Contribution of transit amplifying type-II neuroblast lineages to central complex primordium formation and optic lobe glial cells in *Drosophila melanogaster*

Inauguraldissertation
zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel
von

Nadia Riebli
aus Giswil, OW

Basel 2016

Genehmigt von der Philosophisch- Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Dr Heinrich Reichert (Fakultätsverantwortlicher)

Prof. Dr. Simon Sprecher (Korreferent)

Basel, den 09.12.2014

Prof. Dr. Jörg Schibler
(Dekan)

Acknowledgements

With much gratitude I would like to thank my supervisor Prof. Dr. Heinrich Reichert for giving me the opportunity to work in his laboratory as well as for his advice and continuous support. He continually conveyed a spirit of adventure relating to research and a highly contagious excitement in regard to teaching. Without his guidance and persistent help this dissertation would not have been possible.

I would also like to thank Prof. Dr. Simon Sprecher for helpful and encouraging advice as a member of my PhD advisory committee as well as for being the co-referee at my PhD exam. A big thank you also goes to Prof. Dr. Thomas Mrsic-Flogel for agreeing to be the chairperson at my PhD defense. Furthermore, I would like to thank Prof. Dr. Markus Affolter for interesting discussions during my PhD committee meeting.

An additional huge thank you goes to Dr. Albert Cardona who, together with Prof. Dr. Reichert, gave me the opportunity to approach the search for my neurons from a different angle at HHMI Janelia Farm Research Campus in Ashburn, USA. As a part of this project I would like to express my gratitude to Prof. Dr. Volker Hartenstein for his inspiring input on behalf of *Drosophila* neuroanatomy and for the amazing experience to work with him.

Furthermore, I am truly grateful for the helpful and uplifting colleagues of the lab, especially Dr. Gudrun Viktorin and Susanne Flister with whom the various collaborations and intense discussions about science and life had a profound impact on me. A further big thank you goes to Dr. Philipp Kuert for his much valued friendship and to Dr. Anne-Sophie Laurenson, Dr. Yanrui Jiang, Dr. Yunpo Zhao and Dr. Ricardo Cardoso-Neves for fruitful discussions and for creating a pleasant lab-environment. Additionally, I would like to thank Dr. Sonia Sen for proofreading the introduction and the discussion chapters in terms of language errors.

Most importantly my deepest gratitude goes to my parents, Vreni and Peter Riebli whose unconditional love, support and encouragement is beyond everything a young woman could wish for. Their belief in me during the rough patches of life and science was fantastic and made this work possible in the first place. A huge thank you also goes to my little twin-sister Tini whose sparkly personality lights up my life on a daily basis.
Furthermore I cannot find words to thank my boyfriend Oliver Müller for being my best friend, companion and inspiration on this journey through live and for his belief in me and his endless and loving support and patience.

Additionally, I would like to send a huge thank you to my friends, Andrea, Judith and Andreas, Anna, Merly, Fabian and Faye, Sofia, Felix, Ina and Ida, Florian, Nadja and Pasqual for the fun times we have and for their support in all the important turning points of live.

Summary

In this thesis, we analyzed early born neurons of transit amplifying type-II neuroblast lineages in the central brain of *Drosophila melanogaster*. A subset of these early-born neurons play an important role in building an adult specific neuropil structure called the central complex which is involved in locomotion and visual memory. We studied the embryonic formation of the central complex primordium by type-II neuroblast lineage derived neurons and followed its development through larval live into the adult.

In the first study (Chapter 2), we analyzed the central complex primordium during postembryonic development. First, a genetic driver line was introduced that specifically labels the central complex primordium as well as the neurons which generate this neuropil structure. Second, clonal analysis and immunohistochemistry revealed that the cells which give rise to the central complex primordium are early born, undifferentiated neurons generated by four type-II neuroblasts. Third, flip-out clonal analysis reveald, that these cells innervate the central complex primordium in a highly topologically organized fashion, depending on their lineage affiliation. As a fourth, tracing of these cells through metamorphosis revealed, that these primordium forming cells differentiate and get integrated into the substructures of the adult specific central complex neuropil.

In the second study (Chapter 3), we analyzed the two lateral type-II neuroblast lineages DL1 and DL2 during postembryonic development. First, the DL1 neuroblast was revealed to be a multipotent neuroglial neuroblast which gives rise to adult specific neurons of the central brain and glial cells of the optic lobe. In contrast, the DL2 neuroblast only produces neurons. Second, using Gal4-based clonal lineage tracing, the DL1 derived glial cells were shown to be generated at the end of the second larval instar and their subsequent migration into the optic lobe is delineated. Third, we resolved the adult fate of the DL1 neuroblast lineage derived progeny cells. Clonal labeling revealed that three distinct types of optic lobe glia are produced by DL1, whereas DL1 derived neurons innervate multiple areas of the adult neuropil, one of which is the central complex.

In the third study (Chapter 4), the embryonically born type-II neuroblast lineage derived neurons were investigated by single cell reconstructions undertaken in a serial section electron microscope stack of the early first larval instar brain. First, using light microscopic data as anatomical guide points, we discovered an embryonic origin of the type-II neuroblast derived central complex primordium. Second, single cell reconstructions revealed the specific

topological organization of the primordium to be already present at early first larval instar. Third, morphological analysis revealed that the primordium is made by undifferentiated cells which are devoid of synapses. Fourth, we characterized the embryonic born neurons of one specific type-II neuroblast lineage, revealing other types of undifferentiated neurons as well as a high diversity of differentiated larval functional neurons.

In Chapter 5, the development of the *Drosophila* brain was reviewed in an evolutionary context. The genetic programs involved in brain development as well as complex brain circuits were compared across phyla. Highly conserved neurodevelopmental programs controlled by homologous genes throughout the animal kingdom suggest the last common bilaterian ancestor to have a relatively complex brain.

In summary, type-II neuroblast lineages were shown to produce a high diversity of neurons during embryonic development of *Drosophila melanogaster*. A subset of these neurons gives rise to a neuropil primordium which will develop into the central complex during metamorphosis. The high diversity of functional larval neurons and the production of optic lobe specific glial cells during postembryonic development further emphasize the complexity of these amplifying type-II neuroblast lineages.

Index

1. Introduction

One fundamental question in developmental neurobiology is to understand how a complex brain is built starting from a limited population of multipotent stem cell-like progenitor cells in the embryo. In *Drosophila*, stem cells that produce neurons are called neuroblasts (NB). These NBs are able to divide asymmetrically, thereby producing several progeny cells, all of which remain in close proximity to their NB of origin. This results in the formation of a so called neuroblast lineage, which represents the structural module of the insect brain. All the NB lineages as a whole form the *Drosophila* brain (Hartenstein et al., 2008). The neurons of these lineages are interconnected via synapses, which allow the exchange of information from one to another neuron. Some *Drosophila* neuroblasts were found to resemble the vertebrate neuronal stem cells in their proliferation pattern. Both of these neuronal stem cell types amplify proliferation via intermediate progenitor cells (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Morrison and Kimble, 2006; Brand and Livesey, 2011). These intermediate progenitor cells are able to temporarily divide in a similar way as their neuroblast of origin, therefore generating a NB lineage with more than double the cells found in other canonical NB lineages of *Drosophila*. Based on extensive knowledge accumulated over the last century, *Drosophila* allows easy genetic manipulations and thus has prooven to be an excellent model system to study neural development. In past years, *Drosophila* allowed to investigate the genetic mechanisms involved in the generation of these transit amplifying NB lineages and their intermediate progenitor cells. Furthermore, the morphology of these lineages has been revealed as well as the identity of many neurons within these lineages (for review, see Egger et al., 2008). In this first chapter, we review the most important insights of *Drosophila* neural development with respect to these transit amplifying NB lineages and the brain structures they contribute to.

1.1 *Drosophila melanogaster* neuroanatomy

As it is commonly the case in insects, the *Drosophila* central nervous system (CNS) is a ganglionic structure that comprises the brain and the ventral nerve cord (VNC) (Figure 1.1A, B). In insects, the brain consists of two major subganglions which are distinguishable according to their position to the esophagus that goes right through the neuronal tissue. First, the supraoesophageal ganglion (SPG) can be subdivided into the protocerebral, the deutocerebral, and the tritocerebral neuromere. Second, more ventrally, the suboesophagealganglion (SEG, or

GNG, see Ito et al., 2013) consists of the mandibular, the maxillary and the labial neuromere. In *Drosophila*, the VNC extends posteriorly from the suboesophagealganglion into the body trunk and is further subdivided into three thoracic and eight abdominal ganglia (Campos-Ortega, 1997). Whereas the ventral nerve cord and the suboesophageal ganglion contain the circuits controlling locomotion, flight and feeding, the central brain is mostly a sensory and associative integration center (Figure 1.1A) (Goodman and Doe, 1993; Younossi-Hartenstein 1996, Urbach and Techanu 2003; Truman et al., 2004).

The CNS of *Drosophila* is of a ganglionic type, meaning that the cell bodies of all the neurons are located at the periphery of the brain in the cortex. These neurons send their neurites into the center of the brain towards the neuropil where all the highly branched dendrites and axons of the terminal neurites meet and are interconnected through synapses. As a result, the neuropil is almost free of neuronal cell bodies and hence extraordinarily compact. These synaptic areas build up the different compartments of the neuropil in which incoming sensory information is integrated and the according output response is sent to the periphery (Hartenstein et al., 2008). The only cell bodies found in the neuropil are glial cells that ensheat and delimit the different subcompartments of the neuropil (Figure 1.1C) (Pereanu et al., 2005; Awasaki et al., 2008).

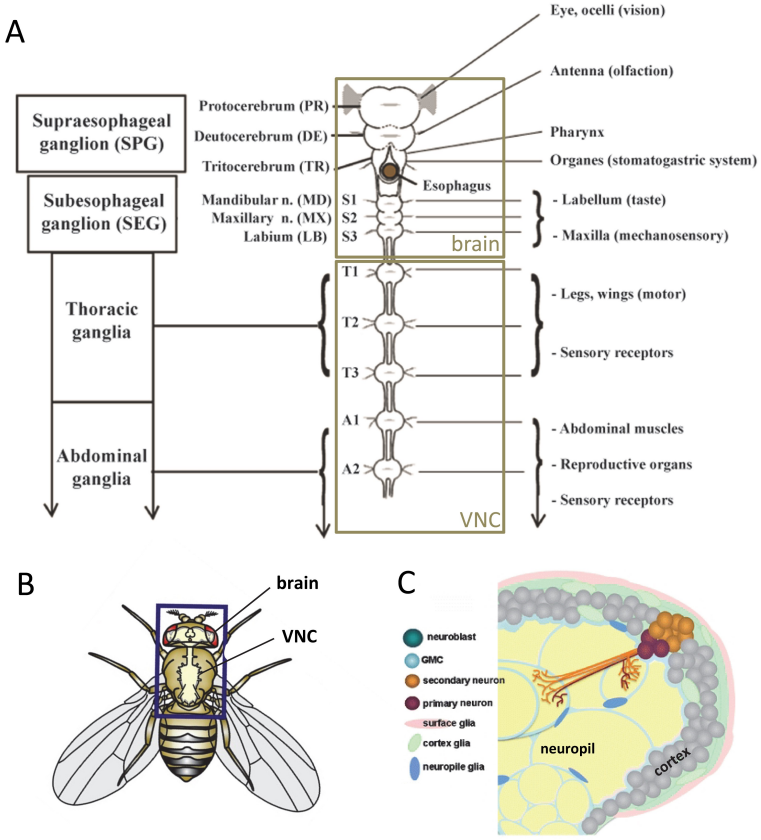


Figure 1.1 Structural organization of the insect CNS and the *Drosophila* brain.

(A) Scheme of the insect CNS illustrating its subdivision into multiple ganglia which further consist of several neuromeres (left side). Innervation and associated functions of CNS regions shown on the right (with complements from Kuert, 2013) **(B)** Sketch of an adult *Drosophila melanogaster*, the brain is framed in a blue box and colored in light beige. Note the localization of the brain in the head capsule whereas the VNC is located in the thorax (adapted from Sánchez-Soriano et al., 2007, with permission from BioMedCentral). **(C)** Sketch of a cross section through one hemisphere of the adult *Drosophila* brain. Cell bodies are located in the peripheral cortex. The neuropil (in yellow) is subdivided into various substructures by glial sheaths (blue). One neuroblast lineage and its projections is shown. At the adult stage, the neuroblast has vanished and the primary as well as the secondary neurons are differentiated, both projecting as one coherent axon tract into specific neuropil compartments (adapted from Spindler and Hartenstein 2010, with permission from Springer).

1.2 Neurogenesis in *Drosophila*

1.2.1 Neuroblast lineages form structural modules

The nervous system of *Drosophila* is formed by a relatively small number of genetically and structurally distinct neuronal cell clusters, the neuronal lineages. Each of these highly invariant lineages is produced by a single stem cell-like neural progenitor called neuroblasts (NB) (Truman and Bate, 1988, Doe, 1992; Schmidt et al., 1997; Lee et al., 1999; Yu et al., 2013; Ito et al., 2013). In total, the *Drosophila* CNS is built up by around 1000 NBs of whom 100 are located in each of the two brain hemispheres (Urbach and Technau, 2004; Technau et al., 2006). Postmitotic neurons produced in a stem cell-like manner by a given NB stay closely together throughout development and extend their projection in a common, lineage specific neurite tract. This feature leads to the formation of a neuroblast lineage where neurons are arranged along a spatio-temporal gradient according to their time of birth. Thus, the NB and the young neurons remain close to the brain surface whereas early born (old) neurons are located deep within the cortex close to the neuropil (Figure 1.1C, Figure 1.2). This clonal organization greatly facilitates the lineage related identification of sibling neurons produced by the same NB and even allows a rough estimate of the time of birth of a given neuron.

During early embryonic development, the NBs delaminate from the bilaterally symmetrical neurectoderm (Urbach and Techanu, 2004; Technau et al., 2006). Studies in the VNC showed that the delamination of NBs follows a stereotyped spatial and temporal pattern, facilitating the identification of individual NBs based on their position (Doe, 1992; Urbach et al., 2003). This highly organized and predictive alignment of NBs is remarkably similar amongst different individual flies and even is comparable to the grasshopper (Zacharias et al., 1993; Urbach and Technau 2003).

In *Drosophila*, closely associated with its holometabolic life cycle, NBs have two proliferative periods. After delamination and during the embryonic period, the NBs undergo a "first neurogenesis" where they start to proliferate in a stem cell- like manner and generate so called primary neurons that differentiate immediately and build up the functional larval central nervous system. These, by default embryonic born, primary neurons (15-20 neurons per neuroblast for most of the lineages), stay closely together in a cluster and their neurites form a coherent bundle called the primary axon tract (PAT) (Nassif et al., 1998; Younossi Hartenstein 2006). The last round of embryonic NB division occurs at embryonic stages 14-15 (Hartenstein et al., 2008). During this embryonic first neurogenesis, the neurons of the primary lineages set up the compartmental map of the brain along which postembryonically born neurons send their axon tracts into their target neuropil compartments (Ito et al., 1997; Larsen et al. 2009).

Around hatching, programmed cell death was reported to induce the apoptosis of 30%-40% of the neurons from the primary lineages (Larsen et al., 2009; Rogulja-Ortmann et al., 2007). At hatching and in the early larval life, neuroblasts undergo a quiescent phase in which they shrink and become mitotically inactive. At this stage, the NB cannot be distinguished by size from neurons (Hartenstein et al., 2008). The entry into quiescence is triggered intrinsically by the same transcription factor cascade that controls neuroblast temporal identity (Isshiki et al., 2001; Tsuji et al., 2008). Most NBs cease embryonic division at stage 14, but the four MB NBs and one lateral NB escape quiescence completely (Prokop and Technau 1991; Ito and Hotta, 1992). Quiescent NBs however endure in this dormant stage until the end of the first larval instar where they awaken and restart their proliferation (Prokop and Technau, 1991; Truman and Bate 1988). This exit of quiescence is triggered by a dietary amino acid threshold which provokes the adipose/hepatic-like fat body to release a signal to glial cells in the brain which subsequently signal the NB to resume proliferation (Chell and Brand 2010; Sousa-Nunes et al., 2011).

With the awakening of the quiescent NBs, the "second neurogenesis" is induced and many immature -so called secondary neurons- are produced. These neurons extend their undifferentiated fasciculated neurites (or secondary axon tracts, SATs) alongside their lineage related PAT and project into the same neuropil compartments. Cell bodies of primary and secondary neurons can be distinguished as primary neurons are larger in diameter, located more deeply in the cortex and are individually wrapped by cortex glia in comparison to secondary neurons which are smaller and are packed more tightly by glia as a group (Pereanu et al., 2005; Larsen et al., 2009). The SATs remain undifferentiated until metamorphosis, where they will enter the neuropil and start to branch and form interconnecting synapses, hence building the functional adult *Drosophila* CNS (Dumstrei et al., 2003; Truman et al., 2004; Zheng

et al., 2006). In addition to the differentiation of secondary neurons during metamorphosis, some primary neurons of the larva are remodeled and integrated into adult specific CNS neuropil structures (Lee et al., 2000; Marin et al., 2005).

Some embryonic born neurons were reported to undergo a different developmental path. First, in the thoracic VNC as well as in the brain, Broad-Z3 expressing neurons are born in the embryo but stay undifferentiated until the onset of metamorphosis (Zhou et al., 2009). Second, the motoneuron MN5, which innervates the adult dorsolongitudinal indirect flight muscle, was reported to be born in the embryo but remains developmentally arrested devoid of synapses until metamorphosis (Consoulas et al., 2002; Ikeda and Koenig, 1988).

In *Drosophila*, in the embryonic brain as well as in the VNC, NB maps were generated based on the individually distinct location and gene expression of each NB (Sprecher et al., 2007; Urbach and Technau 2003; Birkholz et al., 2013; Broadus et al., 1995; Doe, 1992). Furthermore, the invariant trajectories of the SATs as well as the identity coupled location of the NB enabled a complete mapping of all the NB lineages in the late larval brain and thoracic neuromeres as well as in the adult central brain (Dumstrei et al., 2003a, Pereanu and Hartenstein, 2006, Truman et al., 2004; Yu et al., 2013; Ito et al., 2013; Lovick et al., 2013; Wong et al., 2013, Kuert et al., 2014). Despite the extensive knowledge of the individual NB lineage identities at various developmental timepoints, the linkage between the embryonic NB lineages to their corresponding lineage at later stages was not yet made for most of the NB lineages. This is due to the circumstance that the labeling of differentiated primary lineages either with MARCM or flip-out clones revealed to be extraordinary difficult (Larsen et al., 2009). As a consequence, the primary neurons of most of the NB lineages have not yet been described. As one of few exceptions, some studies report the primary lineages of ventral nerve cord NBs in the late embryo by dye-labeling (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). Furthermore, for a few distinct lineages such as the four mushroom body NBs and the five lineages producing olfactory local inter- and projection neurons (in most detail the adPN/Bamv3), the linkage between the embryonically produced primary cells to their corresponding secondary lineage was made and the primary neurons of these lineages described. These studies revealed that primary neurons are individually unique, whereas the secondary neurons of the same lineage are made in blocks with multiple cells acquiring the same fate (Brody and Odenwald, 2000; Kambadur et al., 1998; Kumar et al., 2009; Kunz et al., 2012; Das et al., 2013; Yu et al., 2010). In case of two studies, primary neurons -in contrast to their secondary counterparts- were reported to be of non-intrinsic character meaning that they also innervate outside their postembryonic main target neuropil area. As an example, some

primary neurons of olfactory NB lineages even project into the SEG whereas their secondary counterparts later will not do so and only innervate the antennal lobe and the lateral horn (Kunz et al., 2012; Das et al., 2013; Ramaekers et al., 2005; Thum et al., 2011).

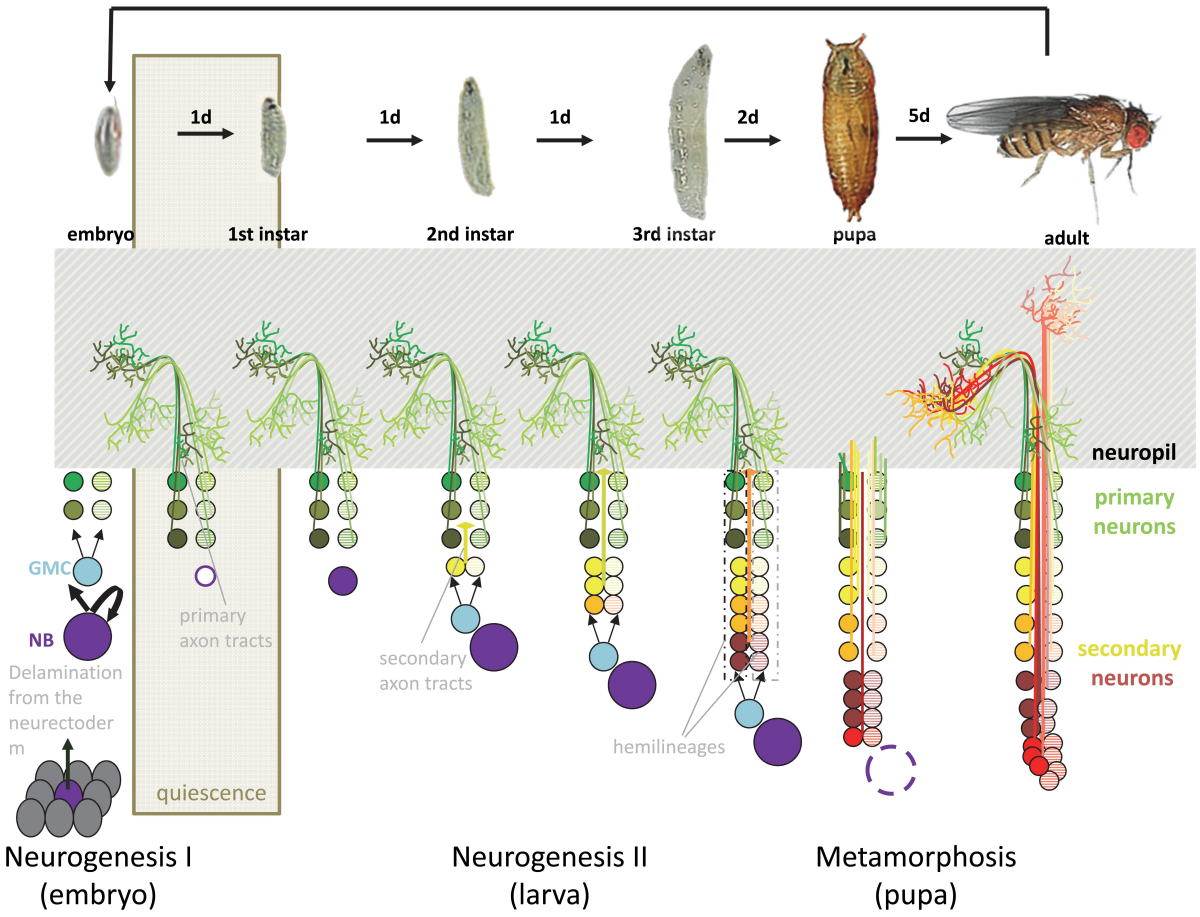


Figure 1.2 *Drosophila* life-cycle and timeline of neurogenesis.
In the embryo, the neuroblast delaminates from the neurectoderm and starts to proliferate, thereby initiating the first neurogenesis which produces all the primary neurons of a NB lineage. At late embryonic stages, the NB enter the quiescent phase and the differentiated primary neurons build up the functional larval brain. The second neurogenesis starts around one day after larval hatching, where the NB resumes proliferation and gives rise to secondary neurons which send their secondary axon tracts along the scaffolding primary axon tracts. During pupal stages, the primary neurons get rewired into the adult circuitry and the secondary neurons differentiate and also form synaptic interconnections. Inspired by unpublished Figure of B. Bello. Fly pictures from FlyMove (Weigmann et al., 2003).

1.2.2 Asymmetric cell division in *Drosophila* neural development

The stem-cell like neuroblasts and their mode of asymmetric proliferation have been studied extensively in *Drosophila* and serve as a model for general stem cell research and tumorgenesis (Bello et al., 2006; Saini and Reichert, 2012; Jiang and Reichert, 2014). During asymmetric cell

division the two sister cells adopt different cell fates such as differences in size, morphology, gene expression or the number of subsequent cell divisions undergone by each of these two sister cells (Horvitz and Herskowitz, 1992). During *Drosophila* neurogenesis, most proliferating NBs undergo sequential cycles of asymmetrical divisions where they self-renew and at the same time produce a smaller daughter cell called ganglion mother cell (GMC). Whereas the renewed NB continues to divide asymmetrically, the GMC then either differentiates into a neuron (Baumgardt et al., 2009; Karcavich and Doe 2005) or divides one last time to produce a pair of post-mitotic cells which then differentiate into neurons (which express Elav), glia (which express Repo) or cells that die prematurely (Truman and Bate, 1988; Schmid et al., 1999; Schmidt et al., 1997; Karcavich and Doe, 2005; Kumar et al., 2009; Lee and Luo, 1999; Lin et al., 2010; Pearson and Doe, 2003; Truman et al., 2010). Most NBs in the *Drosophila* CNS express the proneural gene *ase* as well as the neuroblast self-renewal factor Deadpan (Dpn). During the asymmetric division of the NB, the cell fate determinant Propero gets segregated only into the GMC. The subsequent nuclear localization of Prospero in the GMC leads to a terminal symmetric division of the GMC, thus generating two postmitotic cells which then differentiate (Bello et al., 2008). The NBs that undergo this proliferation mode are called type-I neuroblasts and create neuronal lineages that are made by relatively homogeneous neurons which all project in one, maximum two stereotyped axon tracts to their target area within the neuropil (Figure 1.3A) (Pereanu and Hartenstein 2006).

1.2.3 Type-II neuroblast lineages

In vertebrates, neural stem cells such as radial astrocytes or radial glia produce transient amplifying intermediate neural progenitor cells (INPs) that undergo a limited number of proliferative divisions before they divide terminally to generate a neuron and a glial cell (Kriegstein et al., 2009; Pontious et al., 2008). Mutations of genes that are associated with the generation of INPs have been linked to severe microencephaly and cortical malformation in humans (Baala et al., 2007).

Remarkably, in the *Drosophila* protocerebrum, eight NBs per hemisphere were found to produce transit-amplifying intermediate precursor cells (INPs) whos proliferation pattern resembles the one found in vertebrate radial ascrocytes and glia. Six of these type-II NB lineages, called DPMm1 (DM1), DPMpm1 (DM2), DPMpm2 (DM3), CM4 (DM4), CM3 (DM5), and CM1 (DM6) are located in the dorso-posterior medial area of the brain close to the midline. Two additional type-II NB lineages are located more laterally in the center of the brain hemispheres (Figure 1.3C) (Izergina et al., 2009; Bayraktar et al., 2010). These type-II NBs generate INPs via

asymmetric cell divisions. After a maturation process these INPs themselves undergo limited rounds of self-renewal divisions subsequently creating type-II NB lineages of more than four times as many neurons compared to type-I NB lineages (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Furthermore, type-II NBs were reported to be neuroglioblasts that, in addition to producing neurons, which innervate a defined neuropil structure, also produce glial cells that migrate into the same neuropil compartment and ensheat this area (Izergina et al., 2009, Viktorin et al., 2011). In contrast to type-I NB lineages, the lineages produced by type-II NBs are highly elaborate in their morphology and have more complex projections innervating various neuropil compartments (compare Figure 1.3A', with B').

In recent years several genes involved in the formation and specification of these type-II NB lineages and their INPs have been identified. A type-II NB can be unambiguously distinguished from a type-I NB by its Dpn and Pnt expression and the lack of the expression of the proneural gene *ase* (Figure 1.3 B) (Zhu et al., 2011; Boone and Doe 2008; Bowman et al., 2008; Zhu et al., 2012). During their repeated rounds of asymmetric division, the type-II NBs self-renew, but instead of producing a GMC they give rise to an INP that is also Ase^- and temporarily arrested in cell cycle progression (Bowman et al., 2008). Whereas the expression of self-renewal proteins -such as Dpn- is maintained in the NB, their expression becomes rapidly extinguished in the newly born immature INP (Xiao et al., 2012). The transcription factor PointedP1 is exclusively expressed in type-II NBs, immature INPs and newly mature INPs and is the key molecule that suppresses Ase expression in the NB and with this promotes the generation of INPs (Zhu et al., 2011). During the NB division, the lack of asymmetrical segregation and then nuclear localization of the cell fate determinant Prospero into the INP cell enables its self-renewal capacity (Bello et al., 2008). The newly generated immature INP then undergoes a maturation process to adopt the functional identity of an INP (Bowman et al., 2008; Xiao et al., 2012; Bayraktar et al., 2010). During maturation, the transcription factor *earmuff* gets turned on and restricts the proliferation capacity of the INP by activating Prospero. *Erm* expression also prevents the de-differentiation of INPs back into type-II neuroblasts by diminishing the competence of the INPs to respond to neuroblast self-renewal factors (Weng et al., 2010; Bayraktar et al., 2010; Janssens et al., 2014). With the maturation of the INP, the expression of *ase* and –amongst others- the self-renewal factor Dpn is reactivated whereas the expression of *pointed* and *earmuff* fades (Boone and Doe 2008; Zhu et al., 2011; Janssens et al., 2014). The restrictive susceptibility of the INP to the self-renewal factors just allows a limited number of 4-8 divisions, therefore preventing the adoption of a tumor stem cell-like identity and overproliferation (Bello et al., 2008; Boone and Doe 2008; Bowman et al., 2008; Homem et al., 2013). During the subsequent morphologically symmetric division of the INP, Prospero gets

segregated asymmetrically in only one of the two daughter cells which will become the GMC. As seen in type-I NB lineages, the successive nuclear localization of the Prospero protein in the GMC allows only one terminal division of the GMC producing two postmitotic progeny-cells which in the case of type-II NB lineages can become neurons and/or glial cells.

Based on the amplification of cell number as well as cell diversity by the INP, type-II NB lineages form axon tracts which split into several subsidiary tracts as soon as they reach the brain neuropil (Bayraktar et al., 2010). This stands in contrast to the type-I NB lineages where generally all the cells of one NB lineage contribute to a single axon fascicle or a maximum of 2 tracts that project into the neuropil (Truman et al., 2010; Lin et al., 2010, Pereanu and Hartenstein 2006). In addition to the amplification of proliferation via INPs in type-II NB lineages, approximately a quarter of the produced cells undergo programmed cell death during larval stages, suggestively to prevent INP based overproliferation (Jiang and Reichert 2012). Postembryonic type-II NB lineages have been described exhaustively and the lineages and their projections are known in the larva as well as in the adult (Izergina et al., 2009; Pereanu and Hartenstein 2006; Yu et al., 2013; Ito et al., 2013; Lovick et a., 2013; Wong et al., 2013). For the DPMm1 lineage a detailed single-cell analysis of the first two INP clones generated postembryonically showed that sibling INPs produce a morphologically similar but temporally regulated series of distinct neuron types (Wang et al., 2014).

