the BPM and distal arborizations in the lateral CPLd, the primordium of the LH.

Bar: 25µm

Fig. 11.

Morphogenesis of neuropile compartments during metamorphosis. All panels show digital 3D model of one brain hemisphere in anterior view (top row), lateral view (middle row), and dorsal view (bottom row). Left column represents late third instar larva, second row 12h pupa, third column 24h pupa, fourth column 48h pupa, and right column adult.

Compartments are annotated for the larva and adult (for abbreviations, see legends of Fig.3 and Fig.5). All models are drawn to scale. Arrows in B, G, L point at rapid dorsal and lateral expansion of superior cerebrum (CPLd), covering the lateral inferior cerebrum (CPL).

Arrows in C, H, M point at the emerging optic tubercle (OTU); arrows in D and I label the AMMC which first arises in the 48h pupa.

Bar: 25µm

Table 1**Fiber bundles of the adult brain**

Name	Axon fascicles	Lineages	Compartment Boundaries
Antenno-protocerebral tract	APT	BAla1 BAmv3 BAIc1/2 BAIp4	CCX-IPm/v
Medial equatorial fascicle	MEF	CM	CCX-IPm/v
Lateral root of fan-shaped body	lrFB	CM4	CCX-IPm
Longitudinal superior-medial fascicle	loSM	DPMpm DPMpl DPMl DAMd	SMP-IPm
Longitudinal superior-lateral fascicle	loSL	DPLp DPLl DPLd	SLP-IPl
Lateral equatorial fascicle	LEF		
Posterior division	LEFp	CP	IP-VLP-VMC
Anterior division	LEFa	DALv1	IP-VLP-LAL
Posterior lateral fascicle	PLF		
Posterior division	loILp	BLVp	IP-PLP
Longitudinal ventral fascicle	loV		
Posterior division	loVP	CM	PLP-VMC
Antero-medial subdivision	loVM	BAmv1/2	VMCpr-PONP
Antero-intermediate division	loVI		AL/VMCpr-AMMC
Antero-lateral division	loVL	BAIp	LAL-VLPa
Ellipsoid fascicle	EF	DALv2/3 DALc11/2	IPa-IPm
Transverse superior-anterior fascicle	trSA	DPLal	SLPa-IPl
Transverse superior-intermediate fascicle	trSI	BLAd BLD BLVa	SLPp-IPl
Transverse superior-posterior fascicle	trSP		
Medial division	trSPm	DPLc	SLPp-SMP SMPp-IPm
Lateral division	trSPl		SLPp-LH SLPp-IPl
Great commissure	GC	BLAv BLD5 BLVp	VMCpr-po-in-su
Commiss. of postero-lateral protocerebrum	plpc		
Antennal commissure	aco	BAmd	

Name	Axon fascicles	Lineages	Compartment Boundaries
Supra-ellipsoid commissure	sec	DALv3	SMPa/SLPa-SMPp/SLPp
Subesophageal-protocerebral system	SPS		
Central descending protocerebral fascicle	deCP	DALd	LAL-VMCpr
		DALcm1/2	
Vertical ventro-anterior fascicle	veVA	BAIp2	LAL-VLPa