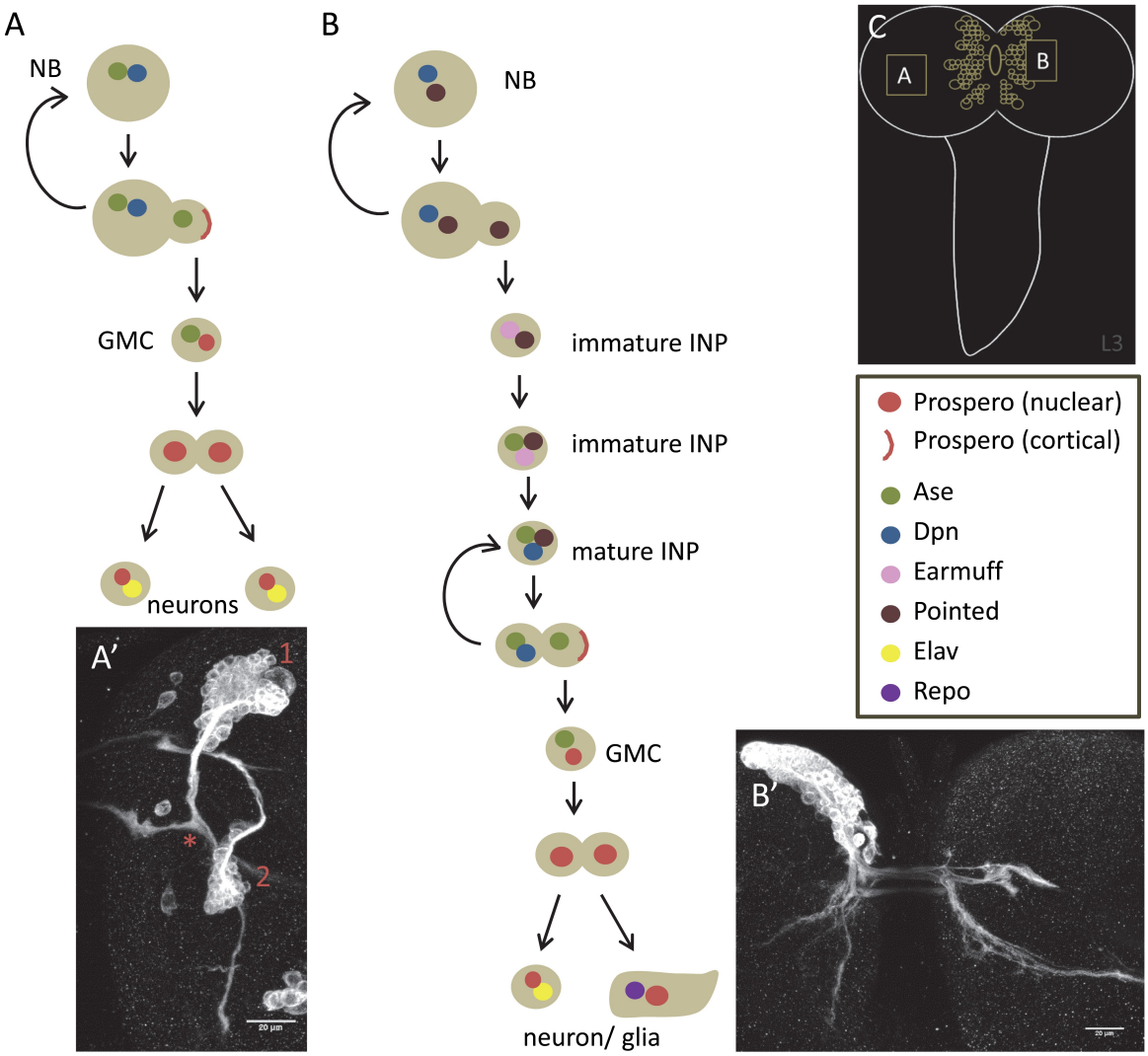


Figure 1.3 Type-II NB lineages amplify their proliferation via intermediate progenitor cells (INPs).
(A) Ase-positive type-I NBs give rise to a GMC via asymmetric division in which Prospero is located cortically in the GMC. Prospero enters the nucleus in the GMC, which leads to the terminal division of the GMC and hence the generation of two post mitotic cells. **(A')** Confocal microscope data of two postembryonic type-I NB lineage MARCM clones. The secondary axon tracts project into the neuropil as a coherent bundle where they bifurcate once at the most (see asterisk of NB clone 1). **(B)** The eight Ase-negative type-II NB lineages per hemisphere express the gene *pointed* and divide asymmetrically to give rise to a transit amplifying intermediate progenitor cell (INP). During the maturation process, the Earmuff-positive INP will turn on *dpn* and *ase* and divide symmetrically for a limited amount of divisions. During division, the INP segregates Prospero asymmetrically into the GMC which divides terminally into two postmitotic cells. Depending on their identity, postmitotic cells express different genes, e.g. *repo* in glial cells and *elav* in neurons (see text for more details). **(B')** MARCM clone as revealed by confocal microscopic analysis of the postembryonic type-II NB lineage DPMm1. At the transition from the cortex into the neuropil, the SAT splits into multiple neuronal fascicles that project ipsi- and contralaterally into various neuropil areas of the third larval instar brain. **(A', B')** Z-projection of multiple optical sections. **(C)** [B] Localization of the six dorso-medial type-II NB lineages as compared to the more abundant type-I NB lineages [A] in the third larval instar brain.

1.3 Generating neuronal diversity

In addition to each NB and its lineage having a different identity, also the neurons within a given NB lineage are phenotypically divers depending on their time of birth. The genetic mechanisms involved in the production of such complex NB lineages of diverse neurons are subject of great interest. So far, four primary mechanisms have been reported to enable neuronal diversification. First, there are two mechanisms by which a parental NB can produce a progeny cell of a different identity; through intrinsic and extrinsic mechanisms. During mitosis, the intrinsic mechanism ensures asymmetrical localization of self-renewal regulator proteins only in one daughter NB. The lack of these self-renewal regulator proteins enables the other daughter cell to adopt a different fate and in the case of the GMC to differentiate (Betschinger et al., 2004; Yu et al., 2006). Alternatively, in case of extrinsic regulation, the stem cell is closely located within a stem cell niche. This niche is responsible for maintaining the potential to self-renew, enabling the daughter cells which are outside the niche to differentiate into a given identity (Xie et al., 2005). Whilst extrinsic niche mechanisms are more common in adult stem cells such as ovarian stem cells, the intrinsic mechanisms are more common in the NBs involved in *Drosophila* nervous system development. Second, each NB in the CNS has an unique identity due to the expression of a specific combination of molecular markers which dictate the number and types of neurons produced by this particular NB (Doe, 1992; Broadus et al., 1995; Urbach and Technau, 2003a; Yu et al., 2010; Hassan et al., 2000; Noveen et al., 2000; Lichtneckert et al., 2007, 2008; Kurusu et al., 2009; Sen et al., 2014). As a third mechanism to create neuronal diversity, NBs sequentially express a cascade of different transcription factors, which specify the temporal identity of their neuronal progeny (Isshiki et al., 2001). Therefore, a given NB produces different types of neurons in an invariant sequence depending on their birth order within the lineage (Li et al., 2013; Kao et al., 2012; Bayraktar and Doe, 2013). In a similar fashion, studies in the vertebrate CNS have shown, that the birth order of cells correlates with their distinct neuronal/ glial identity (Jacob et al., 2008; Maurange, 2012; Pearson and Doe, 2004). As a fourth, during its terminal division, a GMC produces two postmitotic neurons of different identity. This binary cell fate specification is accomplished by the Notch/Numb pathway and leads to a further increase in cell diversity within a NB lineage. Added up to a NB lineage as a whole, this Notch/ Numb dependent identity leads to the formation of hemilineages, which have different projection destinations depending on whether the particular hemilineage expresses either Notch or Numb. In some cases one hemilineage is removed completely by programmed cell death (Truman et al., 2010; Lin and Lee 2012; Lin et al 2010; Kumar et al., 2009). Taken together, each neuron has a distinct identity due to its NB lineage origin, its temporal identity based on the time of birth and the Notch or Numb binary cell fate.

1.4 The central complex in *Drosophila*

The insect brain neuropil consists of various compartments which are delimited from each other by thin glial sheets. Some well-studied examples of these neuropil structures are the mushroom bodies, the antennal lobes and the central complex (Boyan and Williams, 2011; Strausfeld 1976; Young and Armstrong 2010a +b). The central complex was reported to be fairly similar in its overall structure amongst various insect species (Figure 1.4A, B) (Loesel et al., 2002; Strausfeld, 2009). In adult *Drosophila*, the central complex (CC) is prominently located in the midline neuropil of the protocerebrum and plays essential roles in locomotor- and flight control as well as visual pattern-, and spatial orientation memory (Figure 1.4A) (Martin et al., 1999; Strauss and Heisenberg, 1993; Strauss, 2002; Triphan et al., 2010; Neuser et al., 2008; Pan et al., 2009; Poeck et al., 2008).

The CC consists of four substructures including from caudal to rostral the protocerebral bridge (PB), the fan-shaped body (FB), the ellipsoid body (EB) and paired noduli (NO). The PB is located near the dorsoposterior border of the cortex and the neuropile, displaying a curved handlebar-like shape (Hanesch et al., 1989). The FB is a cup-shaped structure whose convex border points dorsoposteriorly toward the PB, whereas the concave anterior side partially encloses the doughnut-like EB. The FB neuropile shows a rectangular array of horizontal layers and vertical segments, whereas the EB ring can be subdivided into multiple concentric zones. Right underneath the FB and EB lie the paired NO (Hanesch et al., 1989; Young and Armstrong, 2010a). Additionally, two laterally adjacent neuropil regions called the bulbs (BU) and the lateral accessory lobe (LAL) are closely associated to the CC in each brain lobe and -together with the PB- are believed to be the main hubs for CC inputs and possibly outputs too (Hanesch et al., 1989; Li et al., 2009; Young and Armstrong, 2010a; Homberg 1994).

Neurons innervating the CC have so far been divided into two categories. So called small-field neurons are intrinsic to the central complex and connect small domains of several CC substructures. In contrast, large-field neurons typically arborize in only a single substructure of the CC and link it to one or two central brain regions outside the central complex (Young and Armstrong 2010a). Research on the lineage affiliation of CC neurons revealed that a total of 15 NB lineages produce postembryonic born neurons that innervate the central complex. Amongst these are seven type-II NB lineages, DPMm1, DPMpm1, DPMpm2, CM4, CM3, CM1 and DL1. The four most dorsoposterior medial type-II NB lineages, DPMm1, DPMpm1, DPMpm2 and CM4 jointly make most types of small-field neurons and innervate all the substructures of the central complex and its accessory compartments in small domains (Yang et al., 2013; Izergina et al.,

2008; Bayraktar et al., 2010). As a contrast, CM3, CM1 and DL1 give rise to large-field neurons that innervate the CC substructures in a broader way and connect it to neuropil structures outside the CC (Yang et al., 2013).

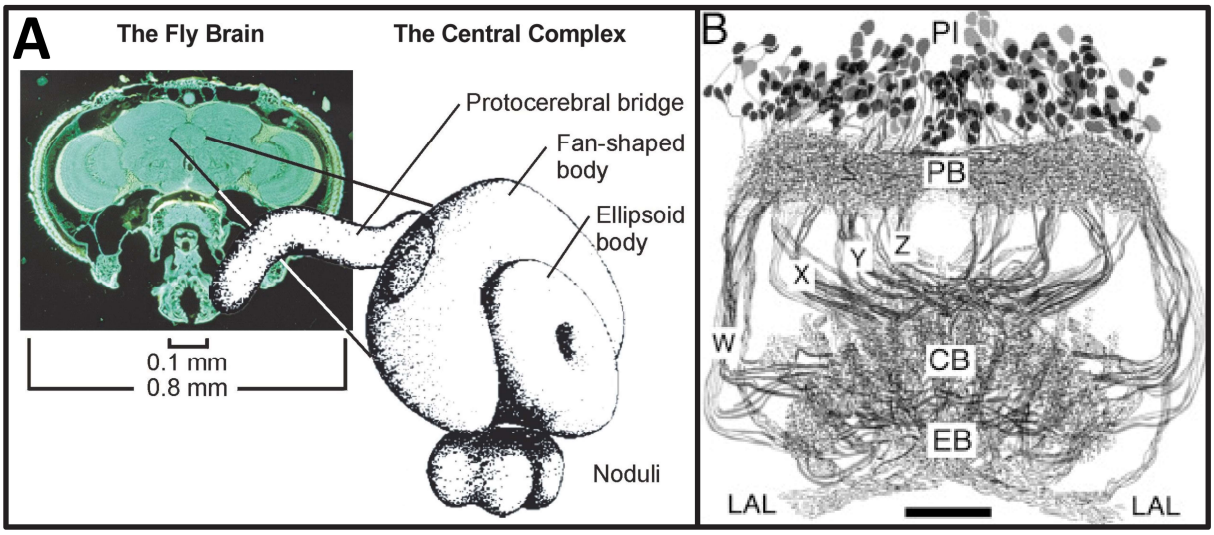


Figure 1.4 The central complex of *Drosophila melanogaster* and *Schistocerca gregaria*
(A) The central complex is located at the midline of the neuropil and dorsal of the esophagus in the *Drosophila* adult. The central complex comprises four interconnected substructures; the ellipsoid body, the fan-shaped body, the noduli and the protocerebral bridge arranged from caudal to rostral in the adult fly brain. Figure A from Strauss R., 2002, "the central complex and the genetic dissection of locomotor behavior in the Drosophila brain" Current Opinion in Neurobiology 12(6): 633-638, with permission from Elsevier. **(B)** Drawing shows the central complex of the locust *Schistocerca gregaria*, which is also subdivided into the protocerebral bridge (PB), the central body (CB) and the ellipsoid body (EB). The central complex of *Schistocera* is generated by neurons derived from eight neuroblast lineages located in the pars incerebralis (PI). In each hemisphere, four NB lineages send their axon tracts (w, x, y, z), to innervate the central complex substructures. (LAL) Lateral accessory lobe. Figure B from Boyan and Williams, 2011, "Embryonic development of the insect central complex: insights from lineages in the grasshopper and Drosophila." Arthropod structure & development 40(4):334-348, with permission from Elsevier.

In hemimetabolous insect species such as grasshoppers, cockroaches and crickets, the central complex develops during embryogenesis and has an adult-like morphology at larval hatching (Panov, 1959). In contrast, most holometabolous insects do not possess any obvious central complex neuropil at larval hatching but first appearance of primordial structures of the protocerebral bridge and the fan-shaped body were reported at late larval stages. As exeptions, some moths and beetles already have a fan-shaped body in which neuroactive substances are expressed, suggesting that they are larval functional (Granger et al., 1989; Wegerhoff and Breidbach, 1992; Wegerhoff et al., 1996). In no holometabolous insect investigated to date was a larval ellipsoid body neuropil found (Ridgel et al., 2007; Homberg and Hildebrand, 1994). The relatively early appearance of a putatively functional fan-shaped body in the larvae of moths

and beetles was suggested to be caused by the presence of legs at larval stages. Therefore, the lack of a functional FB in *Drosophila* is in agreement with the lack of legs during larval stages (Panov et al., 1956).

The CC substructures of the adult *Drosophila* brain can first be seen with neuropil markers at late third larval instar (PB and FB), respectively 12h (NO) and 24h (EB) after pupal formation (Young and Armstrong 2010b). To which extent a corresponding larval CC exists is currently unknown. Due to the relative simple crawling lifestyle as well as restricted visual input during larval stages of *Drosophila*, a complex neuropil center such as the CC might not yet be needed in the larva. However, the larva too has to integrate various sensory inputs and react in an according crawling motion sequence that requires basic neuronal integration. Furthermore, *Drosophila* mutants that were originally isolated for their adult CC abnormalities also showed locomotor defects in the larva speaking for the existence of some representative larval CC structure (Varnam et al., 1996).

Recently, a deep homology was suggested between the vertebrate basal ganglia and the arthropod central complex. In addition to sharing comparable organizational features of neural circuitry, these brain structures also have comparable functional roles in sensorimotor integration as well as in affective behavior. Furthermore, during development, the genesis and specification of these brain structures share genetic programs that involve homologous genes which have comparable expression patterns and fuction (Strausfeld and Hirth, 2013).

1.5 Glia in the *Drosophila* brain

Neuronal cells in the *Drosophila* larval CNS are supported by a complex scaffold of glial cells that is established during late embryonic stages. Glia not only establish and maintain the blood-brain-barrier but also play an essential role in the structural support of neurons, as well as their wrapping and electrical insulating (Jones, 2001; Carlson et al., 2000; Klämbt, 2009). In insects, three types of glia can be distinguished. As a first, the surface glia form a sheath around the surface of the brain and act as the blood-brain barrier (Ito et al., 1995; Carlson et al., 2000). Second, cortex glia are located within the brain cortex and form a scaffold that encapsules neuronal cell bodies, ganglion mother cells and neuroblasts. Third, neuropil glia surround the neuropil and ensheath the individual neuropil compartments as well as major tracts of neurites (Younossi-Hartenstein 2003; Pereanu et al., 2005, Awasaki et al., 2008). Glia play numerous roles in axon pathfinding and targeting during *Drosophila* brain development (Hidalgo, 2003;

Pielage and Klämbt, 2001; Spindler et al., 2009). In the VNC of the *Drosophila* embryo, various type-I neuroglioblasts were described (Beckervordersandforth et al., 2008; Jacobs et al., 1989a+b; Klämbt and Goodman, 1991; Klämbt et al., 1991; Bossing et al., 1996; Broadus et al., 1995). Most of the adult specific glia cells are generated during the second half of larval development, which is in part due to mitotic divisions of glial cells themselves (Pereanu et al., 2005; Viktorin et al., 2011). In the postembryonic central brain of *Drosophila*, five type-II neuroblasts were found to be in fact neuroglioblasts that produce adult specific glia. These glia migrate towards the CC subcompartments which they subsequently ensheath and delimit. Interestingly, these CC substructures are in part formed by the neurons born by the same type-II NB lineages (Izergina et al., 2009; Viktorin et al., 2011).

Glia cell differentiation in the *Drosophila* embryo is initiated by the transiently expressed transcription factor *glial cell missing* (*gcm*). Potential target genes of *gcm* are *reversed polarity* (*repo*), *pointed* (*pnt*), *tramtrack* (*ttk*) and *loco* genes. The transient expression of *gcm* is followed by the maintained *repo* expression in differentiated glial cells. Similarly, *pointed* is also involved in glia cell differentiation and is required for the expression of various glial markers (Hosoya et al., 1995; Vincent et al., 1996; Miller et al., 1998; Campbell et al., 1994; Klämbt and Goodman, 1991).

1.6 The *pointed* gene in *Drosophila* development

The *pointed* (*pnt*) locus codes for two protein isoforms, PntP1 and PntP2 (Klämbt, 1993). Both of these isoforms are members of the ETS DNA-binding domain transcription factor activator proteins executing numerous roles during *Drosophila* development. PntP2 is a downstream target of the RTK/Ras/MAPK (mitogen-activated protein kinase) signaling pathway and requires MAPK phosphorylation for its activation (Rebay et al., 2000; O'Neill et al., 1994). Furthermore, PntP2 is conserved to its mammalian homologue Ets-1 (Wasylyk et al., 1997). As a contrast, Pntp1 constitutively active (O'Neill 1994).

The involvement of *pointed* in the *Drosophila* development is manifold. Pnt has been reported to be required in ventral neurectoderm specification (Chang et al., 2001, Gabay et al., 1996), in oogenesis (Morimoto et al., 1996; Schober et al., 2005), as well as in muscle and heart development (Halfon et al., 2000; Alvarez et al., 2003). Furthermore, *pointed* is involved in cell migration at the tip of the dorsal air sac outgrowth during tracheal development at late larval stages (Cabernard and Affolter, 2005).

During embryonic development, Pnt is required for glial differentiation as well as neuronal-glial cell interactions at the ventral midline. Loss of function mutations lead to a change in the migration behavior of midline glia cells and subsequently to the fusion of the anterior and posterior commissures (Klaes et al., 1994; Klämbt 1993). During eye development *pointed* is involved in several processes via the RTK/Ras/MAPK signaling cascade. In the developing ommatidium, *pointed* induces *hedgehog* expression posterior to the morphogenetic furrow in all developing photoreceptor cells except R8 and therefore enables the recruitment of the future ommatidial cells R1-R7 (Rogers et al., 2005; Ready et al., 1976; Tomlinson, 1985, 1988; Wolff and Ready, 1991; Voas and Rebay, 2004). Furthermore, during photoreceptor R7 cell differentiation, Ras pathway induction via Sevenless receptor tyrosin kinase leads to the phosphorylation of MAPK, which subsequently enters the nucleus and activates the Ets transcription factors Pointed and Yan (O'Neill et al., 1994; Dickson, 1995; Rubin et al., 1997). Via this pathway, MAPK activated *pointed* specifies the photoreceptor R7 fate by activating *prospero* and thus preventing the cell from becoming a cone-cell (Brunner et al., 1994; Treisman, 2013).

Pointed is also involved in the clustering of the chordotonal sense organ cells as a response to the initiation of neural aggregation by the sensory organ precursor (Lage et al., 1997).
In the *Drosophila* central brain, PntP1 has been reported to be specifically expressed in the type-II NBs, immature INPs and newly mature INPs. In the NB, *pointed* expression is sufficient for the suppression of *ase* and consequently enables the generation of INPs in these lineages, assigning a type-II identity to the NB. Consequently, loss of Pointed leads to the reduction or elimination of INPs and additionally to the ectopic expression of *ase* in type-II NB lineages (Zhu et al., 2011).

1.7 The *pointed* gene in vertebrates

In vertebrates, the Ras/MAPK pathway also plays a central role in transducing extracellular signals to intracellular target proteins involved in cell growth and proliferation. As in *Drosophila*, the vertebrate homologue of *pointed*, Ets (v-ets erythroblastosis virus E26 oncogene), is part of this signaling pathway and gets phosphorylated by MAPK (Wasylyk et al., 1996; Albagli et al., 1996). Inappropriate regulation of the ETS transcription factor or the Ras/MAPK pathway leads to a variety of disorders and diseases, including many types of cancers (Wallace et al., 2013; Múnera et al., 2011; Meloche and Pouyssegur, 2007; Yoon and Seger, 2006; Rubinfeld and Seger, 2005; Roux and Blenis, 2004).

1.8 This Thesis

This thesis focusses on the transit amplifying type-II neuroblast lineages of the central brain of *Drosophila*. Previous research contributed comprehensive cellular and molecular data about the development of type-II neuroblast lineages. However, those studies mainly focused on the postembryonic stages of *Drosophila* brain development. A subset of the dorsomedially located transit amplifying type-II neuroblast lineages was shown to be multipotent, giving rise to both glial cells and a high diversity of neurons. Both glia and neurons of these lineages make major contributions to the development of an adult midline neuropil structure called the central complex. Although the central complex primordium was previously described morphologically, the lineal affiliation of the cells giving rise to this neuropil structure was unknown. Furthermore, in contrast to the detailed knowledge about postembryonic stages of type-II neuroblast lineage development, nothing was known about the cells generated by these NB lineages during embryogenesis.

The first part of this thesis reveals the neuroglial nature of a lateral type-II neuroblast lineage and describes the neurons and glial cells produced by this lineage. In contrast to the glial cells produced by dorsomedially located type-II neuroblast lineages, the glial cells of this lateral type-II neuroblast lineage migrate into the optic lobe and differentiate into three different types of glial cells. This is the first report of glial cells generated in the central brain that later contribute to the optic lobe.

Two additional chapters of this thesis describe the cells which give rise to the central complex primordium. Analysis of the early larval brain reveals that type-II NB lineage derived neurons which are born in the embryo contribute to the central complex primordium. These neurons display an undifferentiated morphology. This reveals a novel type of embryonic born cells in the central brain of *Drosophila* which differ from the differentiated primary neurons that assemble the functional larval brain at this stage. We show that the central complex primordium is generated by four type-II neuroblast lineages, and that additional undifferentiated neurons are added to the structure during postembryonic stages. During metamorphosis, the neurons of the central complex primordium differentiate and form synaptic interconnections, thereby integrating into the central complex substructures of the adult fly. Furthermore, focusing on one specific type-II neuroblast lineage, we present the embryonic born differentiated as well as the undifferentiated neurons on a single cell basis. This study reveals novel insights into the ultrastructural characteristics of individual embryonic born neurons generated by a type-II NB lineage.

2. Early-born neurons in type II neuroblast lineages establish a larval primordium and integrate into adult circuitry during central complex development in *Drosophila*

Nadia Riebli[1], Gudrun Viktorin[1], Heinrich Reichert[1]

[1]Biozentrum, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland

Accepted manuscript, Neural Development 2013, 8:6
doi:10.1186/1749-8104-8-6
published by BioMed Central Ltd.

2.1 Summary

The central complex is a multimodal information-processing center in the insect brain composed of thousands of neurons representing more than 50 neural types arranged in a stereotyped modular neuroarchitecture. In *Drosophila*, the development of the central complex begins in the larval stages when immature structures termed primordia are formed. However, the identity and origin of the neurons that form these primordia and, hence, the fate of these neurons during subsequent metamorphosis and in the adult brain, are unknown.

Here, we used two *pointed-Gal4* lines to identify the neural cells that form the primordium of the fan-shaped body, a major component of the *Drosophila* central complex. We found that these early-born primordium neurons are generated by four identified type II neuroblasts that amplify neurogenesis through intermediate progenitors, and we demonstrate that these neurons generate the fan-shaped body primordium during larval development in a highly specific manner. Moreover, we characterize the extensive growth and differentiation that these early-born primordium neurons undergo during metamorphosis in pupal stages and show that these neurons persist in the adult central complex, where they manifest layer-specific innervation of the mature fan-shaped body.

Taken together, these findings indicate that early-born neurons from type II neuroblast lineages have dual roles in the development of a complex brain neuropil. During larval stages they contribute to the formation of a specific central complex primordium; during subsequent pupal development they undergo extensive growth and differentiation and integrate into the modular circuitry of the adult brain central complex.

2.2 Introduction

The highly complex circuitry of the *Drosophila* central brain is established in two developmental steps. The first step takes place during embryogenesis and gives rise to the relatively simple brain of the larva; the second step takes place during postembryonic larval and pupal development and results in the formation of the much more complex mature brain of the adult. Both the embryonically generated neural cell populations that make up the larval brain and the postembryonically generated neural cell populations that form the bulk of the adult brain develop from a set of approximately 100 neural stem-cell-like neuroblasts that derive from the cephalic neuroectoderm in the early embryo (reviewed in Urbach and Technau, 2004; Technau et al., 2006; Hartenstein et al., 2008; Egger et al., 2008).

During embryogenesis, these neuroblasts undergo a first series of stem-cell-like proliferative divisions in which they divide in an asymmetric manner to self-renew and produce secondary precursors, which give rise to postmitotic neural progeny (reviewed in Skeath and Thor, 2003; Doe, 2008; Knoblich, 2008). At the end of embryogenesis, most neuroblasts enter a phase of quiescence, which separates the primary embryonic phase from the subsequent secondary postembryonic phase of neurogenesis (Egger et al., 2008; Isshiki et al., 2001; Tsuji et al., 2008). Most neuroblasts in the central brain resume proliferation during early larval stages in response to factors involving nutritionally activated mitogens and glial-cell-dependent interactions (Chell and Brand, 2010; Sousa-Nunes et al., 2011). The neural cells produced postembryonically during the larval phase differentiate in the subsequent pupal phase and contribute to the functional adult brain circuits (Hartenstein et al., 2008; Truman and Bate, 1988; Prokop and Technau, 1991; Truman et al., 2004; Pereanu and Hartenstein, 2006).

Recent studies have shown that two different types of neuroblast lineages are present in the central brain of *Drosophila* (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008; Reichert, 2011; Chang et al., 2012). Most central brain neuroblasts are type I, and they give rise to lineages that contain on average 100 to 150 cells. These type I neuroblasts divide asymmetrically to self-renew and produce a non-self-renewing progenitor called a ganglion mother cell (GMC), which only divides once to generate two postmitotic neural progeny, neurons or glial cells. In contrast, a small set of neuroblasts in the central brain (8 in each hemisphere) are type II and they give rise to remarkably large lineages averaging 450 cells. These type II neuroblasts divide asymmetrically to self-renew and produce a self-renewing secondary progenitor called an intermediate neural progenitor (INP). This INP acts as a transit amplifying cell which retains its ability to divide several more times and hence can give rise to numerous GMCs each of which divides to produce two neural cells. In consequence, a marked amplification of proliferation takes place in the type II neuroblast lineages.

Clonal analysis of the numerous neuronal progeny generated by each of the type II neuroblasts during postembryonic development indicates that the adult-specific secondary neurons in these lineages form complex and widespread longitudinal and commissural projections in the brain. Furthermore they also demonstrate that a subset of these secondary neurons form major arborizations in all of the compartments of the central complex neuropil (Izergina et al., 2009; Bayraktar et al., 2010; Jiang and Reichert, 2012; Boyan and Reichert, 2011). The central complex of the adult brain is a prominent midline neuropil in the protocerebrum that is involved in multimodal information processing and memory as well as in coordination of motor control in

locomotory behaviors (Strauss and Heisenberg, 1993; Strauss, 2002; Liu et al., 2006). It is composed of thousands of neurons representing more than 50 neural types that are arranged in a stereotyped modular neuroarchitecture in all insects (Hanesch et al., 1989; Loesel et al., 2002; Strausfeld, 2009; Young and Armstrong, 2010a). In *Drosophila*, its principle component modules are referred to as the protocerebral bridge, the fan-shaped body, the ellipsoid body, and the (paired) noduli. All of these modular structures receive major innervation from neurons belonging to type II lineages. Indeed, the amplification of proliferation that characterizes type II neuroblast lineages is thought to be an important factor in generating the enormous number of central complex neurons during the relatively short period of postembryonic secondary neuron proliferation (Boyan and Reichert, 2011). Interestingly, and possibly counterbalancing this amplified proliferation, elimination of excess neurons in these lineages through programmed cell death is required for the formation of correct innervation of the developing central complex neuropil (Jiang and Reichert 2012).

The development of the central complex begins in larval stages, when immature structures termed primordial are formed in a symmetrical manner on either side of the brain midline (Young and Armstrong, 2010b). In early larval stages, the brain midline is formed by numerous thin fascicles that make up the nascent supraesophageal commissure and additional fascicles are added during larval development (Nassif et al., 2003). At the third larval instar stage, but not at the preceding second larval instar stage, primordia of the immature fan-shaped body and the immature protocerebral bridge can be identified using global markers such as DN-cadherin (Pereanu and Hartenstein, 2006; Young and Armstrong, 2010b). However, the identity and origin of the neurons that form these primordia are unknown. Correspondingly, there is no information concerning the fate of these primordium-forming neurons during subsequent metamorphosis in pupal stages and in the adult brain. Thus, although the contribution of secondary neurons from type II lineages to the central complex neuropil has been investigated in some detail, nothing is known about the role of any type II neurons in the formation of the central complex primordia or in the subsequent development of these primordium neurons during central complex development and in the adult.

In the present work, we took advantage of the fact that the P1 isoform of the Ets transcription factor Pointed (Pnt) is specifically expressed in type II lineages (Zhu et al., 2011). We analyzed this type II lineage-specific expression using a *pntP1-Gal4* line to drive reporter gene expression in the late larval brain and observed that recently born adult-specific neurons as well as a set of early-born neurons that innervate the fan-shaped body primordia are labeled. Moreover, we used embryonically induced flip-out methods to demonstrate that the primordium-forming

neural cells are generated by four identified type II lineages DM1, DM2, DM3, DM6 (Izergina et al., 2009). We then screened a collection of *pnt* enhancer-fragment *Gal4* lines (Jenett et al., 2012) and show that the *R45F08-Gal4* line targets reporter gene expression specifically to this population of early-born neurons. Using this specific genetic access we found that these type II neurons generate the primordium of the central complex during larval development in a highly specific and exclusive manner, and we show that this bilaterally symmetric larval primordium already manifests the type of modular subdivision that characterizes the mature central complex. Finally we used the *R45F08-Gal4* driver to document the extensive growth and differentiation of these early-born primordium neurons that occurs during the development of the (unpaired) central complex in pupal stages. Furthermore we show that these neurons persist in the adult central complex where they manifest a layer-specific innervation of the mature fan-shaped body as well as innervation of the ellipsoid body and protocerebral bridge.

2.3 Materials and Methods

2.3.1 Fly strains and genetics

Unless stated otherwise, fly stocks were obtained from the Bloomington Drosophila Stock Centre (Indiana University, Bloomington, IN, USA) and maintained on standard cornmeal medium at 25°C. For visualizing type-II neuroblast lineages *w^{1118}, gcm-lacZ^{rA87}, UAS-mCD8::GFP^{LL5}/CyO,actin-gfp^{JMR1}*; *R09D11-Gal4* (*GMR09D11*; (Pfeiffer at al, 2008)) recombined flies were crossed to *R09D11-Gal4*, *UAS-mCD8::GFP^{LL6}* flies. To generate wild type MARCM clones (Lee and Luo, 1999), we mated female *y, w, hs-Flp^{1}; tubP-Gal4, UAS-mCD8::GFP^{LL5}/CyO,actin-gfp^{JMR1}; FRT82B, tub-Gal80^{LL3}* (Bello et al., 2003) to *gcm-lacZ^{rA87}/CyO, actin-gfp^{JMR1}*; *FRT82B* males. Eggs were collected for 2 to 4 h, grown to first larval instar (22 to 30 h after egg laying), then heat shocked in a 37°C water bath (GFL 1083, Burgwedel, Germany) for 5 minutes. Larvae were then grown to late wandering third instar. The *Gal4$^{14\text{-}94}$* (Zhu et al, 2011) was kindly provided by the Jan lab (University of California, San Francisco, CA, USA). The *R45F08-Gal4* line is the *P{GMR45F08}attP2* enhancer-Gal4 line from Janelia Farm (Ashburn, VA, USA) (Jenett et al., 2012). For the spatiotemporal analysis the Gal4 lines were crossed to *y, w, UAS-Flp; UAS-Flp^{JD1}; UAS-mCD8::GFP^{LL5}/CyO, actin-gfp^{JMR1}; act5C>*>nlacZ,ry506* (*act5C>*>nlacZ* from (Struhl and Basler, 1993); flip-out-lacZ not relevant for this work). To analyze postlarval development, white prepupae were picked and kept at 25°C until they were dissected at the desired timepoints; 12 h pupae, however, were kept for 24 h at 18°C (where development takes twice as long as at 25°C). For analyzing late-born versus early-born type II neuroblast-derived cells, *UAS-mCD8::GFP^{LL5}, Gal4$^{14\text{-}94}$* flies were crossed to *R09D11-CD4::tdTom* (Han et al., 2011).

Flip-out clones were obtained by crossing *y, w, hs-Flp; UAS-FRT>CD2,y^{+}>mCD8::GFP* (G. Struhl provided flies for publication in (Wong et al., 2002)) to the *Gal4$^{14\text{-}94}$* flies. Eggs were collected for 2 h and then heat shocked 2.5 to 4.5 h after egg laying in a 34°C water bath for 15 minutes. Late induced clones were heat shocked at 72 h to 79 h after egg laying in a 37°C water bath for 20 minutes. Then, larvae were grown to late wandering third instar.

2.3.2 Immunohistochemistry

Larval brains were fixed and immunostained as described previously (Viktorin et al., 2011). For all pupal and adult staining and for larval Elav staining, primary and secondary antibodies were incubated for 2 days at 4°C. For all other larval staining, primary antibodies were incubated overnight at 4°C and secondary antibodies were incubated for 3 h at room temperature. In Neurotactin (BP106) staining, a 5-minute methanol step was added after fixation and preliminary washings with PBS. Then 4 × 15 minute washings with PBS/0.5% Triton X-100 were performed before blocking. The following antibodies were used: rabbit anti-β-galactosidase 1:500 (55976, MP Biomedicals, Solon, OH, USA), chicken anti-GFP 1:1,000 (ab13970, Abcam, Cambridge, UK), mouse anti-Neurotactin 1:20 (BP106, Developmental Studies Hybridoma Bank (DSHB), Iowa city, Iowa, USA), rat anti-Elav 1:20 (7E8A10, DSHB), rabbit anti-Repo 1:400 (kindly provided by Veronica Rodrigues), rat anti-DN-cadherin 1:10 (DN-EX #8, DSHB), mouse anti-Nc82 1:20 (Bruchpilot, DSHB), rabbit anti-RFP 1:200 (ab62341, Abcam, Cambridge, UK). Alexa-conjugated secondary antibodies were used 1:300 (A11039, A11031, A21236, A21247, A11077, 21244, Molecular Probes, Eugene, OR, USA) and goat anti-mouse549 (Dylight™, KPL, Gaithersburg, MD, USA).

2.3.3 Microscopy and image processing

All fluorescent images were recorded using a Leica TCS SP5 confocal microscope (Leica microsystems GmbH, Wetzlar, Germany). Optical sections ranged from 0.76 to 1 µm with a picture size of 1,024 × 1,024 pixels. Collected images were arranged and processed using Fiji (Schindelin, 2008). Cell counts were performed with the Fiji plugin 'cell counter' (http://fiji.sc/Fiji). All adjustments were linear and were performed on whole images.

2.4 Results

2.4.1 Specific Gal4-based labeling identifies a bilaterally symmetric central complex primordium in the larval brain

Previous work has shown that specific Gal4 lines that drive expression in type II neuroblasts and/or their progeny can be used in combination with *UAS-GFP* reporters to identify the cellular constituents of these lineages during development. Several useful Gal4 lines of this type have been generated by fusing the cis-regulatory DNA from developmental control genes that act specifically in type II lineages to Gal4 (Jenett et al., 2012; Pfeiffer et al., 2008). The development of type II neuroblast lineages in larval stages has been monitored with the *R09D11-Gal4* line, which specifically labels INPs and a large set of their (recently-born) progeny (Bayraktar et al., 2010). (The *R09D11-Gal4* line represents a fusion to Gal4 of regulatory DNA from the *earmuff* gene that is expressed in mature INPs and is thought to maintain the restricted proliferative potential of these cells and prevent dedifferentiation of these intermediate progenitors to ectopic neuroblasts; (Weng et al., 2010).) Figure 2.1A shows the eight type II lineages in one hemisphere of the third larval instar brain as revealed by the *R09D11-Gal4* line. Six of these type II lineages are located near the midline in the dorsoposterior region of the hemisphere. Each of these six lineages has been individually identified and, based on their spatial arrangement, have been referred to as DM1 (most dorsal/rostral), DM2, DM3, DM4, DM5, and DM6 (most ventral) (Izergina et al., 2009). (Given the fact that the neuroblasts of the type II lineages are located posteriorly and are Asense-negative, the lineages are also referred to as PAN lineages; (Bowman et al., 2008).) The corresponding names given to these lineages by Pereanu and Hartenstein (Pereanu and Hartenstein, 2006) are presented in Table 1. A further set of two type II lineages are located more laterally in the hemisphere. In this study, we focused primarily on the DM1 to DM6 lineages since they can be identified individually and have been characterized in most detail. In the larval brain, the cell bodies of each of the DM lineages are clustered together and neurites from each cell cluster form common axon fascicles that enter the neuropil and then arborize in a complex manner that is characteristic for each identified DM lineage (Izergina et al., 2009). A common feature of all DM lineages in the larval brain is that a subset of their neurites form a complex set of crossed and uncrossed commissural fiber tracts revealed by *R09D11-Gal4* (Figure 2.1A', asterisk).

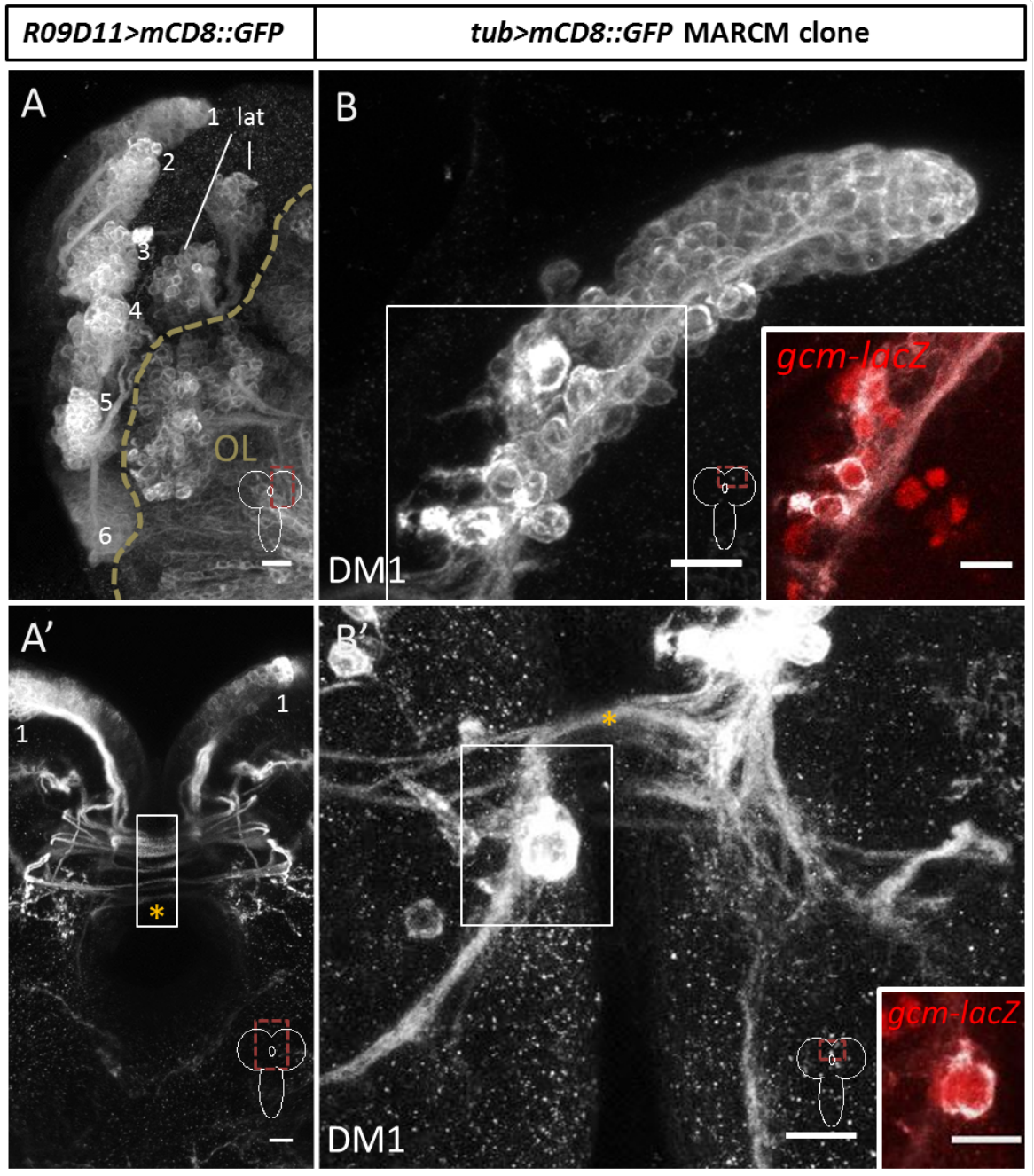


Figure 2.1 Type II neuroblast lineages labeled by *R09D11-Gal4* and mosaic analysis with a repressible cell marker (MARCM) in the late larval brain. (A,A') *R09D11-Gal4* driven labeling of eight dorsomedial (DM) lineages and their commissural fascicles. DM1 to DM6 indicated with numbers; lat, lateral type II lineages. Z-projection *of* multiple adjacent optical sections. **(A)** shows one hemisphere, **(A')** shows the midline regions of the larval brain with the commissure indicated by orange asterisk in **(A')** and part of the commissural region overexposed to show fiber tracts (white box in A'). **(B,B')** *tubulin-Gal4* driven MARCM-based labeling of DM1 lineage shows neurons and glial cells in a single neuroblast clone. Axonal fascicles from the DM1 clone cross the commissure (asterisk in (B')). Z-projection of multiple optical sections. The slightly larger cells at the base of the lineage are glial cells expressing *gcm-lacZ* (insets in (B,B'); red, gcm-lacZ). Scale bars, 10 µm.

Table 2.1 Annotation of type-II NB dorsomedial (DM) lineages by different groups

Group and reference	Annotation					
Bello *et al*. 2008	DM1	DM2	DM3	DM4	DM5	DM6
Pereanu and Hartenstein 2006	DPMm1	DPMpm1	DPMpm2	CM4	CM3	CM1

Although *R09D11-Gal4* labels many of the cells in the type II lineages, it is much more strongly expressed in late-born cells which are located more proximally in the cell body cluster with respect to the neuroblast than early-born cells that are located distal to the neuroblast and closer to the hemisphere neuropil. (In the neuroblast clones of the central brain, late-born (recently born) cell bodies remain located close to their neuroblast of origin and displace the early-born cell bodies that are the most remote from their neuroblast of origin near the neuropil. Thus, the cells in a lineage are clustered and arranged along a spatiotemporal gradient; see (Hartenstein et al., 2008).) A more complete visualization of all of the secondary, adult-specific neurons in each DM neuroblast lineage is possible by using mosaic analysis with a repressible cell marker (MARCM) techniques (Lee and Luo, 1999). Figure 2.1B shows the type of labeled DM clone that can be revealed through MARCM labeling by using a ubiquitously expressed *tubulin-Gal4* driver with a *UAS-mCD8::GFP* reporter if recombination is induced after larval hatching and clones are recovered at late third instar. In the labeled DM1 clone shown, the neural progeny of the DM1 neuroblast are arranged in a spatiotemporal structured array that extends towards the neuropil. Several larger cells derive from this type II neuroblast that are located at the distal end of the lineage. These cells are labeled by both *gcm-lacZ* (Figure 2.1B,B', insets) and Repo, but not Elav, and are thus glial cells, not neurons (Viktorin et al., 2011). The secondary neurons in this MARCM-labeled DM1 lineage initially form a common secondary axon tract (SAT) that enters the neuropil and then arborizes in a highly complex, DM1-specific manner giving rise to interhemispheric commissural tracts, as well as ipsilateral and contralateral ascending and descending tracts (Izergina et al., 2009). Figure 2.1B' shows the commissural tracts that the DM1 lineage neurons form at the brain midline (as well as a labeled glial cell; see inset). As in the case for most other secondary neurons in the larval central brain, all of the commissural and longitudinal tracts comprise immature neurons that only form terminal dendritic/axonal arbors and synaptic neuropil during pupal development (Hartenstein et al., 2008; Izergina et al., 2009; Young and Armstrong, 2010b).

Although both *R09D11-Gal4* labeling and *tubulin-Gal4* driven MARCM labeling allow visualization of the cell bodies and the axon fascicles that derive from these cell bodies, neither method reveals the central complex primordia (immature fan-shaped body, immature protocerebral bridge) that have been identified previously using global markers (Young and

Armstrong, 2010b). For this, other Gal4 lines are required. Recent work has shown that one isoform of the Ets transcription factor Pointed, PntP1, is specifically expressed in type II lineages and functions to promote the generation of mature INPs during larval development (Zhu et al., 2011). Correspondingly, a driver line in which Gal4 is integrated 347 base pairs before the transcription start site of a transcript that encodes PntP1, has been shown to drive reporter gene expression in type II neuroblasts, INPs, and a late-born subset of the secondary neurons specifically in all eight type II lineages of the late larval brain (Zhu et al., 2011); it also labels glial cells in these lineages (Additional file 1, Figure S2.1). (Like *R09D11-Gal4*, this Gal4 line also drives reporter gene expression in the optic lobe; this expression is not related to type II lineages in the central brain and, hence, is not considered further in this report.) Remarkably, we found that this Gal4 line, $Gal4^{14\text{-}94}$, also very strongly labels a third cell type, neurons with large cell bodies, which are clustered near the distal ends of the DM lineages in each hemisphere and give rise to neurites that appear to project to a strongly-labeled structure near the brain commissure (Figure 2.2A). This labeled structure has all of the gross morphological features of the previously identified fan-shaped body primordium in the late larval brain, namely a bilaterally symmetrical, slightly curved bar-shaped structure that straddles both sides of the brain midline (Young and Armstrong, 2010b). Moreover, the two halves of the structure appear to be interconnected by a plexus of commissural fibers, and each half of the structure is subdivided into (barely) discernible substructures. These observations suggest that the $Gal4^{14\text{-}94}$ line may label the cells that contribute to the primordium of the fan-shaped body, in addition to labeling the type II neuroblasts, INPs, and their late-born progeny.

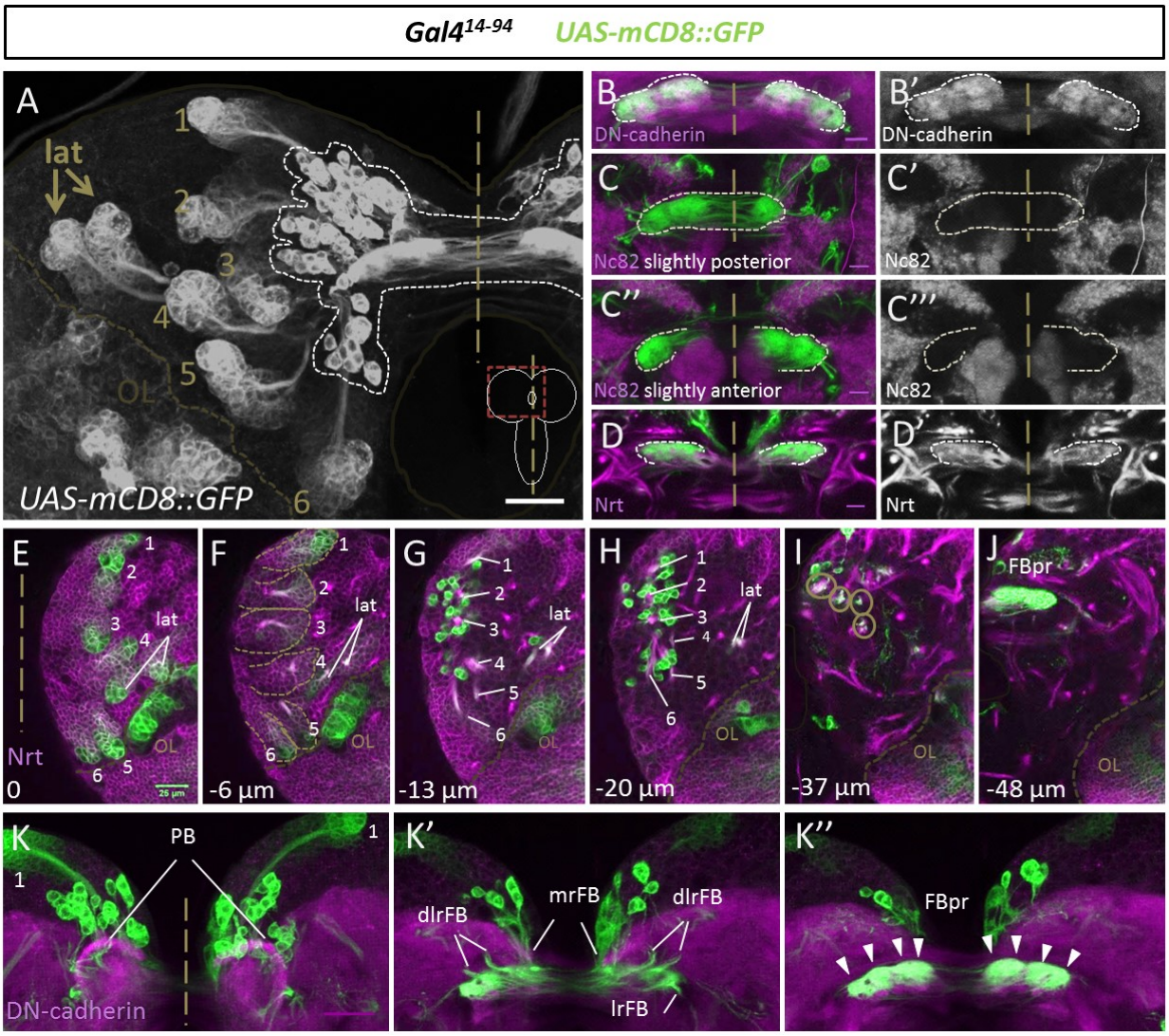


Figure 2.2 Clusters of midline associated neurons and the fan-shaped body primordium are revealed by *Gal4*$^{14\text{-}94}$ driven labeling of dorsomedial (DM) lineages in the late larval brain.

(A) Distal cell clusters corresponding to DM neuroblasts and their recently born progeny as well as a cluster of midline associated cells in close proximity to the fan-shaped body primordium are labeled by *Gal4*$^{14\text{-}94}$. The dotted line indicates midline associated cells and primordium. DM1 to DM6 are indicated with numbers; lat, lateral type II lineages. Maximum projection of all stacks. **(B,B')** The *Gal4*$^{14\text{-}94}$-labeled fan-shaped body primordium (green) is colabeled with the neuropil marker DN-cadherin (magenta/white). **(C-C''')** The *Gal4*$^{14\text{-}94}$-labeled fan-shaped body primordium (green) is not colabeled with the synaptic marker Bruchpilot (magenta/white). **(D,D')** The *Gal4*$^{14\text{-}94}$-labeled fan-shaped body primordium (green) is colabeled with the membrane marker Neurotactin (magenta/white). **(E-J)** Single sections at different depths show the *GAL4*$^{14\text{-}94}$-labeled cells of type II lineages (green) in a brain hemisphere colabeled for Neurotactin (magenta) revealing the relative position of the midline associated cells, the neurite fascicles of the DM lineages, and the fan-shaped body primordium (FBpr). DM1 to DM6 are indicated with numbers; lat, lateral type II lineages. Circles in (I) indicate neurite fascicles. **(K-K'')** *GAL4*$^{14\text{-}94}$-labeled cells and projections of type II lineages (green) at the midline colabeled for DN-cadherin (magenta). The two bilateral clusters of midline-associated cells (K) give rise to four neurite fascicles per hemisphere (K'), which project into the fan-shaped body primordium (K''). The fan-shaped

body primordium is composed of four subcompartments per hemisphere (arrowheads) (K''). FBpr, fan-shaped body primordium; dlrFB, dorsolateral root of fan-shaped body; mrFB, medial root of fan-shaped body; lrFB, lateral root of fan-shaped body; nomenclature according to (Pereanu et al., 2010). (B-D', K-K'') are maximum intensity projections of few adjacent confocal slices. (A) and (E-K'') scale bars, 25 µm, (B-D') scale bars, 10 µm.

2.4.2 The fan-shaped body primordium is innervated by early-born neurons of type II neuroblast lineages

To characterize the *Gal4*$^{14\text{-}94}$-labeled midline structure in more detail, we first used immunocytochemical markers for DN-cadherin, Bruchpilot (NC82) and Neurotactin (BP106). The *Gal4*$^{14\text{-}94}$-labeled midline structure was strongly immunoreactive for DN-cadherin and moderately immunoreactive for Neurotactin, however it was not immunoreactive for Bruchpilot (Figure 2.2B-D). Since the Bruchpilot protein is localized at the presynaptic active zone and is a marker for synaptic neuropil of differentiated neurons, this suggests that the labeled midline structure does not comprise synapses. Furthermore the lack of Bruchpilot staining suggests that the neurons building up the midline primordium are not yet differentiated. Previous studies have shown that DN-cadherin is expressed in primary neurons and their neural processes and synapses as well as in early secondary axons and filopodia, while Neurotactin labels the membranes and axon tracts of secondary axons (Pereanu et al., 2010; Spindler and Hartenstein, 2010). Since Neurotactin and DN-cadherin are expressed in the fan-shaped body primordial structure but Bruchpilot expression is absent, this further suggests that this midline structure is composed of undifferentiated neural processes and filopodia. Indeed, the absence of synapses and the presence of as yet undifferentiated neural processes would be expected properties of the fan-shaped body primordium in the brain.

To study the anatomical relationship between the *Gal4*$^{14\text{-}94}$-labeled cell bodies and the midline primordium structure further, we analyzed optical sections of different depth in late larval brains in which *Gal4*$^{14\text{-}94}$ driven *UAS-mCD8::GFP* labeling was combined either with Neurotactin immunolabeling or with DN-cadherin immunolabeling (Figure 2.2E-K). We also compared *Gal4*$^{14\text{-}94}$ directly with *R09D11-CD4::tdTom* reporter expression and confirmed that, while the two reporters overlap in the lineage proper close to the neuroblast (Additional file 2, Figure S2.2A,B), expression in the fan-shaped body primordium and the large neurons distal to the neuroblast is specific to *Gal4*$^{14\text{-}94}$ (Additional file 2, Figure S2.2C,D). This analysis confirmed the notion that neurite-like processes from the *Gal4*$^{14\text{-}94}$-labeled midline-associated cell clusters project to the labeled primordium structure. It also provided evidence for a fourfold modular organization of the primordium and of the innervating neurite tracts in each hemisphere. Moreover, it demonstrated that the labeled midline-associated cell bodies in each hemisphere

are located adjacent to the nascent neuropil and clearly distant from the clusters of labeled neuroblasts, INPs and late-born secondary neurons in the type II lineages. Finally, this study revealed that a bilaterally symmetrical unfused primordium of the protocerebral bridge was also present at late third instar larval stage, but was not obviously labeled by *Gal4$^{14\text{-}94}$* or innervated by *Gal4$^{14\text{-}94}$*-positive processes.

In view of the spatial location of these labeled midline-associated cell bodies relative to the neuroblasts, INPs and late-born secondary neurons in the type II lineages, and considering the type II-specific labeling of the *Gal4$^{14\text{-}94}$* driver, we hypothesized that these cells might represent early-born neurons of type II lineages. To investigate this, we first determined if these midline-associated cells are lineal members of type II lineages. For this we used flip-out-based labeling of individual type II neuroblast clones in which the *Gal4$^{14\text{-}94}$* driver was coupled with a *UAS-FRT>CD2y^{+}>mCD8::GFP* reporter and *hs-Flp*. For optimal labeling of early-born neurons, heat shock-Flp was induced in the early embryo (between 2 h and 4 h after egg laying) and clones were recovered at the late larval instar stage. (Flip-out labeling was used because early embryonic induction of MARCM-labeled neuroblast clones was not successful, as has also been reported elsewhere (Kunz et al., 2012)). A minimum of seven *Gal4$^{14\text{-}94}$* driver labeled neuroblast clones in otherwise sparsely-labeled brains were recovered for each of the type II lineages DM1 to DM6. For DM1, DM2, DM3 and DM6, these clones invariably comprised both a group of cells consisting of the neuroblast together with its late-born progeny, and a set of more intensely labeled midline-associated neurons at the distal end of the lineages (Figure 2.3A-C,F-G). This indicates that the distal midline-associated cells are lineal descendants of the corresponding four type II neuroblasts, and together with the position of the cells distal to the neuroblast in a given clone implies that these are early-born lineal descendants. Moreover, the neurite tract emanating from the late-born neurons joins with and initially follows the neurite tract from the early-born neurons in each of these four DM lineages as might be expected for clonal descendants of the same neuroblast (Pereanu and Hartenstein, 2006; Spindler and Hartenstein, 2010; Larsen et al., 2009). To confirm that these distal midline-associated cells are indeed early-born members of the lineages, we repeated the flip-out-based labeling of individual type II neuroblast clones but induced heat shock-Flp later at the early third larval instar stage. As expected, all of the clones recovered at the late third instar stages for DM1, DM2, DM3 and DM6 contained exclusively a group of cells consisting of the neuroblast together with its late-born progeny and never comprised the distal midline-associated neurons (Figure 2.4A-C).

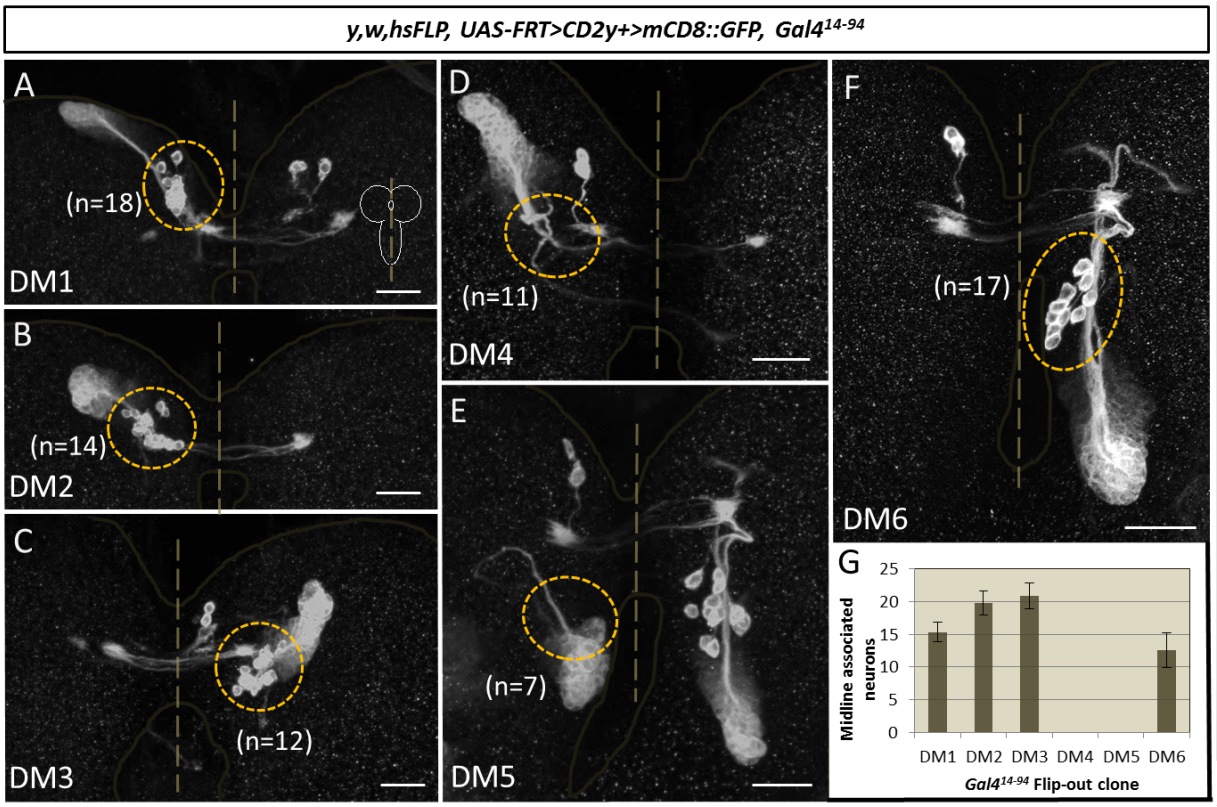


Figure 2.3 The midline-associated cells are lineal descendants of four dorsomedial (DM) neuroblasts. Embryonically induced flip-out clones showing that midline associated cells (cells in yellow dotted circles) are lineal descendants of four DM neuroblasts. **(A)** DM1 flip-out clone, **(B)** DM2 flip-out clone, **(C)** DM3 flip-out clone, **(D)** DM4 flip-out clone, **(E)** DM5 flip-out clone, **(F)** DM6 flip-out clone. Whereas DM1, DM2, DM3 and DM6 neuroblast clones invariably comprise between 10 and 23 midline associated cells, such cells were never found in DM4 and DM5 neuroblast clones. Dotted circles in (A,B,C,F) indicate midline associated cells, which are absent in (D, E). **(G)** Average number of midline cells in DM flip-out clones, error bars are standard deviations. Scale bars, 25 µm.

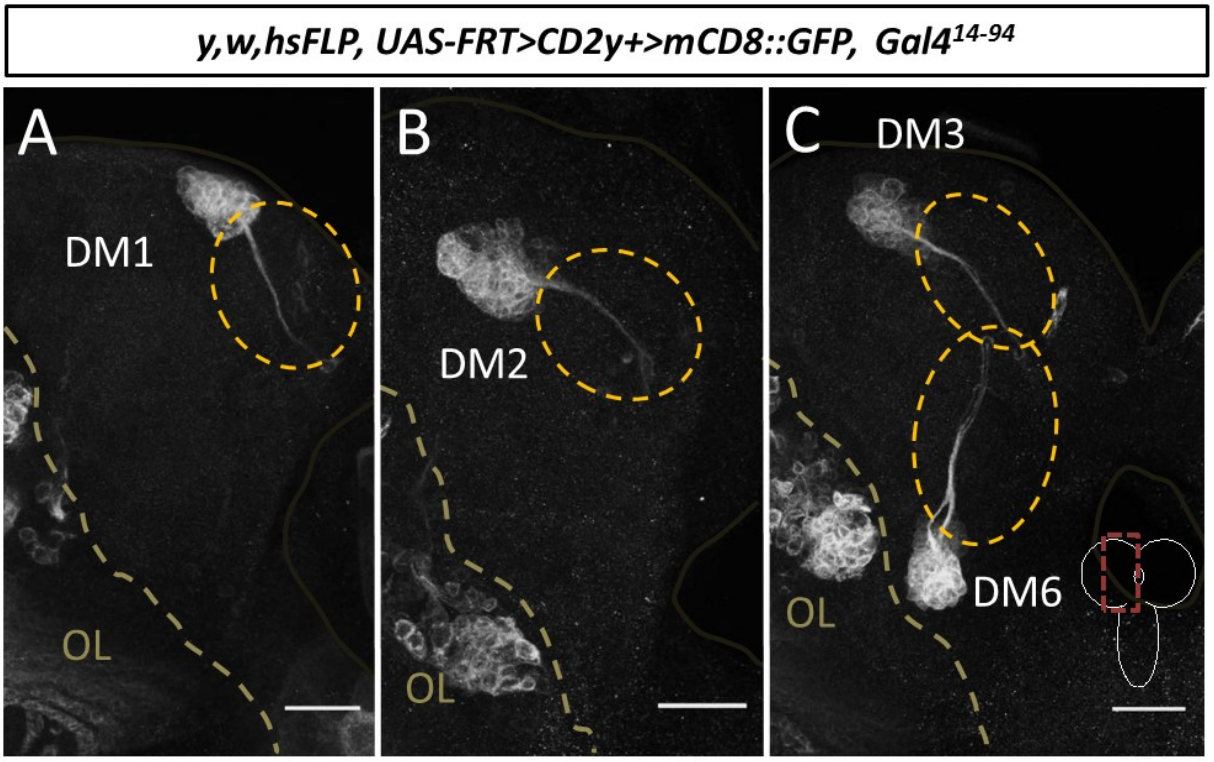


Figure 2.4 The midline-associated cells are early-born lineal descendants of dorsomedial (DM) lineages DM1 to DM3 and DM6.
Flip-out clones induced at early L3 showing that no midline associated cells are produced by DM1 to DM3 and DM6 neuroblasts after this timepoint. However a substantial number of late secondary neurons is produced during the third larval instar stage. **(A)** DM1 flip-out clone, **(B)** DM2 flip-out clone, **(C)** DM3 and DM6 flip-out clone. Dotted circles in (A-C) indicate where midline associated cells would be located, but are absent. Scale bars, 25 µm.

Neuroblast clones of DM4 and DM5 type II lineages never contained the distally located set of intensely-labeled midline-associated neurons and were composed exclusively of the *Gal4*$^{14\text{-}94}$-labeled neuroblast and its late-born progeny (Figure 2.3D,E). Quantification of the number of midline associated neurons in the DM1, DM2, DM3 and DM6 clones was carried out and indicated that each of the 4 neuroblast clones contained between 12 and 20 of these cells, resulting in total of approximately 70 midline-associated cells per hemisphere (Figure 2.3G). Since the total number of midline cells labeled by the *Gal4*$^{14\text{-}94}$ driver corresponds to approximately 90 cells (91 ± 3; n = 4) per hemisphere, this indicates that most, but not all, of the *Gal4*$^{14\text{-}94}$-labeled early-born cells are lineal descendants of the 4 type II neuroblast lineages DM1, DM2, DM3 and DM6. The remaining *Gal4*$^{14\text{-}94}$-labeled early-born cells cannot be progeny of the two lateral type II lineages since all of the cells in these two lineages are positioned more laterally in the brain and show no spatial overlap with the midline cells. However, these midline neurons not generated by DM1 to DM3 and DM6 might be descendants of the DM4 and/or

DM5 lineages that could be resistant to flip-out under the conditions used; due to technical constraints we were not able to determine their lineal origin with certainty.

Given that the primordium-forming neurons are derived from four different neuroblast lineages, we wondered if their processes in the primordium might be topologically organized. To investigate this, we generated flip-out clones using the *Gal4$^{14\text{-}94}$* driver as above, and recovered neuroblast clones or multicell clones from DM1 to DM3 and DM6 at the late larval stage. Up to three neuroblast/multicell clones were recovered in a given preparation. Analysis of their processes in the primordium indicates that there is in fact a topological order (Additional file 3, Figure S2.3). Thus, processes of the DM6 cells arborize most laterally on the ipsilateral side and most medially on the contralateral side of the primordium. Processes of the DM1 cells arborize most medially on the ipsilateral side and most laterally on the contralateral side of the primordium. Processes of the DM3 cells arborize adjacent to those of DM6 (medially adjacent on the ipsilateral side, laterally adjacent on the contralateral side). Processes of the DM2 cells arborize adjacent to those of DM1 (laterally adjacent on the ipsilateral side, medially adjacent on the contralateral side).

2.4.3 A *pointed* enhancer fragment-Gal4 driver specifically labels neurons that exclusively innervate the fan-shaped body primordium

For a precise analysis of the development of the type II-derived midline-associated cells and their contribution to the central complex primordium, more specific genetic access that is restricted exclusively to these cells is required. To obtain this, we screened a set of driver lines in which different *pointed* cis-regulatory DNA fragments are fused to Gal4 (Jenett et al., 2012). Among these, we found that the *R45F08-Gal4* line drives reporter gene expression restricted exclusively to a set of cells that correspond to the type II-derived midline cells (Figure 2.5A). Thus, in the late larval brain *R45F08-Gal4* labels a set of approximately 90 (92 ± 3; n = 4) cell bodies in each hemisphere that are clustered near the midline and that project neurites into a strongly-labeled midline structure which has all of the morphological features of the fan-shaped body primordium. A comparison of larval brains labeled with *R45F08-Gal4* to larval brains labeled with *Gal4$^{14\text{-}94}$* shows that labeling with *R45F08-Gal4* is indeed restricted to the midline-associated primordium-forming cells; neither type II neuroblasts and INPs nor late-born neural cells and their neurite fascicles are labeled (compare Figure 2.2A with Figure 2.5A). The *R45F08-Gal4*-labeled cells are immunoreactive for the neuronal marker ELAV indicating that they are indeed neurons and not glial cells (Figure 2.5B). This was confirmed by the fact that none of the *R45F08-Gal4*-labeled cells showed anti-Repo immunoreactivity (data not shown).

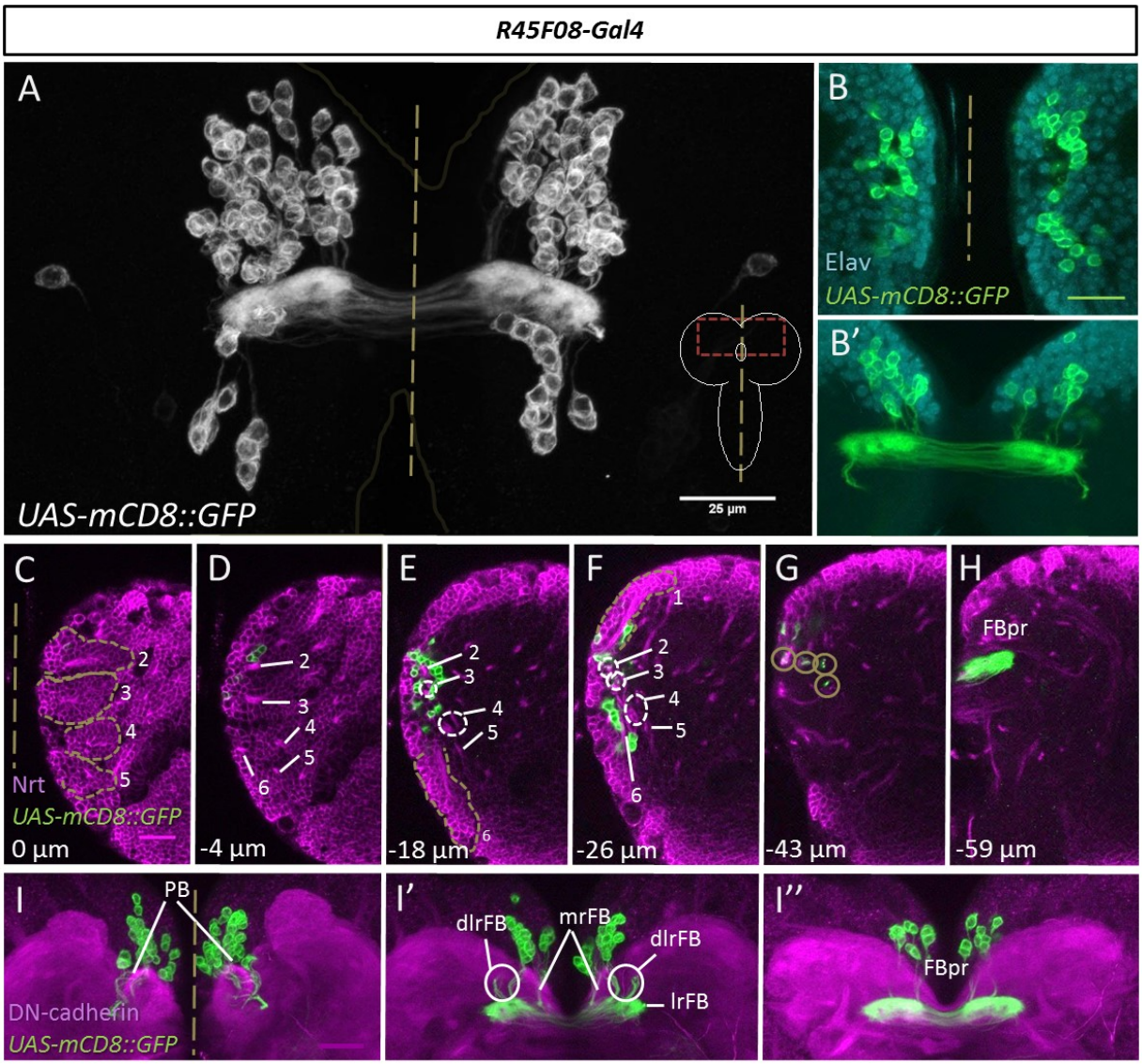


Figure 2.5 ***R45F08-Gal4*** **specifically labels the midline-associated neurons that innervate the fan-shaped body primordium in the late larval brain.**
(A) *R45F08-Gal4* labels two bilateral groups of midline-associated cells as well as the fan-shaped body primordium (FBpr). The number and position of the labeled cell bodies, which project their neurites into the fan-shaped body primordium, correspond to the number and position of the midline cells revealed by Gal4$^{14\text{-}94}$ labeling (compare to Figure 2.2A; cells indicated by dotted line). No other neuronal structures are labeled implying that the labeled cell bodies give rise to the fan-shaped body primordium. **(B)** The *R45F08-Gal4*-labeled cells (green) are colabeled by the neuronal marker Elav (cyan). (B,B') are two Z-projections taken at different focal planes. **(C-H)** Single sections taken at different depths show the *R45F08-Gal4*-labeled cells (green) in a brain hemisphere colabeled for Neurotactin (magenta) revealing the relative position of the primordium cell bodies (D-F), their neurite fascicles (F,G), and the fan-shaped body primordium (FBpr, (H)). Dorsomedial (DM) lineages DM1 to DM6 indicated with numbers. Circles in (G) indicate neurite fascicles. **(I-I'')** *R45F08-Gal4*-labeled cells (green) at the midline of a brain colabeled for DN-cadherin (magenta). In each hemisphere, the group of labeled cell bodies gives rise to four neurite fascicles that project into the fan-shaped body primordium (I''). One fascicle projects to the

medial root of fan-shaped body (mrFB), one fascicle projects to the lateral root of the fan-shaped body (lrFB), and the remaining two fascicles project together to the dorsolateral root of the fan-shaped body (dlrFB); nomenclature according to (Pereanu et al., 2010). (I-I'') are three Z-projections taken at different depths. Scale bars, 25 µm.

To document the morphological relationship among the *R45F08-Gal4*-labeled cells, we analyzed optical sections of different depth in late larval brains in which *R45F08-Gal4* driven *UAS-mCD8::GFP* labeling was combined with either Neurotactin immunolabeling or with DN-cadherin immunolabeling (Figure 2.5C-I). This confirmed that neurite-like processes from the *R45F08-Gal4*-labeled cells project to the labeled fan-shaped body primordium. Moreover, it revealed the fourfold modular organization of the primordium and of the primordium-innervating neurite tracts in each hemisphere. Indeed, a comparison of *R45F08-Gal4*-labeled larval cells to $Gal4^{14\text{-}94}$-labeled larval cells implies that all of the morphological features of the labeled fan-shaped body primordium are due to innervation by the *R45F08-Gal4*-labeled cells and, hence, do not result from innervation by other later-born secondary cells (compare Figures 2.2 and 2.5). Based on these findings, we concluded that the *R45F08-Gal4* line specifically labels the midline-associated type II lineage neurons that project their neurites exclusively into the fan-shaped body primordium in the larval brain, identifying these cells as the hitherto unknown primordium-forming neurons.

The remarkably specific labeling of the *R45F08-Gal4* line, which is restricted to the midline associated type II neurons, makes this driver line optimal for analyzing the subsequent developmental fate of these fan-shaped body primordium neurons during central complex formation and maturation in pupal stages and in the adult. This analysis shows that the primordium neurons undergo extensive growth and differentiation such that layer-specific innervation of the fan-shaped body is formed during metamorphosis, and this persists in the adult central complex. A spatiotemporal documentation of this differentiation process is presented below.

2.4.4 The larval primordium neurons undergo extensive growth and differentiation and are integrated into the mature fan-shaped body of the adult brain

In the following, the development of the larval primordium neurons was analyzed throughout early metamorphosis (Figure 2.6). Moreover, the primordium forming neurons and their arborizations were also analyzed during more advanced metamorphosis and into the adult (Figure 2.7). At 12 h after puparium formation (apf) the developing fan-shaped body has increased in size relative to the larval primordium, and its two halves have fused at the midline forming a seemingly unpaired midline structure that begins to bend ventrolaterally. Moreover,

the protocerebral bridge primordium has grown larger and the noduli have become visible. (For a more detailed description of overall central complex development during metamorphosis, see (Young and Armstrong, 2010b).) At this stage, the *R45F08-Gal4*-labeled cell bodies of the primordium neurons are located at the dorsomedial midline of the central brain and project their neurites through a complex commissural chiasma into the developing fan-shaped body, where they form eight (2 × 4) prominent columns of innervation in two layers (Figures 2.6A and 8A). At 18 h apf, the overall development of the fan-shaped body, as well as its innervation by *R45F08-Gal4*-labeled processes, are largely similar to 12 h apf (Figure 2.6B). At 24 h apf, the fan-shaped body has further increased in size and become more bent. A slender, unfused ellipsoid body is visible rostral/ventral to the fan-shaped body, the protocerebral bridge has enlarged further, and the associated noduli have moved medially towards each other. At this stage, the *R45F08-Gal4*-labeled neurons continue to project through a midline plexus and their innervation of the fan-shaped body has grown but retains its modular arborization pattern in eight columns and two layers (Figure 2.6C and 2.8B). Very sparse *R45F08-Gal4*-labeled innervation of the ellipsoid body is seen at this stage (Figure 6C’’’).

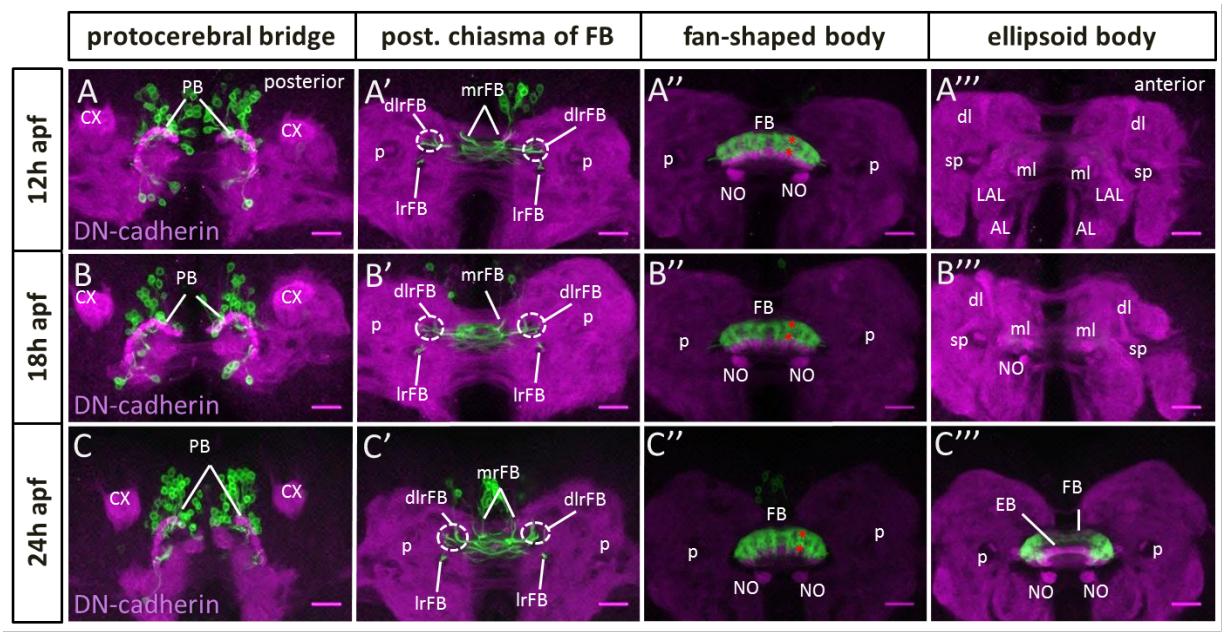


Figure 2.6 Differentiation of larval fan-shaped body primordium-innervating neurons begins during early metamorphosis.

Spatiotemporal analysis of the *R45F08-Gal4* expressing cells and neurites as well as their innervation of developing central complex substructures during the first 24 h of metamorphosis. *R45F08-Gal4* labeling (green), DN-cadherin labeling of neuropil (magenta). Maximum intensity projections of few adjacent confocal slices taken at different depths. **(A-A’’’)** At 12 h after puparium formation (apf) the *R45F08-Gal4*-labeled cells, located at the dorsomedial region of the brain cortex, bypass the protocerebral bridge and their fascicles project through midline chiasma and enter the developing fan-shaped body, whose two halves have fused, via the medial root of fan-shaped body (mrFB), the lateral root of the fan-shaped body (lrFB), and the dorsolateral root of the fan-shaped body (dlrFB). The labeled fibers arborize in the

developing fan-shaped body and form eight (2 × 4) columnar innervation domains. Labeled fibers are not evident in other neuropils. **(B-B''')** At 18 h apf the *R45F08-Gal4*-labeled cells as well as their innervation of the developing fan-shaped body are largely unchanged as compared to 12 h apf. **(C-C''')** At 24 h apf the *R45F08-Gal4*-labeled cells continue to project via midline chiasma and innervate eight columnar domains of the fan-shaped body, which has grown and become more bent. In addition sparse labeling is seen at the lateral edges of the developing (and yet unfused) ellipsoid body. In (A''-C'') asterisks indicate one labeled columnar domain in two layers of the fan-shaped body. AL, antennal lobe; CX, calyx; dlrFB, dorsolateral root of fan-shaped body; EB, ellipsoid body; FB, fan-shaped body; LAL, lateral accessory lobe; lrFB, lateral root of fan-shaped body; ml, medial lobe; mrFB, medial root of fan-shaped body; NO, noduli; PB, protocerebral bridge; p, peduncle; sp, spur of mushroom body. Neuroanatomical nomenclature according to (Hartenstein et al., 2008; Pereanu et al., 2010). Scale bars, 25 µm.

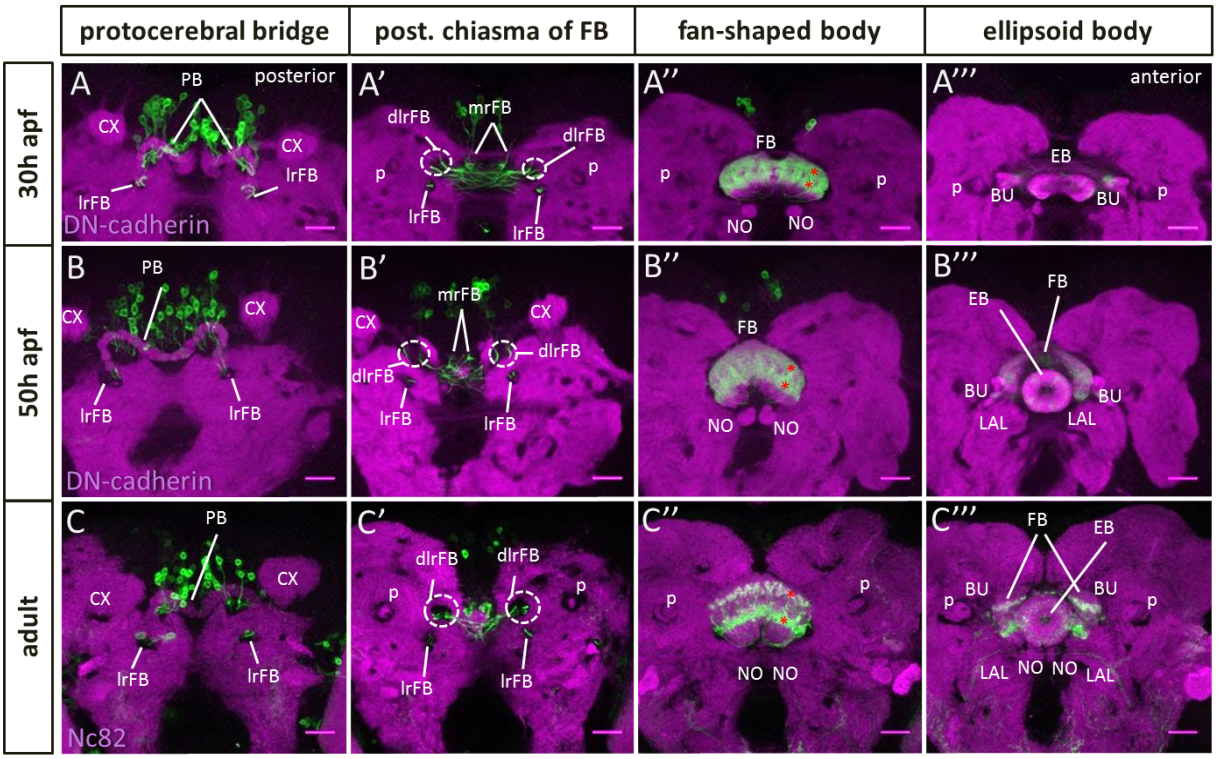


Figure 2.7 Differentiation and integration of larval fan-shaped body primordium-innervating neurons into the mature central complex.

Spatiotemporal analysis of the *R45F08-Gal4* expressing cells and neurites as well as their innervation of developing central complex substructures at 30 h and 50 h of metamorphosis and in the adult. *R45F08-Gal4* labeling (green), **(A-B''')** DN-cadherin labeling of neuropil (magenta), **(C-C''')** Nc82 labeling of neuropil (magenta). Maximum intensity projections of few adjacent confocal slices taken at different depths. (A-A''') At 30 h after puparium formation (apf) the *R45F08-Gal4*-labeled cells as well as their innervation of the developing fan-shaped body are similar to that observed at 24 h apf. (B-B''') At 50 h apf, the innervation of the developing fan-shaped body by *R45F08-Gal4*-labeled cells continues to manifest eight columnar domains and labeled processes are also seen in the inner and outer layers of the ellipsoid body and in the bulbs. (C-C''') In the adult the innervation of the mature fan-shaped body by *R45F08-Gal4*-labeled cells is largely restricted to two well separated layers, each of which is subdivided into the eight major columnar domains. Labeled processes remain visible in the ellipsoid body layers and in the bulbs. The noduli are not innervated by *R45F08-Gal4*-labeled cells at any stage. In (A''-C'') asterisks

indicate one labeled columnar domain in two layers of the fan-shaped body. BU, bulbs, CX, calyx; dlrFB, dorsolateral root of fan-shaped body; EB, ellipsoid body; FB, fan-shaped body; LAL, lateral accessory lobe; lrFB, lateral root of fan-shaped body; mrFB, medial root of fan-shaped body; NO, noduli; PB, protocerebral bridge; p, peduncle. Neuroanatomical nomenclature according to (Hartenstein et al., 2008; Pereanu et al., 2010). Scale bars, 25 µm.

At 30 h apf, morphogenesis of the fan-shaped body is comparable to that seen at 24 h apf. The pattern of its innervation by *R45F08-Gal4*-labeled processes is also largely similar to that seen at 24 h apf. However, the ratio of labeled to unlabeled innervation of the fan-shaped body begins to be lower implying that other neurons are starting to contribute more, in relative terms, to the nascent neuropil of the fan-shaped body (Figure 2.7A). The first *R45F08-Gal4*-labeled processes are also visible in the bulbs, central complex-associated neuropil structures that appear around 30 h apf. Additional labeling is also apparent in the ellipsoid body (data not shown). At 50 h apf, the fan-shaped body has acquired the typical 'fan-like' form of the mature adult structure. The two halves of the protocerebral bridge as well as the two halves of the ellipsoid body have fused and the noduli have reached the midline. At this stage, the *R45F08-Gal4*-labeled cells continue to form eight columns in two layers in the fan-shaped body (Figures 2.7B and 2.8C-E). Labeled processes are also prominent in the inner and outer layers of the ellipsoid body as well as in the bulbs. In the adult brain, approximately 80 cells (76 ± 6; n = 3) per hemisphere are labeled by *R45F08-Gal4* as compared to approximately 90 cells per hemisphere that are *R45F08-Gal4*-labeled in the late larval brain. This indicates that most of the neurons that form the larval primordium survive and are integrated into the adult neuropil of the fan-shaped body. In the mature fan-shaped body of the adult, the *R45F08-Gal4*-labeled cells form two distinct layers that are clearly separated by relatively large domains of unlabeled neural processes that now represent to most of the neuropil (Figures 2.7C and 2.8F-I). The 8-fold repeated pattern shows a further subdivision suggestive of a 16-fold modular organization, notably in the upper layer of the fan-shaped body. Labeled processes remain present in the ellipsoid body layers and in the bulbs. It should be mentioned that very weak innervation of the protocerebral bridge by *R45F08-Gal4*-labeled cells is observed throughout pupal development but is not obvious in the adult.

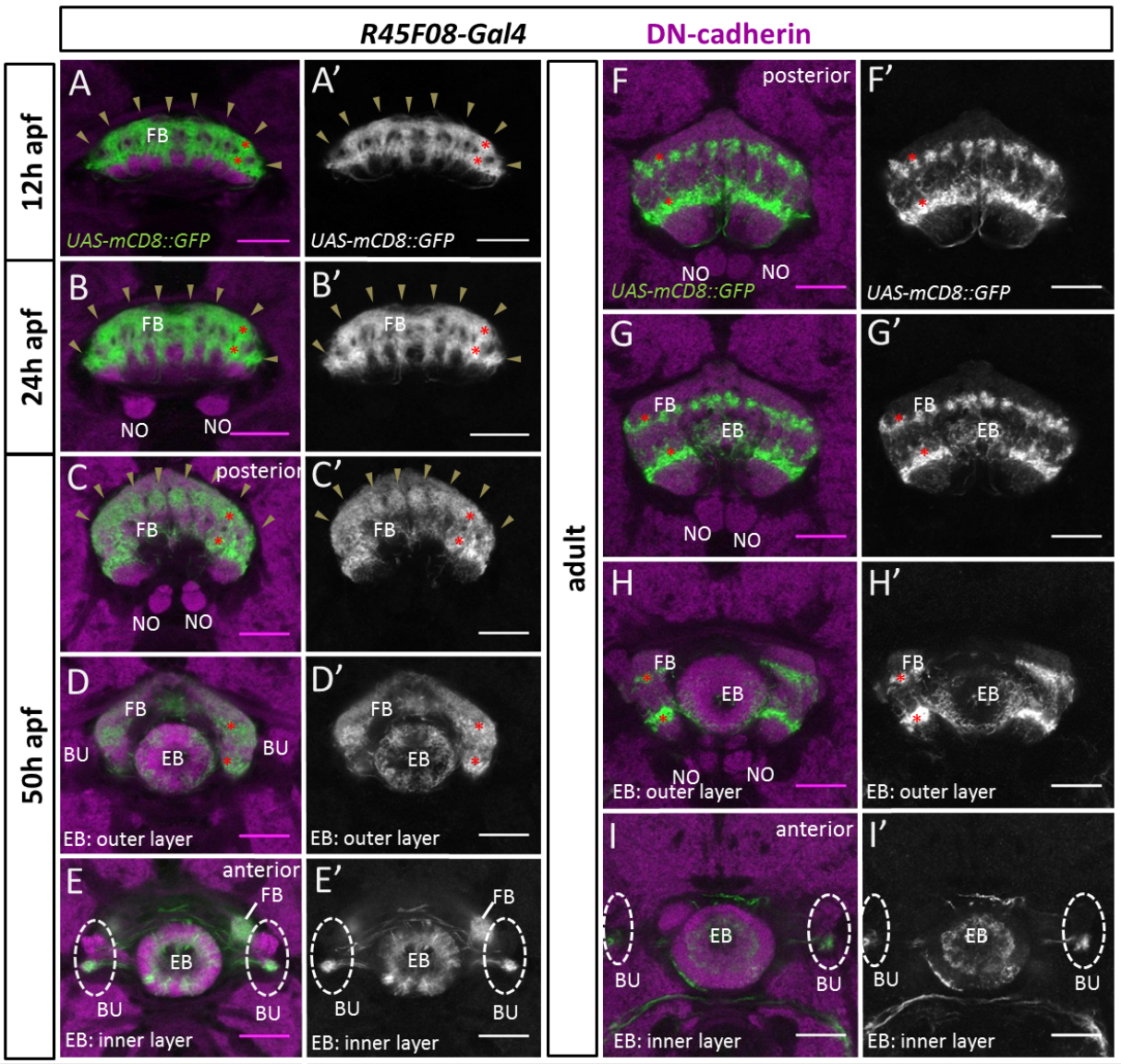


Figure 2.8 Innervation of modular subdomains in the developing central complex neuropil by *R45F08-Gal4*-labeled neurons.

Spatiotemporal analysis of the modular innervation pattern of *R45F08-Gal4* expressing cells in the developing central complex during metamorphosis and in the adult. *R45F08-Gal4* labeling (green), DN-cadherin labeling of neuropil (magenta); all panels show single confocal sections. **(A,A')** At 12 h after puparium formation (apf) *R45F08-Gal4*-labeled innervation of the developing fan-shaped body manifests eight columnar domains (arrowheads) arranged in two closely apposed horizontal layers. **(B,B')** At 24 h apf *R45F08-Gal4*-labeled innervation of the developing fan-shaped body has grown but continues to show eight columnar domains (arrowheads) arranged in two closely apposed horizontal layers. **(C-E')** At 50 h apf *R45F08-Gal4*-labeled innervation of the fan-shaped body has expanded further but is still seen in eight columnar domains (arrowheads) arranged in two horizontal layers. Labeled innervation is also seen in the inner and outer layer of the ellipsoid body, and innervation is seen in the ventral part of the bulbs. (C,C',D,D',E,E') are taken at different focal planes along the A/P axis in the developing central complex. **(F-I')** In the adult *R45F08-Gal4*-labeled innervation of the mature fan-shaped body is seen in two distinct layers that are clearly separated by unlabeled neuropil. Whereas in the ventral layer the

eight columnar domains seem to have fused, in the dorsal layer the columns appear to be further subdivided into a 16-fold modular organization. Labeled innervation remains in the inner and outer layer of the ellipsoid body and the ventral part of the bulbs. (F,F',G,G',H,H',I,I') are taken at different focal planes along the A/P axis in the central complex. In (A-D',F-H') asterisks indicate one labeled columnar domain in two layers of the fan-shaped body. BU, bulbs; EB, ellipsoid body; FB, fan-shaped body; NO, noduli. Neuroanatomical nomenclature according to (Hartenstein et al., 2008; Pereanu et al., 2010). Scale bars, 25 µm.

Given the lineage-specific topological order of the early-born neuron processes in the fan-shaped body primordium of the larval brain, we wondered if this same topological order might be retained by these neurons in the developing fan-shaped body during pupal development. To investigate this, we generated flip-out clones using the *R45F08-Gal4* driver, and recovered neuroblast clones from DM1 to DM3 and DM6 at 50 h apf. Analysis of their arborizations in the protocerebral bridge, the fan-shaped body and the ellipsoid body indicates that there is indeed a topological order of these early-born neurons (Figure 2.9). Moreover, this lineage specific topological order is the same as that observed for the early-born neurons of the DM1 to DM3 and DM6 lineages in the larval primordium of the fan-shaped body. Thus, arbors of the DM6 cells arborize most laterally on the ipsilateral side and most medially on the contralateral side of the fan-shaped body. Processes of the DM1 cells arborize most medially on the ipsilateral side and most laterally on the contralateral side of the fan-shaped body. Processes of the DM3 cells arborize adjacent to those of DM6 (medially adjacent on the ipsilateral side, laterally adjacent on the contralateral side). Processes of the DM2 cells arborize adjacent to those of DM1 (laterally adjacent on the ipsilateral side, medially adjacent on the contralateral side). Topologically ordered innervation by these lineages is also seen in the protocerebral bridge and in the ellipsoid body at this 50 h apf stage (Figure 2.9).

Taken together, these findings indicate that the same R45F08-Gal4-labeled cells that form the bilaterally symmetric larval primordium of the fan-shaped body also contribute to the unpaired midline neuropil of the mature fan-shaped body. This occurs because the innervation of the fan-shaped body primordium that these cells form in larval stages, undergoes growth and differentiation during pupal development such that a highly patterned and restricted innervation of two layers of the adult fan-shaped body results.

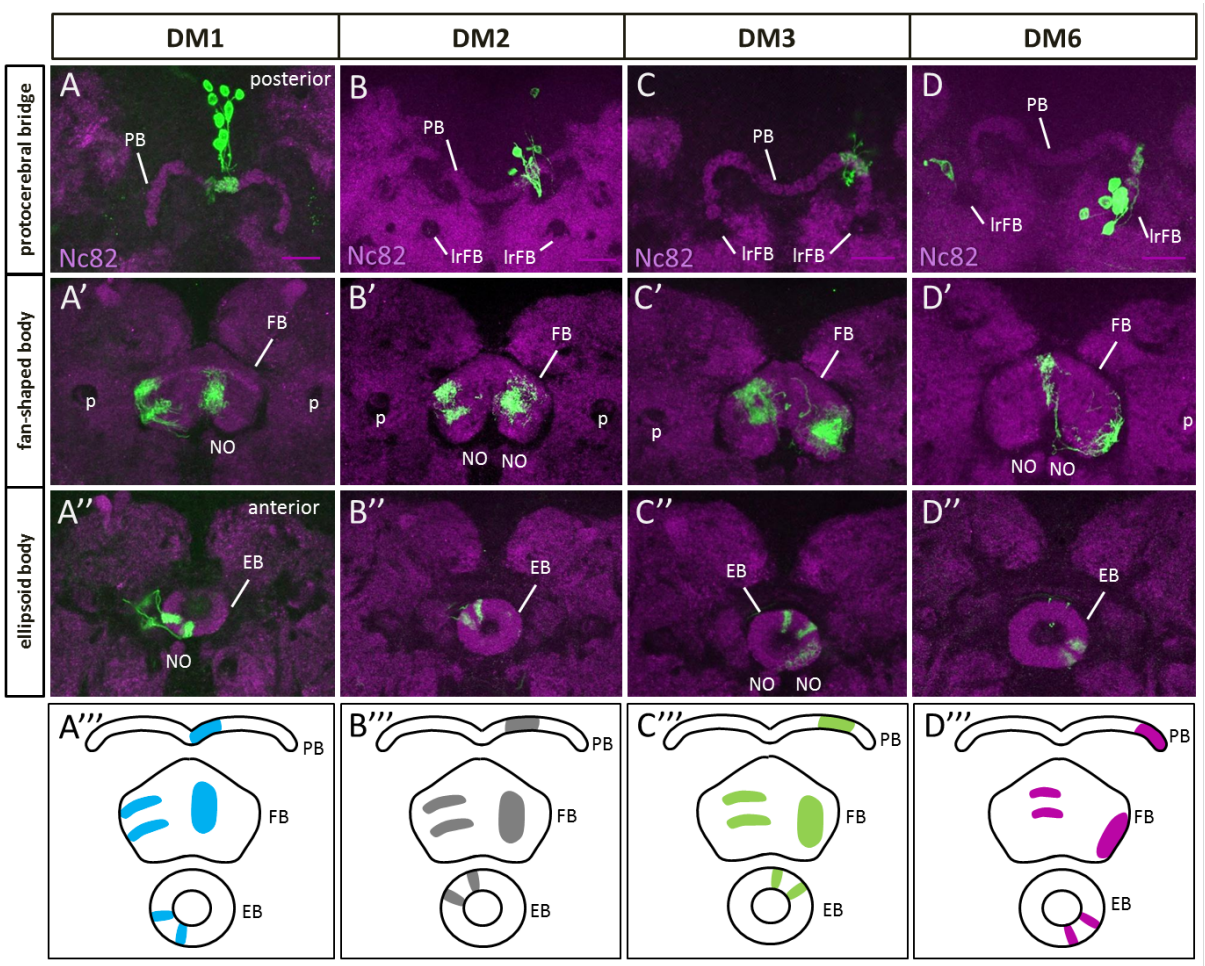


Figure 2.9 The processes of *R45F08-Gal4*-labeled neurons display a topological organization in the central complex at 50 after puparium formation (apf).
Embryonically induced flip-out clones of dorsomedial (DM) lineages DM1 to DM3 and DM6 showing distinct arborization pattern by *R45F08-Gal4*-labeled neurons in the protocerebral bridge, the fan-shaped body and the ellipsoid body of the central complex. *R45F08-Gal4* flip-out clones labeling (green), Nc82 labeling of neuropil (magenta). Maximum intensity projections of few adjacent confocal slices taken at different depths. **(A-A''')** DM1-derived midline associated cells projecting most medially on the ipsilateral side of the protocerebral bridge and the fan-shaped body and additionally innervating on the contralateral side of the fan-shaped body (most laterally) and the ellipsoid body. **(B-B''')** DM2-derived midline associated cells projecting adjacent to DM1 more laterally on the ipsilateral side of the protocerebral bridge and the fan-shaped body and additionally innervating on the contralateral side of the fan-shaped body (medially adjacent to DM1) and the ellipsoid body. **(C-C''')** DM3-derived midline associated cells projecting adjacent to DM2 more laterally on the ipsilateral side of the protocerebral bridge and the fan-shaped body and additionally innervating on the contralateral side of the fan-shaped body (medially adjacent to DM2). DM3 innervates the ellipsoid body on the ipsilateral side. **(D-D''')** DM6-derived midline associated cells projecting most laterally on the ipsilateral side of the protocerebral bridge and the fan-shaped body and additionally innervating on the contralateral side of the fan-shaped body (most medially). DM6 innervates the ellipsoid body on the ipsilateral side. EB, ellipsoid body; FB, fan-shaped body; lrFB, lateral root of fan-shaped body; NO, noduli; PB, protocerebral bridge; p, peduncle. Neuroanatomical nomenclature according to (Hartenstein et al., 2008; Pereanu et al., 2010). Scale bars 25 µm.

2.5 Discussion

In this report we used *pnt*-Gal4 lines to identify a specific subset of early-born neurons in four type II neuroblast lineages that contribute to a larval primordium of the central complex. Moreover, we demonstrated that this population of primordium neurons undergoes extensive growth and differentiation during metamorphosis, which results in a layer-specific innervation of the mature fan-shaped body of the adult. These findings reveal novel insights into the neural development of a larval brain primordium and well as into the integration of primordium neurons into the complex neuropil of the mature brain.

Previous studies have shown that a larval precursor of the fan-shaped body is present at the third larval instar stage (Pereanu and Hartenstein; 2006; Young and Armstrong, 2010b). However, neither the neurons that innervate this primordium nor the details of its structural organization were known. Using specific *pnt*-enhancer-Gal4 lines we have shown that neurons belonging to four identified pairs of type II neuroblast lineages innervate the larval fan-shaped body primordium. Remarkably, in the larval brain these neurons are involved exclusively in primordium innervation. The approximately 90 neurons identified by *R45F08-Gal4* labeling do not innervate any other part of the larval brain. This highly restricted innervation contrasts with the widespread innervation of many parts of the larval brain that other neurons in type II lineages form (Izergina et al., 2009; Bayraktar et al., 2010; Jiang and Reichert, 2012). This, in turn, suggests that the primordium neurons represent a distinct, highly specified set of type II lineage cells that differ in their developmental genetic program from the other neurons in these lineages. It is tempting to speculate that the specific activation of a *pnt*-enhancer subunit in these cells, reflected by the highly specific nature of *R45F08-Gal4* labeling, is a consequence of this developmental genetic program.

Our data indicate that the neurons labeled by *R45F08-Gal4* correspond to early-born cells in each of their lineages. This implies that the neurons that are born first in the lineage also establish the precursor of the mature neuropil, which subsequently becomes innervated by that lineage during later development. Interestingly, the type II primordium neurons do not appear to form functional synapses during larval stages, since the neural processes in the larval primordium do not express the synaptic marker Bruchpilot. This implies that the primordium consists of immature neural processes from the type II early-born cells that, therefore, are not likely to be involved in larval brain function. Hence, we posit that the main function of the larval primordium is to serve as a scaffold for innervation and differentiation by later-born type II lineage neurons during metamorphosis in pupal stages.

It is noteworthy that the larval primordium is already subdivided into modular units, notably a fourfold subdivision of the hemiprimordium located in each hemisphere. This modularization of the primordium could serve as a structural basis for establishing the more complex (16-fold) columnar modularization of the mature fan-shaped body. The fourfold organization of the two hemiprimordia is reflected in the four innervating neurite tracts. Each of these four tracts derives from one of the four identified type II neuroblast lineages in each hemisphere. This modularization can be documented due to the highly restrictive labeling of the primordium neurons by *R45F08-Gal4*, which also makes it possible to deduce further organizational features of the primordium and its neural innervation. For example, the fact that the two hemiprimordia are interconnected by a prominent set of commissural fascicles indicates that neurons, whose cell bodies are located in one hemisphere, might innervate modular units on both sides of the primordium. Clonal labeling of the primordium-forming neurons indicates this is indeed the case. Moreover, there appears to be a lineage-specific topological order of projections from these early-born neurons to the primordium, and this topological order is retained by these neurons during subsequent fan-shaped body development. This general type of 'bilateral' innervation is typical of many of the numerous small-field neurons that connect small subdomains of compartments such as the fan-shaped body into an ordered array in the mature central complex (Hanesch et al., 1989; Young and Armstrong, 2010a).

A remarkable feature of the primordium neurons is that they persist throughout metamorphosis and become integrated into the mature central complex, where they form prominent layer-specific innervation of the fan-shaped body. This requires a marked growth and differentiation of these neurons' innervation pattern from the non-layered innervation seen in the fan-shaped body primordium through successive stages of innervation expansion manifest as the central complex neuropil grows in early pupal stages, to the restricted pattern of innervation in two layers of fan-shaped body in the mature central complex at the end of metamorphosis. This restricted laminar innervation pattern in the adult suggests that these neurons might play specific functional roles in adult circuitry. Examples for the laminar regionalization of function are the neurons arborizing in specific horizontal strata of the fan-shaped body that have been shown to play important roles in visual memory (for example, (Liu et al., 2006). Given the highly specific genetic access to the fan-shaped body primordium neurons provided by *R45F08-Gal4*, it should be feasible to investigate the role of these cells in behaving adult animals through targeted transgenic activation/inhibition and optogenetic methods. It is noteworthy that the number of (*R45F08-Gal4*-labeled) neurons that persist in the adult is comparable to that observed in the late larval brain. This implies that little, if any, cell death occurs in these early-born type II lineage neurons during metamorphosis. This contrasts with the pronounced degree

of programmed cell death that has been reported to occur during postembryonic development in the overall ensemble of type II lineage cells (Jiang and Reichert, 2012), and provides further evidence for the notion that the early born differ in their developmental genetic program from other neurons in the type II lineages. The growth and differentiation of the primordium innervating neurons during metamorphosis is accompanied by a marked morphogenetic reorganization of the central complex itself. In addition to the overall growth of the neuropil due to increasing innervation and synapse formation, the most prominent aspect of this morphogenesis is the fusion of initially paired, bilaterally symmetrical hemiprimordia into an (apparently) unpaired midline neuropil, and this is seen both for the fan-shaped body and the protocerebral bridge.

2.6 Conclusions

Taken together, these findings indicate that early-born neurons from type II lineages have dual roles in the development of complex brain neuropil. During larval stages they contribute to the formation of a specific central complex primordium. During subsequent pupal development they undergo extensive growth and differentiation and integrate into the modular circuitry of the central complex of the adult brain. Thus, in addition to generating a large number of structurally diverse neurons, some of which comprise the intrinsic neurons of the central complex, and giving rise to specific glial cells, some of which ensheath the neuropil components of the central complex (Izergina et al., 2009; Bayraktar et al., 2010; Viktorin et al., 2011), the type II neuroblasts also appear to generate neurons that establish a larval scaffold-like structure for the mature central complex. This provides further support for the notion that type II neuroblasts are remarkably multipotent neural stem cells that can generate the neural primordium, the mature neuronal cells, and the glial cells for one and the same complex brain structure.

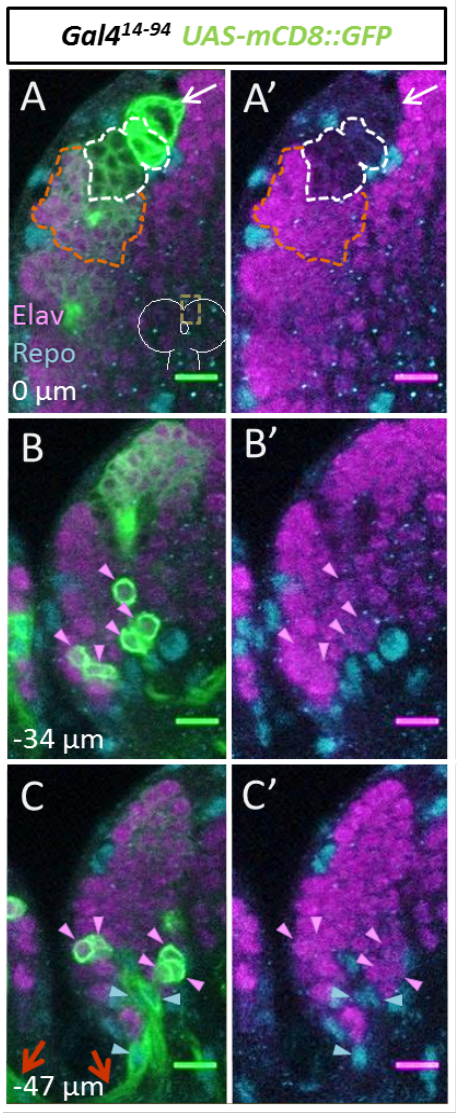
2.7 Authors' contributions

NR and GV carried out all the experiments. HR conceptualized the project. NR, GV and HR analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

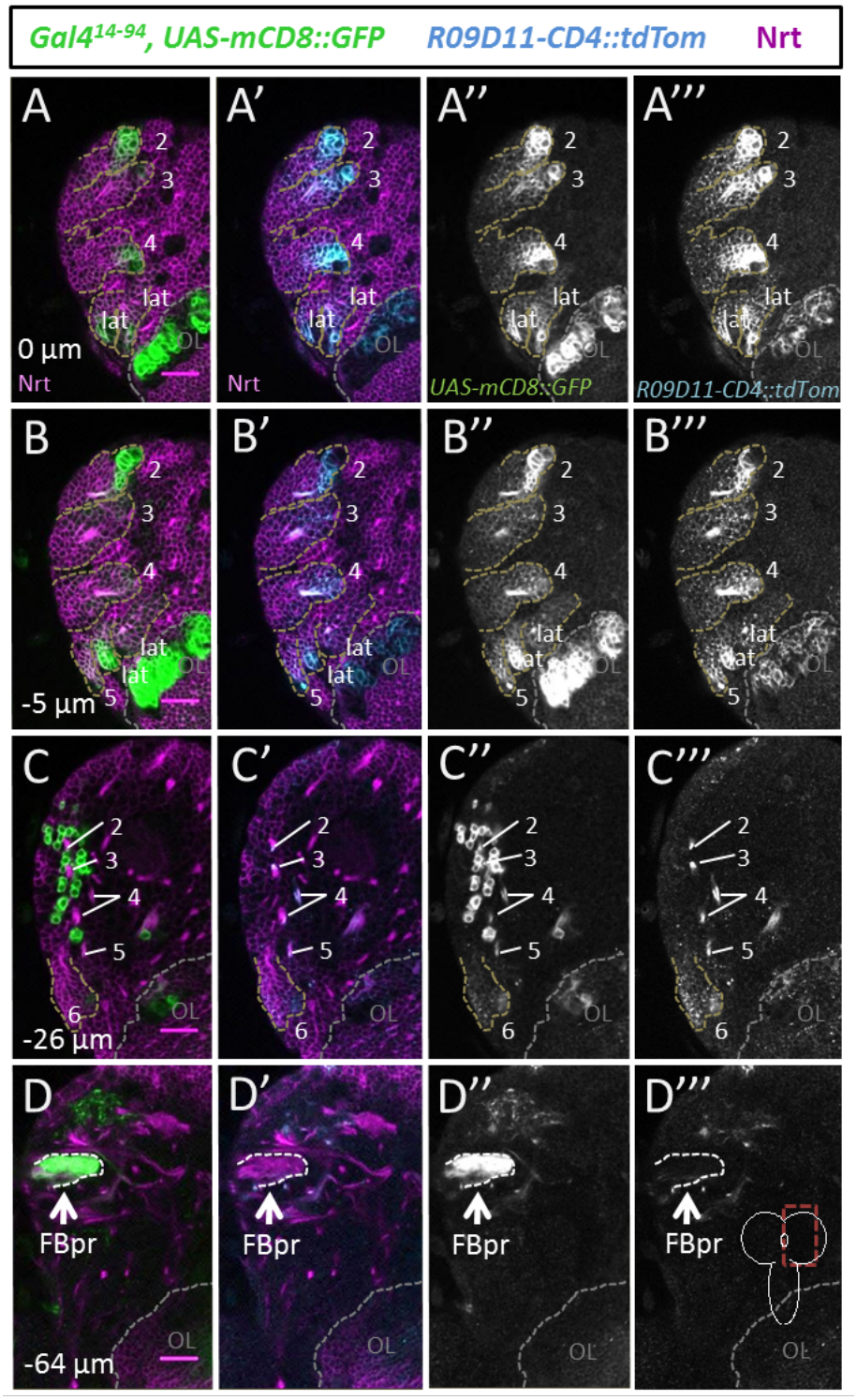
2.8 Acknowledgements

We thank Arnim Jenett, Bruno Bello, Yuh-Nung Jan, Veronica Rodrigues, Reinhard Stocker, Gerald Rubin, Barrett Pfeiffer, the Bloomington Stock Center and the DSHB for strains and antibodies. We also thank Susanne Flister for excellent technical help. This work was supported by the Swiss NSF.

2.9 Additional files



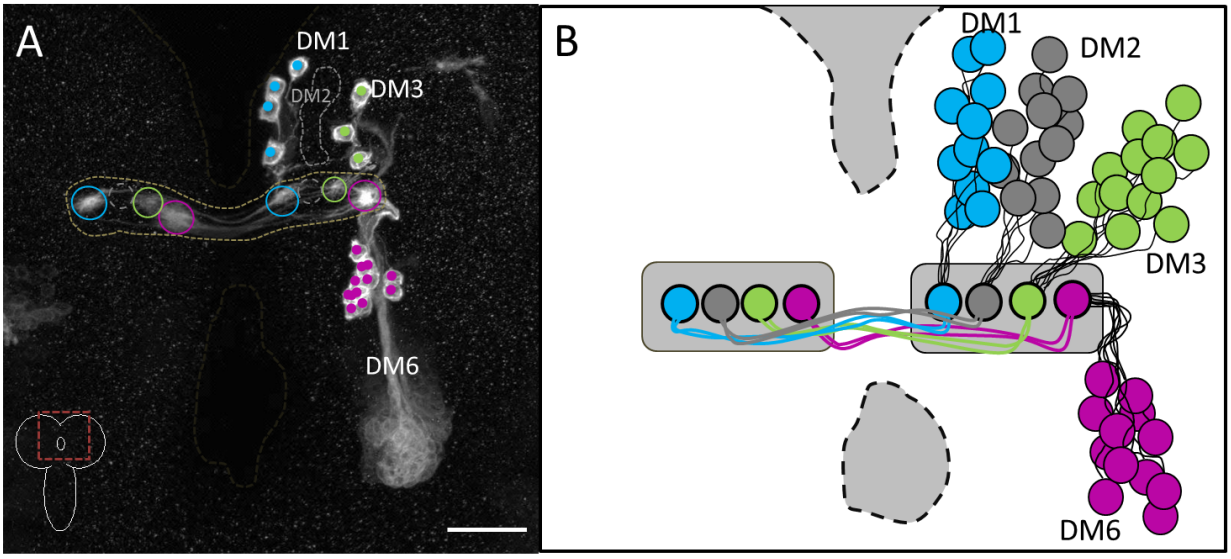
Additional file 1, Figure S2.1 *Gal4*$^{14-94}$ expressing neuroblast lineages contain neurons as well as glia cells.
(A-C) Dorsomedial (DM)1 lineage cells of *Gal4*$^{14-94}$ (green) at three different focal planes. The marker for differentiated glia (Repo) is in cyan and the marker for differentiated neurons (Elav) is in magenta. **(A)** Proximal to the considerably bigger neuroblast (arrow) there are closely associated, Elav-negative precursors (ganglion mother cells (GMCs) and intermediate neural progenitors (INPs), white dotted line), while many Elav-positive neurons are located more distally in the lineage (orange dotted line). **(B,C)** Closer to the commissure and even more distal to the neuroblast, the midline associated cells appear and show Elav expression (magenta arrowheads in (B) and (C)). At the level of the commissure (tracts crossing the commissure are indicated by red arrows in (C)) and even closer to the midline some glia are located (cyan arrowheads in (C)). Scale bars, 10 µm.



Additional file 2, Figure S2.2 Midline associated cells and fan-shaped body primordium are revealed by *Gal4^14-94* labeling.

Comparison of *Gal4^14-94* driven *mCD8::GFP* labeling and *R09D11-CD4::tdTom* labeling of type II neuroblast lineal cells in a late larval brain hemisphere**. (A-A''',B-B''',C-C''',D-D''')** Single confocal slices taken at four

different depths of the same brain. *Gal4*$^{14\text{-}94}$ labeling in green in (A-D) and in white in (A''-D''), *R09D11-CD4::tdTom* expression in cyan in (A'-D') and in white in (A'''-D'''), and neurotactin labeling of neuropil in magenta. (A-B''') Dorsomedial (DM) neuroblasts are labeled by *Gal4*$^{14\text{-}94}$ and not by *R09D11-CD4::tdTom* but newly born cells located closely to the neuroblast are labeled by both *Gal4*$^{14\text{-}94}$ and *R09D11-CD4::tdTom*. (C) At the level of the central brain neuropil, *Gal4*$^{14\text{-}94}$ but not *R09D11-CD4::tdTom* labels the midline associated cells that are arranged around the fascicles of the DM lineages. (D) At the commissural midline the fan-shaped body primordium is labeled by *Gal4*$^{14\text{-}94}$ but not by *R09D11-CD4::tdTom*. The numbers 2 to 6 correspond to lineages DM2 to DM6; DM1 is located in between the focal planes (C) and (D). FBpr, fan-shaped body primordium; lat, lateral DMs. Scale bars, 25 µm.



Additional file 3, Figure S2.3 The processes of midline associated neurons display a topological organization in the fan-shaped primordium at third larval instar.
(A) Embryonically induced flip-out clones showing one dorsomedial DM6 neuroblast clone and two multicell clones of DM1 and DM3 and their fan-shaped primordium processes revealing a highly ordered arborization pattern within the forming central complex structure. Primordium-forming neurons are indicated by colored dots and arborization areas within the fan-shaped primordium by circles. Different colors are assigned to the different DM-derived cells and processes (magenta for DM6, green for DM3, blue for DM1 and dotted grey for prospective DM2-derived cells and arborization pattern). **(B)** The topological order of arborizations of the DM1 to DM3 and DM6-derived primordium-forming cells shown in a schematic. Different colors are assigned to the different DM-derived cells and processes (blue for DM1, grey for DM2, green for DM3 and magenta for DM6). Scale bar, 25 µm.

3. A multipotent transit-amplifying neuroblast lineage in the central brain gives rise to optic lobe glial cells in Drosophila

Gudrun Viktorin*, Nadia Riebli, and Heinrich Reichert

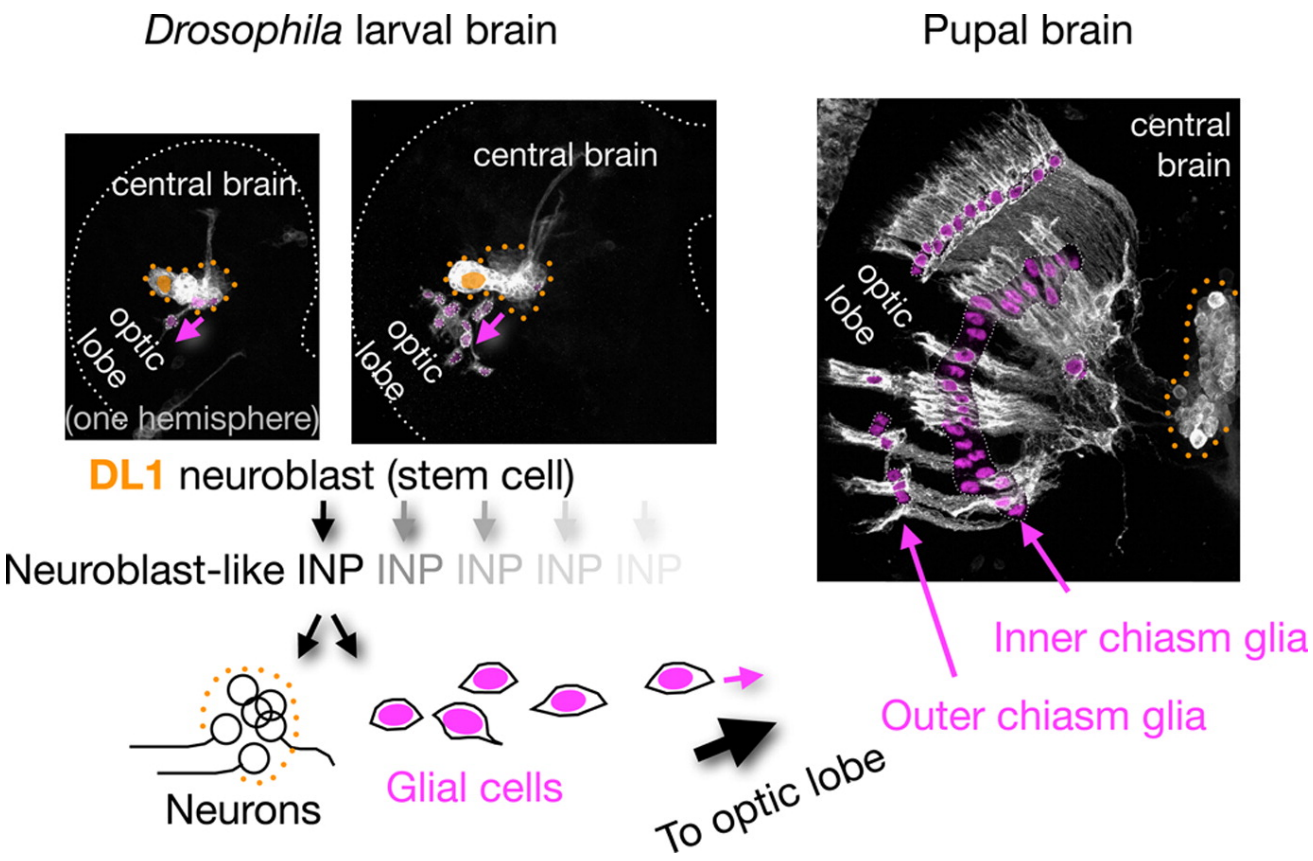
Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

*Corresponding Author

Accepted manuscript, Developmental Biology 2013, 379:2
doi: 10.1016/j.ydbio.2013.04.020
published by Elsevier

3.1 Summary

The neurons and glial cells of the Drosophila brain are generated by neural stem cell-like progenitors during two developmental phases, one short embryonic phase and one more prolonged postembryonic phase. Like the bulk of the adult-specific neurons, most of glial cells found in the adult central brain are generated postembryonically. Five of the neural stem cell-like progenitors that give rise to glial cells during postembryonic brain development have been identified as type II neuroglioblasts that generate neural and glial progeny through transient amplifying INPs. Here we identify DL1 as a novel multipotent neuroglial progenitor in the central brain and show that this type II neuroblast not only gives rise to neurons that innervate the central complex but also to glial cells that contribute exclusively to the optic lobe. Immediately following their generation in the central brain during the second half of larval development, these DL1 lineage-derived glia migrate into the developing optic lobe, where they differentiate into three identified types of optic lobe glial cells, inner chiasm glia, outer chiasm glia and cortex glia. Taken together, these findings reveal an unexpected central brain origin of optic lobe glial cells and central complex interneurons from one and the same type II neuroglioblast.



3.2 Introduction

During development, neural stem cells as primary progenitor cells proliferate through different modes of symmetric and asymmetric divisions to self-renew and initiate lineages that comprise the differentiated neuronal and glial cell types of the brain. The differentiated neural and glial cells are, however, not always generated directly by neural stem cells, they can also be produced by intermediate progenitor cells, also referred to as intermediate neural progenitors (INPs). INPs are secondary progenitors of more restricted proliferative potential that derive from the parent stem cell and that act as transit amplifying cells to generate the enormous number and diversity of cells required for the formation of complex brain circuitry (reviewed in Kriegstein and Alvarez-Buylla, 2009; Lui et al., 2011). In mammalian cortical development, numerous types of neurons and of glial cells, including oligodenrocytes or astrocytes are generated via INPs although the neurogenic and the gliogenic phases are usually separate (reviewed in Kriegstein and Alvarez-Buylla, 2009; Miyata et al, 2010). However due to the vast number of progenitors (neural stem cells and INPs) in the developing mammalian brain, it is not clear if the neurons, oligodendrocytes and astrocytes of the brain arise from distinct fate-restricted progenitors or if multipotent progenitors contribute progeny to glial as well as neuronal lineages.

Drosophila neural stem cells, called neuroblasts for historical reasons, are similar to vertebrate neural stem cells in many aspects of asymmetric cell division, self-renewal, and cell fate determination. Indeed, they are currently one of the best-understood models for neural stem cell biology (reviewed in Doe, 2008; Knoblich, 2008; Brand and Livesey, 2011; Homem and Knoblich, 2012). Recent work has shown that the neuroblasts of the Drosophila brain can be divided into two classes based on their proliferation pattern, called type I and type II. Type I neuroblasts generate their neural progeny through non self-renewing ganglion mother cells (GMCs) which divide only once to produce two postmitotic neural cells, neurons or glial cells. Type II neuroblasts generate their progeny through self-renewing INPs which have features of transit amplifying cells. Since an INP undergoes several rounds of proliferative cell divisions that each result in self-renewal of the INP and in the generation of a GMC which produces two neural progeny, a marked amplification of proliferation occurs (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008; Weng et al., 2010; reviewed in Weng and Lee, 2011; Saini and Reichert, 2012; Homem and Knoblich 2012). Most of the 100 central brain neuroblasts correspond to type I and each of these generates neural lineages consisting of 100-150 neural cells. In contrast, 8 identified brain neuroblast pairs correspond to type II and, due to transit

amplification of proliferation via INPs, each of these type II neuroblasts generates neural lineages consisting of an average of 500 cells.

Taken together, the 8 amplifying type II neuroblast pairs generate approximately one-fourth of the total number of neural cells in the Drosophila central brain. Thus, similar to the situation in the developing mammalian cortex, a significant proportion of the neural cells in the fly brain are generated by neural stem cells via transit amplifying INPs. However, until recently, the neural phenotypes of these INP lineage-derived cells as well as the circuitry to which they contribute were unknown. Recent studies that focussed on the 6 medial pairs of type II neuroblasts, referred to as PAN or DM1-6 neuroblasts (see Bello et al., 2008; Bowman et al., 2008), demonstrate that five of these type II neuroblasts, DM1-5, are multipotent neuroglial progenitors that contribute both neuronal and glial cells to a highly complex multimodal neuronal integration center called the central complex (Izergina et al., 2009; Bayraktar et al., 2010; Viktorin et al., 2011; Jiang and Reichert, 2012; reviewed in Boyan and Reichert, 2011).

Multipotent neuroglial progenitors have been characterized during embryogenesis of the ventral nerve cord in Drosophila (Beckervordersandforth et al., 2008; Jacobs et al.,1989; Klämbt and Goodman, 1991; Klämbt et al., 1991; Bossing et al., 1996; Broadus et al., 1995; Schmidt et al., 1997). Together with glioblasts, which generate exclusively glial cells, these (type I) neuroglioblasts produce several subtypes of ventral nerve cord glial cells comprising neuropil glia, cell body (cortex) glia and surface glia. During embryogenesis, neuroglioblasts also generate the glial cells of the larval central brain (Hartenstein et al., 1998; Hartenstein, 2011). However, most glial cells found in the central brain of the adult are generated postembryonically (Pereanu et al., 2005; Awasaki et al., 2008). While some of these adult-specific glial cells amplify their cells numbers through glial mitosis, the only neuroglioblasts identified to date in postembryonic brain development are the five type II progenitors that give rise to the substantial number of central complex glia through transient amplifying INPs (Viktorin et al., 2011).

In contrast to the 6 pairs of medially located type II lineages (DM1-6), which have been characterized in detail, the remaining two, more laterally located pairs of type II neuroblasts have not been studied further. Thus neither the proliferative properties of these type II neuroblasts, nor the phenotypes of the cells in their lineages, nor the role of these cells in the developing brain are currently known. In this report, we identify the lineages produced by the two laterally located type II neuroblasts, DL1 and DL2. We show that the DL2 lineage contains only neurons while the DL1 lineage comprises both neurons and glial cells, indicating that the

DL1 neuroblast, as well as its neurogliogenic INPs, are multipotent neuroglial progenitors. We then focus on the DL1 lineage and demonstrate that the neurons in this lineage contribute to the central complex while the glial cells in this lineage contribute exclusively to the optic lobe. Immediately following their generation in the central brain during the second half of larval development, these DL1 lineage-derived glial cells migrate into the developing optic lobe, where they differentiate into three identified types of optic lobe glial cells, including the prominent glia of the inner and outer chiasm. Taken together, these findings identify the type II DL1 neuroblast as a novel multipotent neuroglial progenitor that gives rise both to central brain interneurons and to optic lobe glial cells via transit amplifying INPs.

3.3 Materials and Methods

3.3.1 Fly strains and genetics

Flies were maintained on standard cornmeal-yeast-agar medium at 25°. *R09D11-CD4-tdTomato* (Han and Jan, 2011) in combination with *gcm-lacZ^{rA78}* (Jones et al., 1995) were used for identifying and distinguishing DL1 and DL2 neuroblast lineages. To generate wild type MARCM clones (Lee and Luo, 1999), we mated *y w hs-flp^{122}; tubP-Gal4, UAS-mCD8GFP^{LL5}/CyO, act-gfp^{JMR1}; FRT82B, tub-Gal80^{LL3}* (Bello et al., 2003) to *gcm-lacZ^{rA87}/CyO, act-gfp^{JMR1}; FRT82B* or *gcm-lacZ^{rA87}/CyO, act-gfp^{JMR1}; FRT82B, R09D11-CD4-tdTomato* males. For sparse clone induction, the time of heat shock was titrated to 6-8 minutes immersion in a 37°C water bath using a grape juice plate or bottle with an equal amount of cornmeal-yeast-agar medium. Eggs were collected for 2 hours, grown to first larval instar (22-26 hours after egg laying, h AEL), heat shocked, and grown to the desired stage at 25°C. To generate *R38H02-Gal4*-driven Flp-out clones expressing mCD8GFP or nuclear beta-galactosidase, we mated either of *UAS-flp; UAS-Flp^{JD1}/ CyO, act-gfp^{JMR1}; Actin>CD2>Gal4^{S}, UAS-mCD8GFP^{LL6}* females, or *UAS-flp, Actin>CD2>Gal4^{S}*; *UAS-Flp^{JD1}/CyO,act-gfp^{JMR1}; UAS-mCD8GFP^{LL6}*, *R09D11-CD4-tdTomato* females, or *UAS-flp; UAS-Flp^{JD1}, UAS-mCD8GFP^{LL1}/ CyO, act-gfp^{JMR1}; Actin>CD2>Gal4^{S}, UAS-mCD8GFP^{LL6}* females to *R38H02-Gal4* males (Jenett et al., 2012) or *gcm-lacZ^{rA78}*; *R38H02-Gal4* males. *Act5C>>Gal4* insertions were from Pignoni and Zipursky (1997), *hs-flp^{122}* and *Act5C>>nlacZ* from Struhl and Basler (1993). Eggs were collected for 1-2 hours and raised at 25°C to the desired stage. Larvae were kept at a maximum density of 170 larvae per bottle to avoid developmental delay due to food competition and to ensure exact staging. First and second instar larvae were distinguished according to air inflation of their tracheae, and confirmed after dissection according to the size and shape of the mouth hooks (Park et al., 2002).

3.3.2 Immunohistochemistry

Brains were prepared as previously described (Viktorin et al., 2011), including a ten minute Methanol incubation after fixation for larval brains labelled with anti-Neurotactin. We used chicken anti-GFP 1:500 (ab13970, Abcam, Cambridge, UK), rabbit anti-RFP (ab62341, Abcam) rabbit anti-beta-Galactosidase 1:500 (55976, MP Biomedicals, Solon, Ohio, USA), mouse anti-Neurotactin 1:20 (BP106, DSHB, Iowa City, Iowa, USA) (Hortsch et al., 1990), mouse anti-Neuroglian 1:10 (BP104, DSHB) (Bieber et al., 1989), mouse anti-Fasciclin III 1:20 (7G10, DSHB) (Patel et al., 1987; Ito and Awasaki, 2008), mouse anti-Repo 1:30 (8D12, DSHB) (Alfonso and Jones, 2002), mouse anti-Bruchpilot 1:10 (nc82, DSHB) (Wagh et al., 2006), rabbit anti-Repo 1:1000 (kindly provided by Veronica Rodrigues), mouse anti-phospho histone H3 1:500 (9706, Cell signalling technology, Danvers, MA, USA), rat anti-Deadpan monoclonal, undiluted (a gift from Cheng-Yu Lee) (Weng et al., 2010), and Alexa-conjugated secondary antibodies 1:300 (A11039, A11077, A11036, A21236, Molecular Probes, Eugene, OR, USA).

3.3.3 Microscopy and image processing

Fluorescent images were taken on a Leica TCS SP5 confocal microscope, and processed using Fiji (Schindelin et al., 2012). All adjustments were linear and were performed on whole images. Left-right orientation of brains was not preserved. Cells were counted using the CellCounter plugin for Fiji/ImageJ (Kurt De Vos).

3.4 Results

3.4.1 Identification of the two lateral type II neuroblast lineages DL1 and DL2

Among the total of eight transit amplifying type II neuroblasts in each brain hemisphere, six are located at the posterior medial edge, and these have been referred to as PAN or DM1-6 (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008). Because these six lineages are easier to identify, most of the previous studies of type II neuroblasts and their lineages have focused on DM1-6 (Fig. 3.1A, arrowheads). In addition to these six lineages, there are two further type II neuroblast lineages located more laterally in each brain hemisphere. These two more laterally located type II lineages, like the DM1-6 lineages, are selectively labeled by an *earmuff* genomic enhancer-fragment driven reporter *erm^{R09D11}-CD4-tdtomato* (Pfeiffer et al., 2008; Han et al., 2011); their location in a third larval instar brain hemisphere is shown in Fig. 3.1A (arrows). Secondary axon tracts (SATs) from both of these lateral lineages initially project towards each

other, join, and then branch apart again (Fig. 3.1B-F). Confirming their identity as type II lineages, both of these lateral lineages contain Deadpan-positive mature INPs (Fig. 3.1G-H). These two lineages tentatively correspond to the CP2/3 lineage pair, however, based on SAT trajectory alone it has not been possible to distinguish further between the two (Pereanu and Hartenstein, 2006).

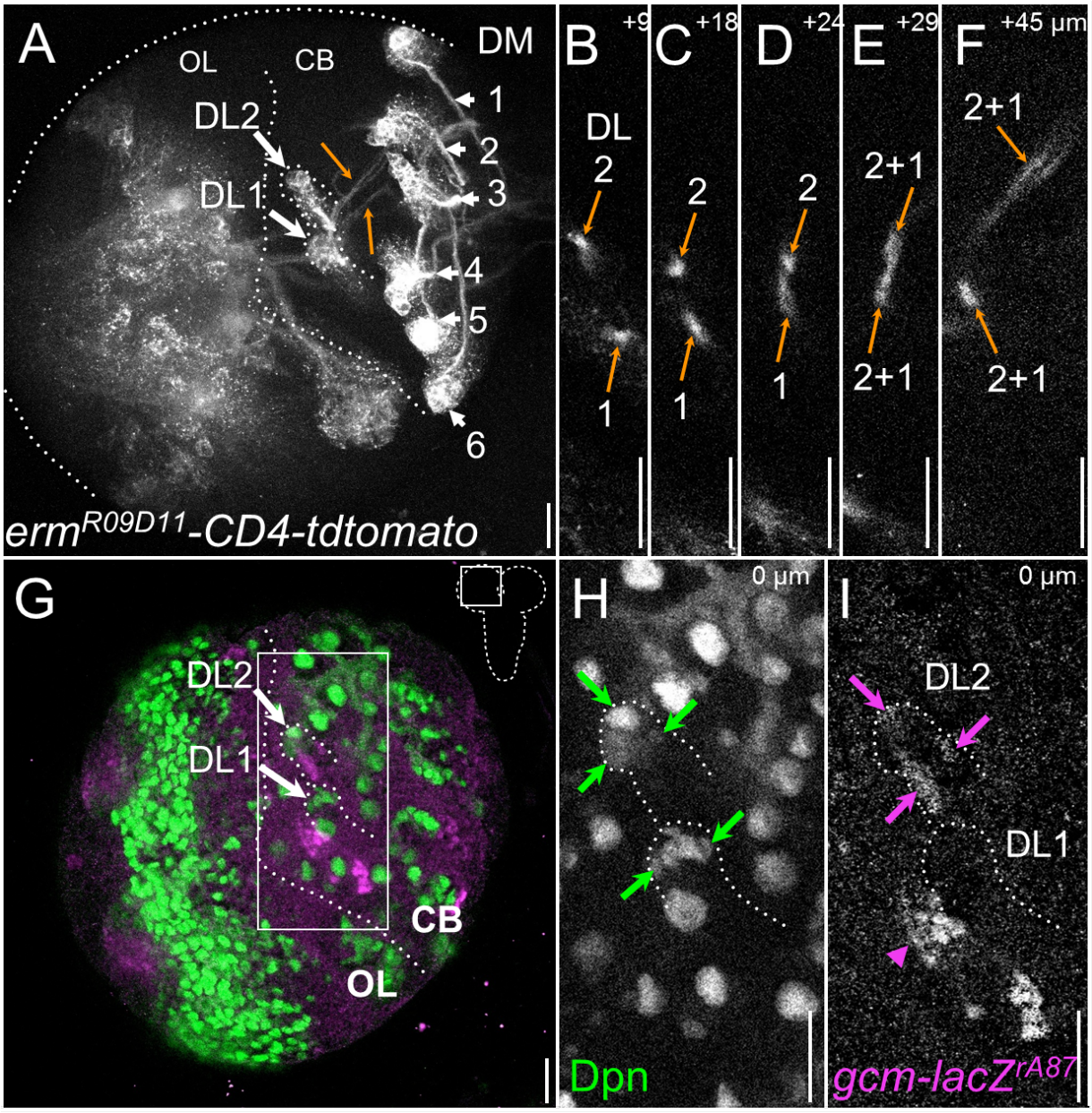


Figure 3.1 The two lateral type II lineages DL1 and DL2 are distinguished by expression of *gcm-lacZ^{rA87}*. **(A-I)** Hemisphere of a *gcm-lacZ^{rA87}/+ ; ermR09D11-CD4-tdtomato/+* transgenic larva at wandering third instar, labeled with anti-Dpn (green) and anti-beta-galactosidase (magenta); CD4-tdtomato expression is shown in white in (A-F). **(A)** Maximum intensity projection of confocal stacks of CD4-tomato expression.

Arrowheads point to medial type II lineages named DM1-6, arrows point to lateral type II lineages DL1 and DL2. **(B-F)** Single confocal slices following the secondary axon tracts (SATs) of DL1 and DL2 by CD4-tomato expression. The SATs fuse (B-D), then each lineage branches in the same manner (E-F). **(G-I)** Single confocal slice of Dpn-expressing INPs (green, green arrows) and *gcm-lacZ* expressing cells (magenta, magenta arrows) close to the neuroblasts of the DL1 and DL2 lineages (dotted outlines, white arrows). **(H-I)** Closeup of Dpn (H) and *gcm-lacZ* (I) expression within lineages DL1 and DL2. Both lineages have Dpn-positive INPs (green arrows in G) , but only DL2 has a band of *gcm-lacZ*-expressing cells in the proximal part of the lineage (magenta arrows in H). Magenta arrowhead in (I) points to the strongly *gcm-lacZ*-expressing lineage from Soustelle et al. (2007) that is not a type II lineage. CB, central brain. OL, optic lobe. Scale bars, 20 µm.

For individual identification of the two lineages in the late larval and early pupal brain, we took advantage of our finding that one of the two lineages reliably expresses *gcm-lacZ*^{rA87} (Jones et al., 1995) in a band of cells located near the outer lineage surface close to the neuroblast while the other does not (Fig. 3.1G,I). (Both lateral type II lineages lie close to a type I lineage with a similar, but consistently stronger, band of *gcm-lacZ* expressing cells (Fig. 3.1I, Fig. 3.2B-C) that has been previously described by Soustelle and Giangrande (2007), who also showed that the *gcm* gene does not have a gliogenic role in this lineage.) We designate the lateral type II lineage, which does not have the proximally located band of *gcm-lacZ*-labeled cells, to be the DL1 lineage and, correspondingly, we refer to the other lateral type II lineage as the DL2 lineage. The relative positions of the DL1 and DL2 lineages show some variability in different preparations. In 87% of wandering third instar larval brain hemispheres the DL2 lineage lies dorsolateral to the DL1 lineage and in the remaining 13%, this orientation is reversed (n>30 hemispheres).

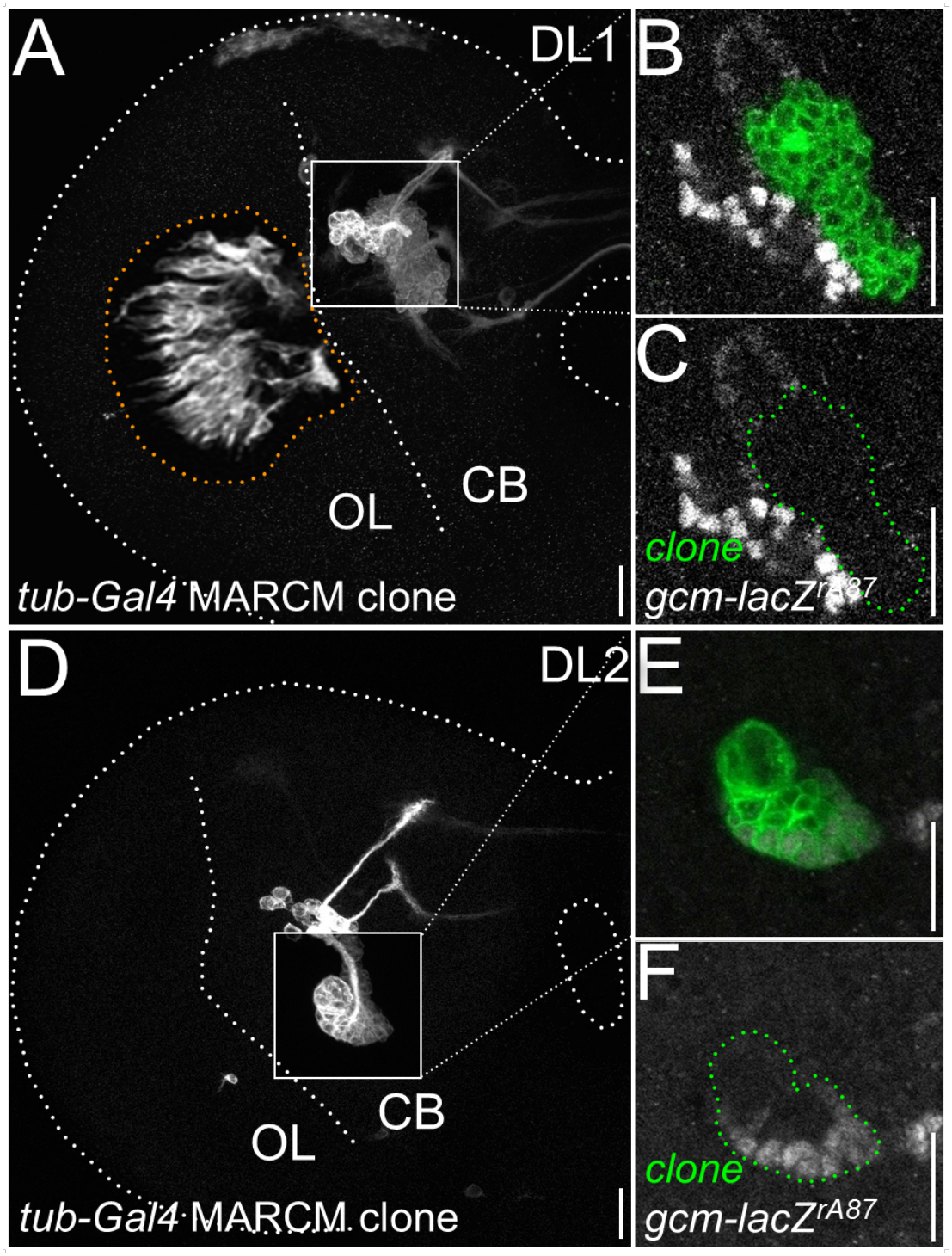


Figure 3.2 DL1, but not DL2, neuroblasts generate a large array of optic lobe glia.
tub-Gal4 MARCM clones induced at hatching and recovered at wandering third instar of DL1 **(A-C)**, and DL2 **(D-F)**. DL1 is associated with a large array of optic lobe glia (orange dotted outline in A), but no *gcm-lacZ* expression in the neuronal part of the lineage (B,C). The DL2 lineage is not associated with any other cells apart from the neuronal lineage itself, but has *gcm-lacZ* expressing cells in the neuronal part of the lineage (F). Large panels **(A,D)** are maximum intensity projections of the whole clone, small panels

(B-C, E-F) are single confocal slices at the level of *gcm-lacZ* expression, taken from the inset in (A,D); brightness and contrast is adjusted to different levels in insets to visualize a large variation in fluorescence intensities between neuronal part of the lineage and axon tracts. CB, central brain. OL, optic lobe. Scale bars, 20 μm.

3.4.2 The DL1 lineage contributes glial cells to the developing optic lobes, the DL2 lineage does not

Although their SAT trajectories in the larval brain are very similar, the DL1 and DL2 neuroblasts give rise to very different sets of neural progeny during postembryonic development. This is manifest in mosaic-based MARCM labeling experiments using *tub-Gal4* to drive *UAS-mCD8GFP* (Lee and Luo, 1999), in which recombination is induced after larval hatching and neuroblast clones are recovered at wandering third instar. Strikingly, DL1 neuroblast clones invariably comprised a cluster of cells in the central brain as well as a large array of large cells in the adjacent developing optic lobe (Fig. 3.2A). (100% of the DL1 neuroblast clones recovered from sparsely labeled MARCM brains (n=26) contained a central brain cell cluster and an optic lobe cell cluster, and no optic lobe cell clusters were found without a labeled DL1 lineage). We confirm their common origin by observing gliogenesis in young DL1 clones below (see Fig. 3.5). In contrast, DL2 neuroblast clones, identified by their proximal band of *gcm-lacZ*-labeled cells, only had central brain progeny and never contained optic lobe cells (Fig. 3.2D). (100% of the DL2 neuroblast clones recovered (n=15) contained cells in the central brain but not in the optic lobe.) These findings were independent of the relative position of DL1 and DL2 lineages, underscoring the fact that the proximal band of *gcm-lacZ*-expressing cells is an identifying characteristic of DL2 versus DL1 lineages.

The morphological features of the DL2 cell cluster in the larval central brain indicate that the cells in this lineage differentiate into typical secondary, adult-specific interneurons; their fate was not studied further in this report. The morphology of the two separate cell clusters generated in the DL1 lineage suggests that the central brain cluster corresponds to secondary, adult-specific interneurons and that the cluster of cells in the optic lobe corresponds to glial cells. The cells in the central brain manifest a tight cluster of their somata and have two prominent SATs that project dorsomedially towards the main commissural region of the brain. In contrast, the cells in the optic lobe are arranged in extensive arrays, have elaborated the elongated processes typical of glial cells, and appear to correspond to three different morphological types; their nuclei are relatively large and all express the glial marker Repo (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995), confirming their glial cell nature (see Fig. 3.3).

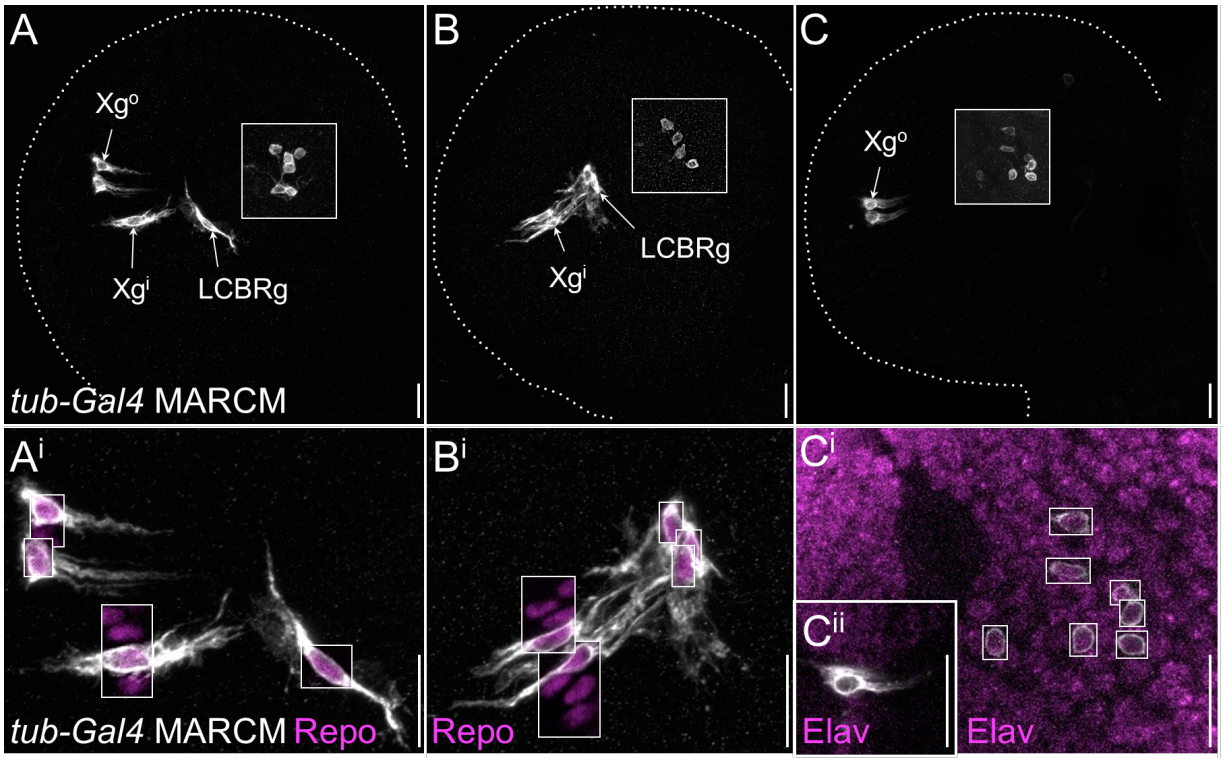


Figure 3.3 INP lineages are mixed neuronal and glial.
(A-C) Three examples of DL1 INP MARCM clones induced at hatching and recovered at wandering third instar; all three clones contain both neurons (white insets, enhanced contrast) and glial cells. Glial cell types (for discussion see Fig. 3.5) and numbers of glia vary between different INPs. Panels **(A-C, A^{i}-B^{i})** show maximum intensity projections. **(C^{i})** and insets in lower panels show single confocal sections with anti-Repo staining in glial nuclei (A^{i}, B^{i}), and anti-Elav staining in neuronal nuclei (C^{i}) but not glial nuclei (C^{ii}). Hemispheres are outlined in white dotted lines. Dorsal is to the top, medial to the right. Scale bars, 20 µm.

Like all other type II neuroblasts, the DL1 neuroblast generates its progeny through INPs that act as transient amplifying cells. To determine if these INPs can give rise to mixed sublineages containing both neuronal cells of the central brain and glial cells of the optic lobe, MARCM labeled INP clones were induced at larval hatching and recovered in late third instar brains. In all cases (n=20), these INP clones contained both glial cells in the optic lobe as well as neuronal cells in the central brain implying that INP sublineages contain cells of mixed fate. INP clones that contained glial cells but not neuronal cells were never seen. Closer inspection of the glial cells in these INP clones indicates that three different glial cell types can be generated; INP clones containing one, two, or all three of these glial cell types together with neuronal cells expressing the neuronal marker Elav were recovered (Fig. 3.3). This in turn implies that INPs have multipotent progenitor potential, at least for those clones labeled in out experiments, i.e. induced at larval hatching, or glial fate may not be entirely determined by lineage.

Based on their morphology and location in the third instar larval brain, we hypothesize that two of these glial cell types correspond to the outer chiasm glia (Xg^o, frequently referred to as medulla glia), and inner chiasm glia (Xg^i), both large glial cells that wrap the axon tracts of the two optic chiasmata (Tix et al., 1997; Chotard and Salecker, 2007; Hofmeyer et al., 2008; Edwards and Meinertzhagen, 2010; Hartenstein, 2011; Edwards et al., 2012). Xg^o were readily identified in late wandering third instar larvae and early pupae as the proximal most of three adjacent, dorso-ventral rows of glial nuclei, with distinctly larger nuclei than the two distal rows (compare Fig. 3.4A-D). The two distal rows of glial nuclei correspond to the epithelial and marginal glial cells of the lamina, in between which the R1-R6 photoreceptor axons terminate (Winberg et al., 1992; Poeck et al., 2001). We confirmed our recognition of these glial cells in MARCM clones of the eye disc that contain photoreceptor axons terminating between epithelial and marginal glial cells, as well as with two Gal4 drivers known to be expressed in Xg^o glial cells, *R25A01-Gal4* (Edwards and Meinertzhagen, 2012) and *mz97-Gal4* (Poeck et al., 2001). The single row of Xg^i glial cells with intercalated nuclei was easily recognizable from late third instar on, based on their very large oblong nuclei and their position in the center of the developing optic lobe where no other glial cells are present (Tix et al., 1997; Edwards and Meinertzhagen, 2012). We confirmed our recognition of Xg^i glial cells using *mz97-Gal4* expression (Poeck et al., 2001). The characteristic arrangements of Xg^o and Xg^i glial cells is most clearly visible in slowly moving, late third instar larvae close to pupariation and beyond. The third glial cell type that is derived from the DL1 neuroblast lies closest to the central brain and does not correspond to a known glial cell type at wandering third instar, but is hypothesized to develop into the cortex glia-like cells that are associated with adult DL1 clones (described in Fig. 3.9).

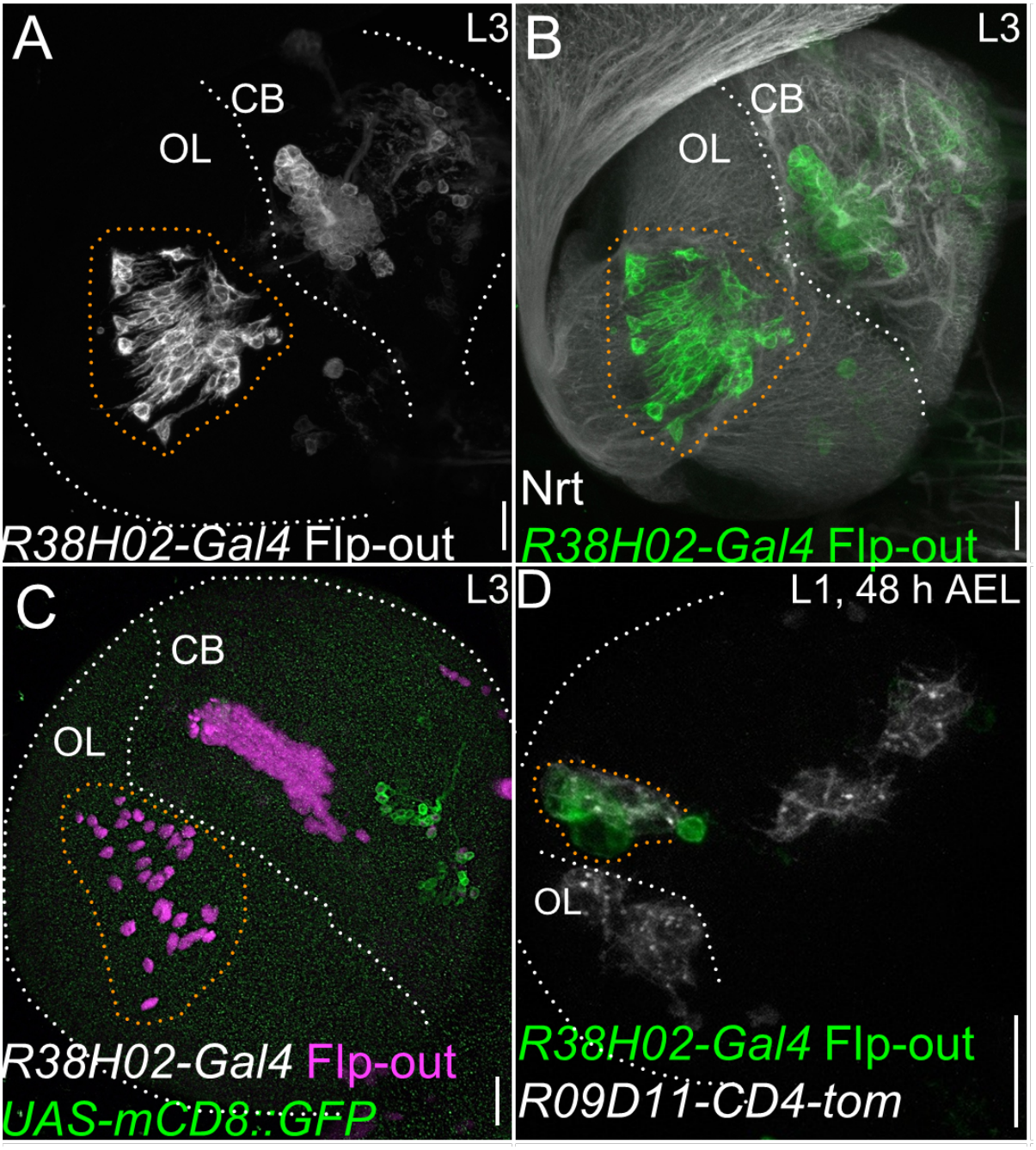


Figure 3.4 R38H02-Gal4 give rise to DL1 Flp-out clones.
(A-D) *R38H02-Gal4* driven DL1 Flp-out clones in late wandering third instar larval brain hemispheres using *act>>Gal4, UAS-mCD8::GFP* (white in A, green in B,D) and *UAS-mCD8::GFP; act>>nlacZ* (magenta in C). **(B)** is counterstained with anti-Nrt for orientation and identification of DL1 secondary axon tracts. **(C)** Expression of the *R38H02-Gal4* driver alone is limited to a few medial INP-like clusters at wandering third instar (green in C). **(D)** shows a young lateral type II clone at 48 hours AEL, close to the L1/L2 molt. The lineage is identified by its overlap with *R09D11-CD4-tomato* expression; the distinguishing features of DL1 and DL2 are not yet present at that stage. All panels show maximum intensity projections of whole brain hemispheres. Scale bars, 20 µm.

3.4.3 Gal4-based lineage tracing allows specific labeling of developing DL1 progeny

To confirm the glial cell types generated by the DL1 lineage, we first sought to identify molecular markers that would allow us to follow the development of the DL1 lineage cells through pupal stages and into the adult. To this end, we screened diverse Gal4 collections for drivers that might be appropriate for Flp-out based lineage-tracing experiments specific to DL lineages (see Materials and Methods). In these Flp-out experiments, screened Gal4 drivers were used in combination with *actin>>Gal4* (Pignoni and Zipursky, 1997), *UAS-mCD8::GFP* (Lee and Luo, 1999), and two copies of *UAS-flp* to visualize both current and lineage-traced expression of a given Gal4 driver. Alternatively, to see the current expression with UAS-mCD8GFP separate from the flp-out history of expression, *act>>nlacZ* was used as Flp-out reporter (Struhl and Basler, 1993).

In lineage tracing experiments of this type, the *R38H02-Gal4* line, which represents a neurotactin enhancer fragment (Jenett et al., 2011), allowed stochastic, but very specific labeling of DL1 progeny throughout postembryonic development. In approximately 12% of the late larval brain hemispheres recovered, *R38H02-Gal4* Flp-out clones target the DL1 lineage (n=31 DL1 lineages from 236 hemispheres) and reveal both the neuronal cell cluster in the central brain and the glial cells in the optic lobe, in many cases with little other brain expression (Fig. 3.4). Importantly, the DL1 lineage cell clusters labeled by *R38H02-Gal4* Flp-out and those labeled by MARCM clonal methods are very similar (compare Fig. 3.2A, 3.4A). In MARCM, labeling of only the DL1 lineage is achieved by adjusting the time of heat shock-driven clone induction such that only one or very few lineages are labeled in any one brain (see Material and Methods). In *R38H02-Gal4* Flp-out, induction of DL1 clones is a feature of the *R38H02* enhancer fragment in combination with the Flp-out stock used. (In this sense, the *R38H02-Gal4* Flp-out used behaves analogous to the direct *ey*-Flp and *repo*-Flp fusion constructs that have been used to generate eye disc and glial cell clones (Newsome et al., 2000; Silies et al., 2007). All *R38H02-Gal4* Flp-out clones that comprised an optic lobe glial array could be identified unambiguously as DL1 by the same means as the randomly induced MARCM clones, namely as the lateral type II lineage that shares an SAT with the other lateral type II lineage expressing *gcm-lacZ* (n=22 clones). This result confirms that *R38H02-Gal4* Flp-out labels DL1 and not a similar lineage that may also produce optic lobe glial cells, but may not be easily recovered by our MARCM conditions. The DL1 lineages labeled by *R38H02-Gal4* Flp-out (in wandering third larval instar brains) have a slightly higher number of glial cells in the optic lobe (32±6) than corresponding MARCM labeled DL1 lineages (27±4), but are otherwise indistinguishable from MARCM clones. Moreover, labeled INP-like sublineages appear as well at high frequency in *R38H02-Gal4* Flp-out

experiments, indicating that the *R38H02* enhancer fragment can also be active in DL1 lineal INPs.

To identify the onset of expression of *R38H02-Gal4* Flp-out clones in DL1, we dissected brains of newly hatched larvae and at the L1/L2 transition, 48 h AEL. At 0-4 hours after larval hatching, we found no cells or clones that overlapped with *R09D11-CD4-tdTomato* expression. At 48h AEL however, in L1 as well as L2 larvae, numerous lineages coincided with *R09D11-CD4-tdTomato* expression in the lateral central brain (Fig. 3.4D). These data suggest that *R38H02-Gal4* Flp-out DL1 clones label the postembronic progeny of the DL1 neuroblast and sets the onset of expression to the first instar.

3.4.4 DL1 derived glial cells are generated in the central brain and migrate into the optic lobe

From the late third larval instar onward through pupal development and in the adult (compare Fig. 3.8,3.9), the putative DL1- derived glial cells are located in the optic lobe and, are clearly separated from the DL1 derived neuronal cells in the brain hemisphere. However, since we hypothesize that both the glial cells and the neuronal cells are lineal progeny of the same brain neuroblast, the glial cells like the neuronal cells in the DL1 lineage are likely to be generated in the central brain. This, in turn, implies that the DL1 glial cells (or their intermediate progenitors) translocate from their site of origin in the central brain into the nascent optic lobes during larval development.

To investigate this, we again used *R38H02-Gal4* Flp-out labeling as well as MARCM clonal labeling to study the spatiotemporal development of DL1-derived glial cells during larval stages. Our experiments indicate that the initiation of gliogenesis in the DL1 lineage occurs in the late second larval instar (Fig. 3.5, Fig. S3.1, Table 3.1). At 61h AEL (after egg laying), corresponding to the late second instar, we recovered MARCM labeled DL1 neuroblast clones (n=7) and INP clones (n=5), and found that the neuroblast clones did not contain Repo-positive glial cells whereas the INP clones did (Fig. 3.5A,B). (Note that MARCM recombination in a type II neuroblast stochastically labels only one of its two daughter cells - either the neuroblast, or the INP, and their respective progeny.) The presence of Repo-positive glial cells in INP clones but not in their sister neuroblast clones at 61 h AEL indicates that at that time, gliogenesis has been initiated in the first INPs but not yet in the subsequently generated INPs contained within a labeled neuroblast lineage. This sets the time of onset of gliogenesis in the DL1 lineage at around 61 h AEL, or approximately 5-10 hours prior to the L2/L3 transition.

Table 1
Timing of gliogenesis in DL1 neuroglioblast lineages.

stage and age of animals [hours AEL or APF]	Repo+ cells total	Repo+ cells in neuronal part of lineage	Repo+ cells, migrating or optic lobe	number of clones	Method
L2 61 h	0	0	0	7	MARCM
L2 66–72 h	3 ± 2	1–2	0–1	10	MARCM
L3 66 h	3 ± 1	4 ± 2	0–2	7	MARCM
L3 72–80 h	11 ± 4	8 ± 2	3 ± 3	7	MARCM
L3 72 h	10 ± 2	5 ± 2	6 ± 2	7	Flp-out
L3 84–90 h	27 ± 5	0.8 ± 0.6	26 ± 6	10	Flp-out
L3 96–102 h	32 ± 5	0	32 ± 5	7	Flp-out
L3 wandering	32 ± 6	0	32 ± 6	17	Flp-out
L3 wandering	27 ± 4	0	27 ± 4	12	MARCM
Pupa 0–15 h	29 ± 6	0	29 ± 6	6	MARCM
Adult	27 ± 7	0	27 ± 7	4	MARCM

Table 3.1 Timing of gliogenesis in DL1 neuroglioblast lineages.
Counts of Repo-positive cells within DL1 neuroglioblast clones at different stages; values are mean ± standard deviation. Gliogenesis in the DL1 lineage begins at late second instar, stops abruptly before mid-third instar, and glial numbers do not increase further until the adult. Newly formed glia migrate away immediately, so that the number of glial cells located within the neuronal part of the lineage (containing the neuroblast and neuronal cell bodies) remains low throughout the period of gliogenesis.

Gliogenesis in the DL1 lineage continues rapidly during development around the L2/L3 (second larval instar/third larval instar) molt. At 66-68 h AEL, shortly before the molt, all DL1 neuroblast clones (7 MARCM clones, 5 *R38H02-Gal4* Flp-out clones) contained 1-3 Repo-positive cells (Fig. 3.5C-D; Fig. S3.1A-B) and in some cases, one of these Repo-positive cells had already extended a cytoplasmic process and appeared to translocate towards the optic lobe (data not shown). In MARCM and *R38H02-Gal4* Flp-out labeled neuroblast clones recovered at 72h AEL, shortly after the L2/L3 molt, the number of Repo-positive cells had increased and most of these glial cells appeared to be migrating out of the central brain and into the optic lobe (Fig.3.5E-H, Fig. S3.1C-D). Througout the early L3 stage, numerous new glial cells were formed and migrated out rapidly, as seen by the large increase in glial cell numbers from L2 to L3 (Table 3.1). All of these migrating glial cells entered the optic lobe between the posterior borders of the IPC (inner proliferation center), the structure that abuts the central brain and forms part of the optic lobe neuroblasts (see Egger et al., 2007 for posterior view). However, the number and spatial

arrangement of the glial cells seen migrating through the IPC was variable in different preparations (compare Fig. 3.5F-H). At 84-85 hours AEL, many migrating glial cells had passed between and beyond the IPC, and had begun to spread out laterally along the distal surface of the IPC (Fig. 3.5I-J, Fig. S3.1E-F). All of these migrating glial cells appeared to be interconnected by (*mCD8-GFP*-labeled) cellular processes, some of which also extended to the cluster of DL1-derived neuronal cells in the brain hemisphere (orange arrows in Fig. 3.5F,H,J and Fig. S3.1D,F). The processes that connected glial cells with neuronal cells in the lineage were observed in all brains up to 90 h AEL; afterwards, these processes began to detach, and were not detectable at 108 h AEL and beyond (data not shown).

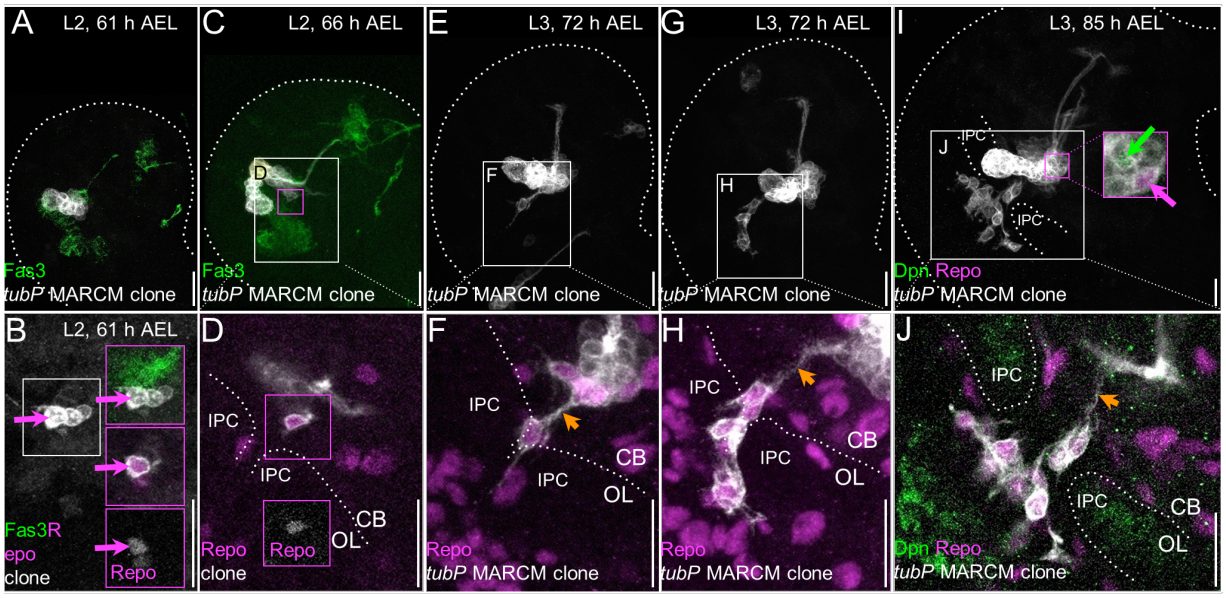


Figure 3.5 Time line of glia formation and their migration into the optic lobe.
(A-J) Maximum intensity projections of *tub-Gal4* MARCM clones (white) in larval brains. Clones were induced at hatching and recovered at the stages and time points indicated in the top right corner of each panel. Hemispheres are outlined (dotted lines). (D,F,H,J) as well as insets in (B) are either magnified single confocal slices or maximum intensity projections of few slices to show Repo+ glial cells (magenta) within the clones shown in the panels above, regions outlined in white. Anti-Fas3 (green in A-C) or anti-Dpn (green in I,J), together with anti-Repo, identify DL1 lineages and the optic lobe proliferative epithelia (IPC, inner proliferation center); dotted lines in (D,F,H,J) delineate the border between optic lobe (OL) and central brain (CB). - **(A,B)** Two clones from 61 hour AEL L2 larval brains; the DL1/2 neuroblast clone (A) has no Repo+ cell yet, but the DL1 INP clone (B) consists of one Repo+ glial cell in addition to four other cells. **(C,D)** DL1 neuroblast clone in an L2 larval brain shortly before the L2/L3 molt; the clone contains one Repo+ cell that has not yet migrated away from the lineage (inset). **(E-H)** Two DL1 neuroblast clones from L3 larval brain shortly after the L2/L3 molt with several Repo+ cells migrating away from the base of the lineage between the two posterior ends of the IPC epithelia into the optic lobe. The glial cells remain physically connected to the lineage (orange arrowheads). **(I-J)** At 85 hours AEL, more glial cells have formed and the glia have spread inwards and sideways beneath the inner surface of the IPCs. Scale bars, 20 µm.

These glial cells may be formed either by differentiation of cells from the lineage or by cell division of newly formed glial cells. In order to examine the contribution of glial proliferation to the increase in glial cells, we labeled *R38H02-Gal4* Flp-out clones with anti-pH3 antibody. The largest increase in glial cells occurs in early third instar, between 72 h and 84 h AEL (Table 3.1). If the observed increase was due to proliferation of newly formed glial cells, we should observe many pH3-positive glial cells derived from DL1 clones at early third instar. However, at 72 h AEL we found only one pH3-positive glial cell among 77 migrating glial cells from 17 lineages, and 59 Repo-positive cells within the lineage were pH3-negative. Likewise, between 84 and 108 hours AEL, less than 1% of glial cells in >30 DL1 Flp-out clones were pH3-positive. These data indicate that newly formed glial cells can divide, but mitotic proliferation is a minor contribution to the observed increase of DL1-derived glial cells during early third instar.

Since we did not observe any glial cells within the neuronal DL1-derived cell cluster located in the central brain of wandering third instar larva, gliogenesis presumably ceases during the second half of the third larval instar. To determine this more precisely we used *R38H02-Gal4*-based lineage tracing to quantify the number of glial cells that had already migrated and the number of glial cells that were still located in the central brain at different time points (Table 3.1). At 84h and 90 hours AEL, the number of glial cells migrating or already in the optic lobe had increased to almost the same number found in wandering third instar larvae, and only single glial cells remained in the central brain cluster. From 96h AEL onward no new nascent glia were found within the central brain cell body cluster; the number of labeled glial cells in the optic lobe then remained the same until the adult (MARCM clones again had a slightly lower number of glial cells compared to *R38H02-Gal4* based lineage tracing).

Soon after migrating into the optic lobe, the chiasm glia started to extend their processes around axon tracts. Between 102 and 106h AEL, outer chiasm glial cells were beginning to extend processes around nascent axon tracts between the proximal medulla and the lobula primordia (Fig. 3.6A,B). Inner chiasm glial cells started wrapping inner chiasm axon tracts between 114 and 120 h AEL (Fig. 3.6C,D). In contrast, the glia of the lateral cell body rind were seen to envelop the first cortex cell bodies only in the second half of pupariation (data not shown).

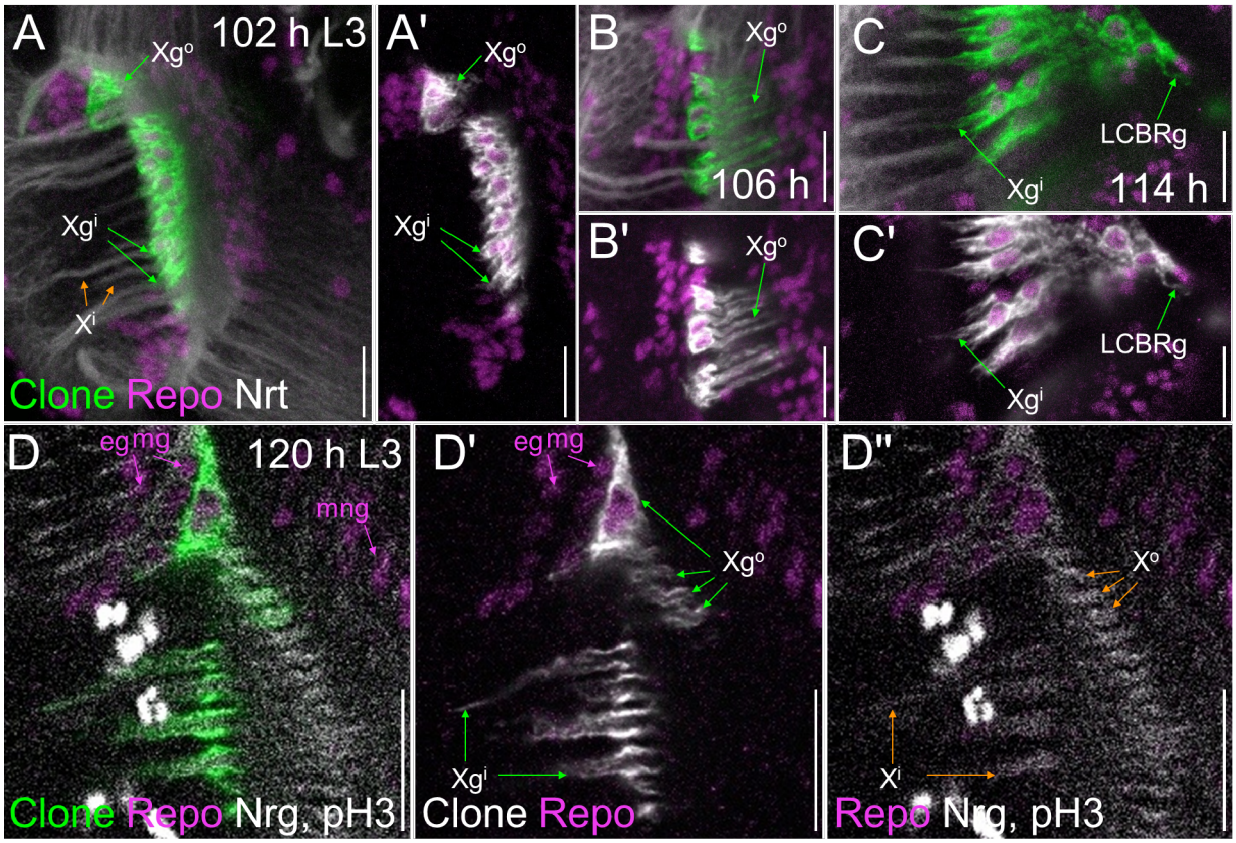


Figure 3.6 Chiasm glia differentiate during the second half of third instar.
(A) Inner and outer chiasm glia from *R38H02-Gal4* Flp-out clones have aligned in rows at the base of their axon tracts by 102 h AEL, and extended very short processes (green arrows) along the chiasm axon tracts that have already developed (orange arrows, developing inner chiasm tracts). **(B)** Outer chiasm glia (Xg^o) differentiate first and have begun to wrap axon tracts by 106 h AEL (green arrow). **(C)** Inner chiasm glia (Xg^i) only begin to extend processes along inner chiasm axon tracts at 114 hours AEL (green arrow in C). **(D)** Both Xg^i and Xg^o have wrapped most of the length of their tracts by 120 hours AEL. Panels show single confocal slices (D) or maximum intensity projections of few adjacent slices (A-C). Scale bars, 20 µm.

The gliogenesis period in DL1 begins slightly earlier than that of other type II lineage-derived glial cells such as the central complex glial cells generated by DM1-3 (Viktorin et al., 2011). Like most other glial cells, the DL1-derived optic lobe glia initially express *gcm-lacZ* shortly before expressing Repo (Fig. 3.7A), and this expression of *gcm-lacZ* gradually diminishes. Thus, at wandering third instar, *gcm- lacZ* is almost absent from DL1-derived optic lobe glia, while the later formed central complex glia as well as many surrounding optic lobe glia of different origin, such as medulla neuropil glia and the lamina glia from the glia precursor center, still express it (Fig. 3.7C-D).

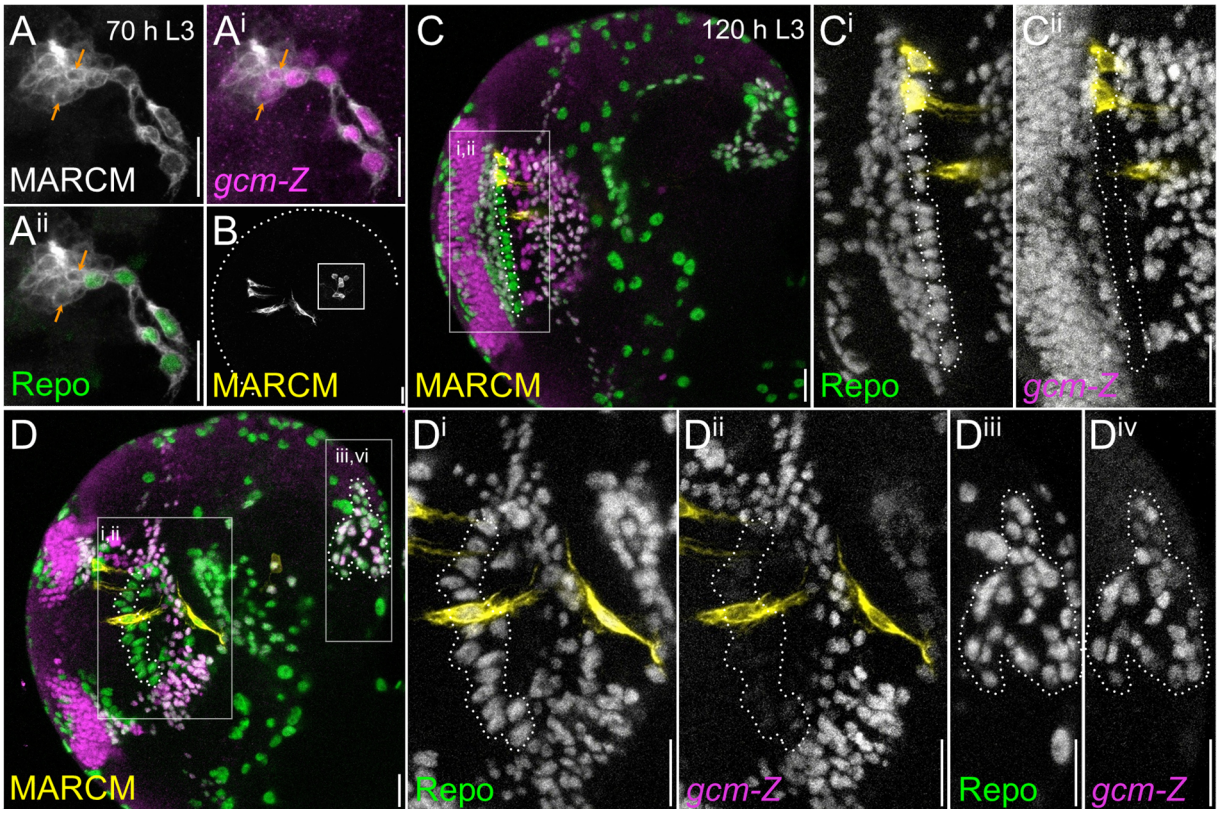


Figure 3.7 *gcm-lacZ* expression reflects the early formation of DL1-derived optic lobe glia.
(A-A^{ii}) In early third instar, all Repo-positive cells (green in A'') in DL1 MARCM clones (white) also express *gcm-lacZ* (magenta in A^{i}), but several cells per clone express only *gcm-lacZ* but not (yet) Repo (orange arrows). By 120 hours AEL, in late wandering third instar larvae, *gcm-lacZ* expression is lost (C^{ii}), or almost lost (D^{ii}) from all outer and inner chiasm glia (dotted outlines in C-D^{iv}), which distinguishes these glia clearly from the surrounding glia in the optic lobe, as well as from the central complex glia (D^{iii}) that originate from medial type II lineages at a later time. The DL1 INP clone in (B-D^{iii}) (yellow) is the same clone as in Fig. 3.4A. Scale bars, 20 µm.

Taken together, these results indicate that gliogenesis in the DL1 lineage begins at the end of L2 and is most prolific during the first half of L3, with most glia being generated within a few hours after the L2/L3 molt. Their cell division rate is low, indicating that DL1-derived glial cells are primarily generated by differentiation of neuroblast progeny, rather than by mitotic proliferation of newly formed glial cells. Moreover, the spatiotemporal features of DL1 gliogenesis imply that once the glial cells are generated, they rapidly migrate out of the central brain and into the developing optic lobe and begin to wrap around axon tracts.

3.4.5 The DL1 lineage gives rise to neuronal cells in the central brain and three identified types of glial cells in the optic lobe

Given their excellent correspondence, we used *R38H02-Gal4* Flp-out clonal labeling together with MARCM clonal labeling to follow the lineal fate of the two DL1 cell clusters during metamorphosis in the pupa and in the mature brain of the adult. Fig. 3.8 shows labeled DL1 clones in pupae at 10h and 24h APF (after puparium formation) co-immunolabeled with the glial cell specific marker anti-Repo and antibodies against neural cell adhesion molecules BP106/anti-Nrt and BP104/anti-Nrg to label secondary axon tracts. At both time points, the DL1 lineage is clearly composed of the two cell clusters, one in the central brain and the other in the optic lobe (Fig. 3.8A,E).

The most distal layer of glial cells corresponds to outer chiasm glia (Xg^o). At the pupal stages examined, their cell bodies are aligned in a curved row that extends from dorsal to ventral along the distal edge of the medulla, immediately adjacent to two rows of lamina glia called epithelial glia and marginal glia (Fig. 3.8B-D). Outer chiasm glial cells have three principal processes; one extends distally into the lamina (Fig. 3.8H), one extends caudally and distally along the edge of the lamina and at the border to the medulla (Fig. 3.8I), and the longest process follows axon tracts that run from the distal to the proximal medulla that are labeled by anti-Nrg but not anti-Nrt antibodies (Fig. 3.8B,F,G). The more proximal layer of glial cells corresponds to inner chiasm glia (Xg^i). Their nuclei are also aligned in a curved row that lies between the medulla and the lobula and runs parallel to the row of outer chiasm glia (Fig. 3.8C-D,G-H). These glial cells extend processes distally and proximally, wrapping the thick axon bundles of the inner optic chiasm (Fig. 3.8G-H), as well as caudally into the developing lobula complex (Fig. 3.8D,I). A third glial cell type is located at the border between optic lobe and central brain and extends thin processes along axon tracts that interconnect optic lobe and central brain in the pupa (LCBRg; Fig. 3.8C,G-H). We tentatively refer to this type of glia as LCBRg since they subsequently acquire features of cortex glia of the lateral cell body rind (LCBR) found in the adult (see Fig. 3.9).

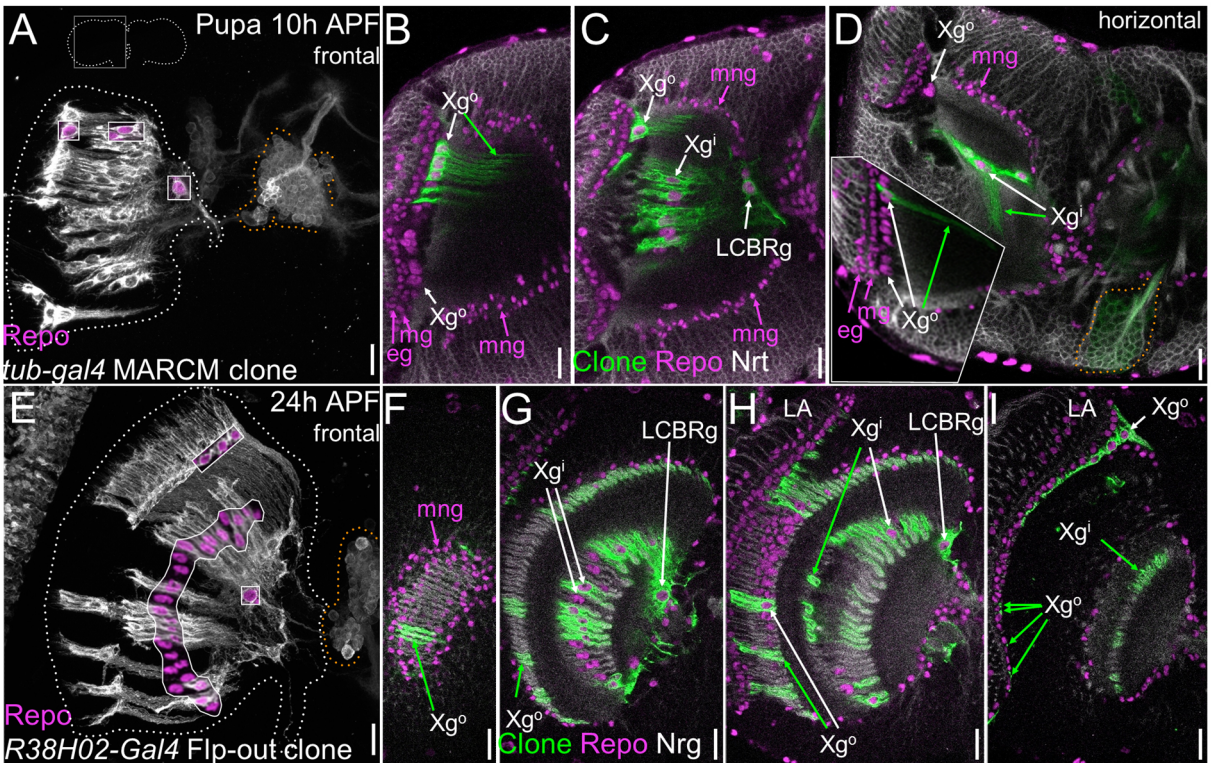


Figure 3.8 Morphology of DL1-derived neuronal and glial cells during metamorphosis.
(A-D) tub-Gal4 MARCM clones from two 10 h APF pupae in frontal view (A-C, dorsal to the top, medial to the right) and horizontal view (D, rostral to the top, medial to the right), counterstained with anti-Repo (magenta) and anti-Nrt (white in B-D). **(A)** Maximum intensity projection of the entire clone. The glial and neuronal cell clusters are outlined in white and orange dotted lines, respectively. Insets show single confocal slices through nuclei of the three glial cell types to indicate their positions. **(B,C)** Single confocal slices from the clone in (A) at the level of the glial cell bodies and processes of the outer chiasm glia (Xg^o; B-C), inner chiasm glia (Xg^i, C) and the most proximal glia labeled LCBRg (lateral cell body rind glia, C) that are assumed to be identical to the proximal most glial type in the adult lateral cell body rind (compare with Fig. 3.9E-H). The horizontal view in **(D)** shows three processes of an Xg^i cell, as well as the rows of epithelial, marginal, and outer chiasm glia (inset). **(E-I)** Maximum intensity projection (E; insets and outlines as in (A)) and single confocal slices (F-I) of an *R38H02-Gal4* Flp-out clone at 24 hours APF, counterstained with anti-Repo (magenta) and anti-Nrg (white in F-I). White arrows point to glial nuclei, green arrows to glial processes. The longest processes of Xg^o run from distal to proximal along the anterior surface of the medulla neuropil (F), at the level of the medulla neuropil glia nuclei (F, mng). The three parallel processes shown in (F) (green arrow) belong to a single outer chiasm glial cell. All Xg^o processes (green arrows in F-I) wrap around outer chiasm axon tracts that are labeled by anti-Nrg (F-H) but not anti-Nrt (B). Beneath and parallel to the layer of outer chiasm glia lie the thicker axon bundles of inner optic chiasm that are labeled by both anti-Nrt and anti-Nrg (C,G,H). Xg^i wrap these axon bundles along their whole length (G,H), and project additional processes into the developing lobula complex (D,I, green arrow) that come to lie between the lobula and lobula plate in the adult (compare with Fig. 3.9D). Scale bars, 20 µm.

In the adult brain, the DL1 lineage cells have completed their differentiation and assumed their mature morphology and position in the brain (Fig. 3.9). The neuronal cells in the mature central

brain have differentiated into two subclusters of interneurons, one of which arborizes in the superior protocerebrum and in two lamina of the fan-shaped body of the central complex, while the other arborizes more diffusely in the ventrolateral protocerebrum (Fig. 3.9A). The DL1-derived Repo-positive glial cells in the mature optic lobe have differentiated into three clearly distinguishable optic lobe glial cell types, all of which form elaborate processes. Fig. 3.9B-H show examples of isolated cells of the three types from DL1 INP clones and DL1 neuroblast clones. In addition to the outer chiasm (Fig. 3.9B) and the inner chiasm glial cells (Fig. 3.9C-E), which correspond to optic lobe tract glia (Edwards and Meinertzhagen, 2010), the third type of DL1 lineage glial cell in the adult optic lobe has developed into cortex glia-like cells that are located in the lateral cell body rind (LCBR). DL1-derived LCBR glial cells had variable morphologies in the adult; Fig. 3.9E-F shows an example of an LCBR glial cell with its nucleus located at the edge of the medulla and lobula neuropils, that wraps around several nuclei in the overlying cortex and extends one process into the space between medulla and lobula neuropils at the level of the inner chiasm glia. Fig. 3.9G-H shows two examples of glial cells that are located deeper in the lateral cell body rind, wrapping around many nuclei of the LCBR. One of them extends a short process into the central brain along an axon tract that interconnects the optic lobe and central brain.

Taken together, these data indicate that the mature DL1 lineage comprises interneurons of the central brain and multiple types of glial cells of the optic lobe. This in turn implies that the type II DL1 neuroblast is a novel multipotent neuroglial progenitor that generates both central brain interneurons and optic lobe glial cells through transit amplifying INPs.

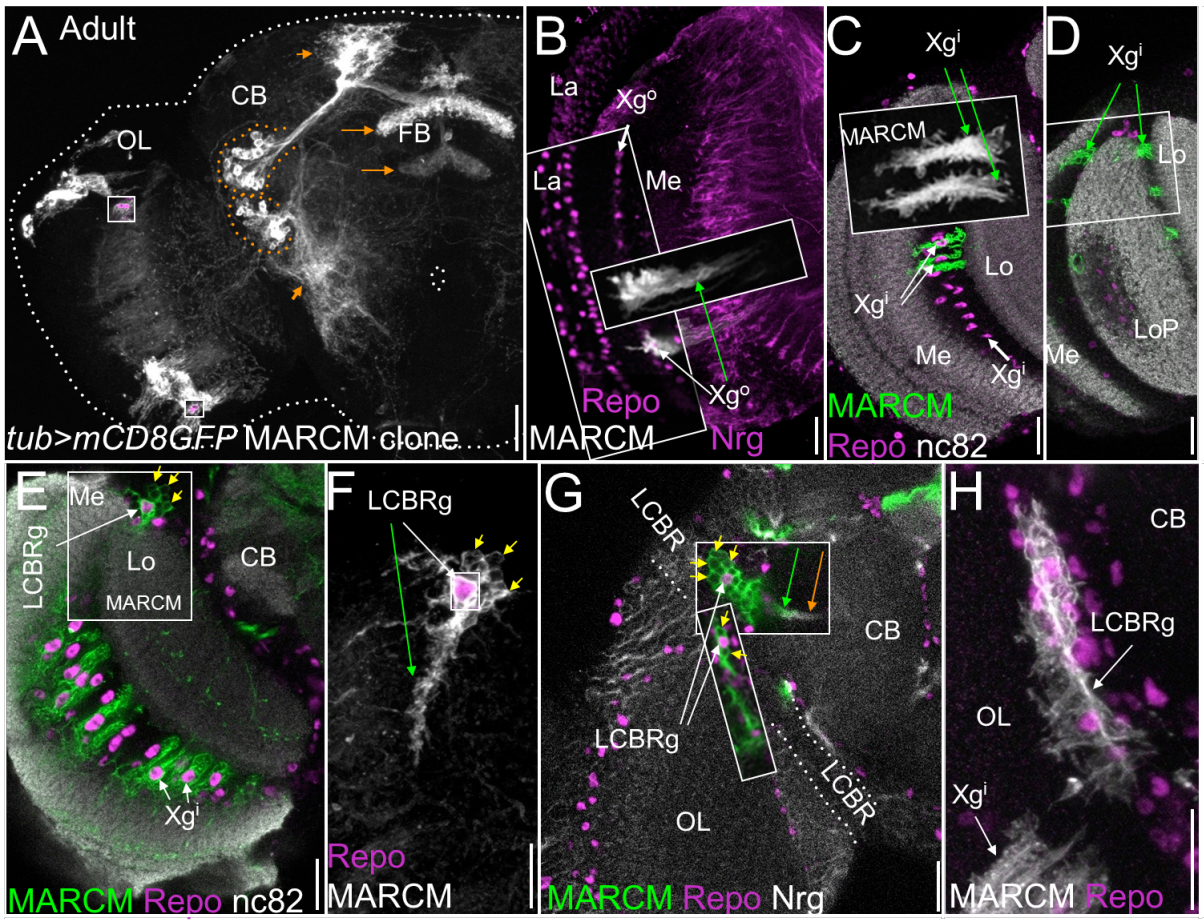


Figure 3.9 Morphology of DL1-derived neuronal and glial cells in the adult.
tub-Gal4, UAS-mCD8GFP labeled MARCM clones of DL1 **(A,E-F)** and DL1 INPs **(B-D,G-H)** in adult brains to reveal the morphology of DL1-derived secondary axon tracts (A), outer chiasm glia (B, Xg^o), inner chiasm glia (C-E, Xg^i), and two examples of cortex glia in the lateral cell body rind (E-F and G-H, LCBRg). All panels show frontal views, dorsal is to the top, medial is to the right. The brains were counterstained with anti-Repo (magenta) and either nc82 (white in C-E) to reveal neuropil structures, or anti-Nrg (magenta in B, white in G) to reveal axon tracts. **(A)** Maximum intensity projection of an isolated adult DL1 neuroblast clone. The neuronal part of the clone is composed of two cell clusters (orange dotted outlines); the dorsal cluster arborizes in the intermediate-medial region of the superior protocerebrum (short orange arrow) and projects to two layers of the fan-shaped body (FB, long orange arrows). The ventral cluster arborizes broadly in the posterior ventrolateral protocerebrum (orange arrowhead). Two glial cell nuclei of the DL1 neuroblast clone are shown in insets. **(B)** Adult morphology of two Xg^o cells; the inset shows the top cell in a maximum intensity projection (green arrow), the bottom cell is shown in a single slice at the level of the nucleus (long white arrow) that is located within the single row of Xg^o nuclei (short white arrow) at the edge of the medulla, adjacent to the lamina (La). **(C,D)** Adult morphology of four Xg^i cells; the inset in (C) shows the top two cells in a maximum intensity projection (green arrows), and the bottom two cells in a single slice at the level of their nuclei (white arrows) that lie within the row of Xg^i nuclei (short white arrow). **(D)** Apart from their proximodistal orientation, the Xg^i cells also extend processes posteriorly between the lobula plate (LoP) and lobula (Lo), and the lobula plate (LoP) and medulla (Me) (green arrows). **(E-H)** Two cortex glial cells with different morphology in the lateral cell body rind (LCBR), the region of neuronal cell bodies between the optic lobe and central brain. The LCBR glial cell (LCBRg) in (E,F) has its cell body at the dorsal edge of the lobula and medulla

(long white arrow), wraps around several adjacent Repo-negative cell bodies in the cortex (yellow arrows), and extends a diffuse process (long green arrow in F) with fine branches into the space between medulla and lobula at the level of the Xg^i cells (short white arrows in E). The LCBR glial cells in (G,H) wraps around many more Repo-negative cell bodies in the LCBR (yellow arrows in G), and extend short processes into the central brain (green arrow in G) along axon tracts that interconnect the central brain and optic lobe (orange arrow in G). **(H)** Maximum intensity projection of one LCBRg cell from (G); Repo channel included (magenta) to visualize its position between central brain (CB) and optic lobe (OL). Scale bars, 20 μm.

3.5 Discussion

In this report, we focus on the two lateral type II neuroblast lineages of the central brain in which amplification of proliferation is mediated through transit amplifying INPs (reviewed in Boyan and Reichert, 2011; Brand and Livesey, 2011; Homem and Knoblich, 2012). While one of these lineages, DL2, generates exclusively interneurons of the central brain, the other, DL1, generates both central brain interneurons and glial cells of the optic lobe. Thus, while DL2 functions as a neuroblast, DL1 has neuroglioblast function. Like the five other type II neuroglioblasts, DM1-5, the interneurons generated by DL1 contribute to the central complex neuropil (Izergina et al., 2009; Viktorin et al., 2011). However, in contrast to DM1-5, the glial cells generated by DL1 do not contribute to the central complex. Indeed, they do not contribute to the central brain at all. Although they are generated in the central brain, they rapidly migrate out into the developing optic lobes, where they subsequently differentiate into outer chiasm glia, inner chiasm glia and cortex glia. This is the first example of a central brain lineage that gives rise to cells of the optic lobes and is at the same time the first identification of the developmental origin of optic lobe chiasm glia.

The observation that distinct glial cells types in the optic lobe can have their developmental origin outside of the optic lobe primordia is remarkable but not unique. The optic stalk of the larval eye-disc also gives rise to glial cells that migrate into the optic lobe where they differentiate into specific surface glial types (Perez and Steller, 1996; Chotard and Salecker, 2007; Edwards and Meinertzhagen, 2010; Hartenstein, 2011; Edwards et al., 2012). Thus, in addition to specialized glial precursor zones located within the optic lobes (Perez and Steller, 1996; Dearborn and Kunes, 2004), the DL1 neuroglioblast and as yet unidentified glial precursors are essential for the formation of the numerous distinct subpopulations of optic lobe glial cells in Drosophila. This dependence of optic lobe glial cell formation on progenitors located both within and outside of the optic lobe primordia likely reflects the more basal condition in hemimetabolous insects in which retina, optic lobe and central brain are generated

in intimate spatial and temporal association during embryogenesis. It will be interesting to investigate if specific subsets of optic lobe glial cells in hemimetabolous insects such as the grasshopper also derive from retinal and central brain precursors.

In most cases studied so far, a central feature of glial cells is their ability to migrate during the course of development in order to establish their specific relationship with neuronal cells (see Klämbt, 2009). Accordingly, the central brain origin of DL1-derived glial cells of the optic lobe requires the migratory displacement of these cells from their site of origin into the optic lobe. Remarkably, all of the DL1-derived glial cells appear to migrate into the optic lobe; we did not find glial cells that remain in the central brain or migrate into other regions of the central brain. Thus, although there are numerous glial cells located near the DL1 lineage in the central brain, we have not found them to be DL1-derived. On the other hand, DL1-derived cells do not appear to give rise to the entire set of inner chiasm glia or outer chiasm glia; labeled DL1 glial cell clones never comprised all the glia of a given type, and their spatial distribution in the array is not fixed. Thus, some of the chiasm glial cells in the optic lobe may come from other unknown sources, unless both the tubulin (MARCM) and actin (Flp-out) promoters are not ubiquitously expressed in chiasm glial cells. Our analysis of early larval *R38H02-Gal4* Flp-out clones suggests that we labeled only postembryonic DL1 progeny. It is therefore possible that some glial cells are derived from the embryonic part of the lineage. In our extensive analyses of MARCM clones and Flp-out analyses of Gal-4 drivers, we have not recovered additional sources for these very conspicuous types of glia than DL1 neuroblasts or INPs (compare Dearborn and Kunes, 1994; Perez and Steller, 1996; Chotard and Salecker, 2007). The developmental mechanisms which may integrate DL1-derived glial cells with glial cells of other origin into seemingly homogeneous arrays of chiasm glia are currently unknown.

The central complex associated glial cells produced by DM1-3 increase their number approximately four-fold through local proliferation based on glial cell mitosis during pupal stages (Viktorin et al., 2011). In contrast, our experiments provide evidence for only minor mitotic activity of DL1-derived glial cells during their migration into the optic lobe, and no mitotic activity in pupal stages. However, it is noteworthy that all of these glial cells manifest exceptionally large nuclei compared to surrounding non-DL1 glial cells in the optic lobe. This suggests that while the DL1 lineage glial cells do not divide, they might nevertheless undergo DNA replication and become polyploid in the optic lobe. This polyploid state may be a prerequisite for an insulating function in wrapping large axon tracts, as polyploidy is necessary in subperineurial glia to maintain the integrity of the blood-brain barrier (Unhavaithaya and Orr-Weaver, 2012).

Together with DM1-5, DL1 is the sixth identified neuroglioblast that generates neuronal and glial cells during postembryonic development of the central brain. Thus, all of the postembryonically acting neuroglioblasts in the brain identified to date are type II neural stem cells that amplify their proliferation through INPs. The neuronal and glial cells of the DL1 lineage do not originate directly from the DL1 neuroblast, rather they are generated via INPs, multipotent secondary progenitors with features of transit amplifying cells that can give rise to both neuronal and glial cells (Viktorin et al., 2011; this report). Remarkably, in mammalian brain development, many neuronal and glial cells also originate from transit amplifying intermediate progenitors and not directly from neural stem cells (see Kriegstein and Alvarez-Buylla, 2009). Indeed, in the mammalian cortex, the majority of neural cell-generating proliferative divisions occur through intermediate progenitors at all stages of development, implying that the major role of cortical neural stem cells is to generate intermediate progenitors (Kowalczyk et al., 2009; Lui et al., 2011). The intriguing parallels between the INP-generating type II neural stem cell lineages in the Drosophila brain and the intermediate progenitor-generating neural stem cell lineages in the mammalian brain suggest that comparable lineage types might be present in other developing brains and, hence, represent a common and phylogenetically conserved feature in the development of complex brain architecture (Boyan and Reichert, 2011).

3.6 Authors' contributions

GV carried out all the experiments. HR and NR conceptualized the project. GV and HR analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

3.7 Note added in proof

While this paper was in review, two studies described adult DL1 and DL2 lineages (Ito et al., 2013; Yu et al., 2013), one of which mentions DL1-associated glia in the optic lobe (Yu et al., 2013). The lineages in the present report were named accordingly.

3.8 Acknowledgments

We thank Philipp Kuert and Susanne Flister for help with screening Gal4 lines, Volker Hartenstein, Holger Apitz, and Iris Salecker for assistance in identifying lineages and glial cells and for discussions, as well as Bruno Bello, Angela Giangrande, Yanrui Jiang, Cheng-Yu Lee, Veronica Rodrigues, Iris Salecker, the Developmental Studies Hybridoma bank, and the Bloomington stock center for reagents and fly stocks, and an anonymous reviewer for useful suggestions on the manuscript. We thank Susanne Flister for excellent technical assistance. Supported by the Swiss NSF 31003A 140607.

3.9 Additional files

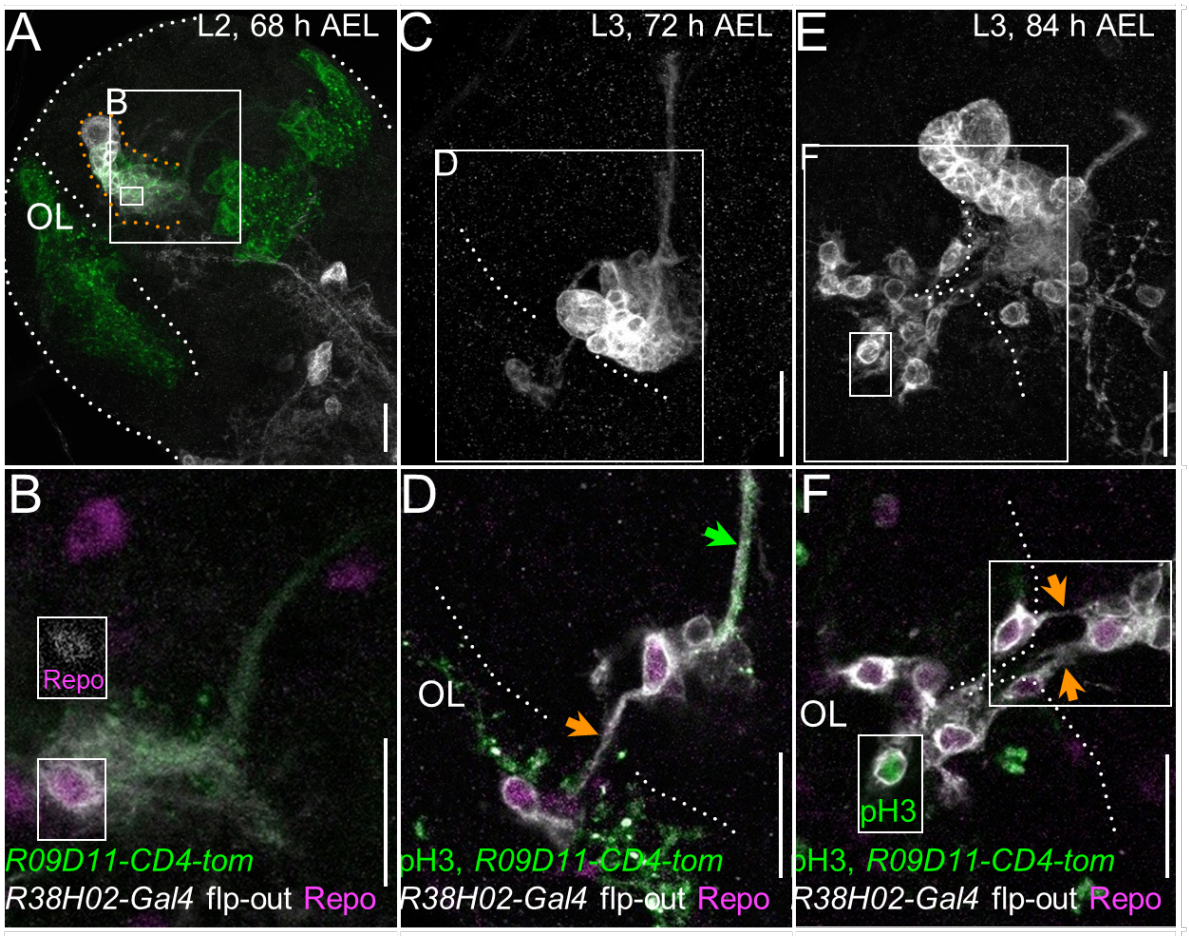


Figure S3.1 Time line of glial cell formation and division in R38H02-Gal4 Flp-out clones.
(A-F) In DL1 clones generated by *R38H02-Gal4* Flp-out (white, outlined by orange dotted lines), the timing of development of Repo-positive (magenta) glial cells and their translocation into the optic lobe (OL, border indicated by white dotted lines) are identical to that seen in MARCM clones (compare with Fig. 3.5). **(A-B)** Late second instar larval DL1 clone, recovered at 68 hours AEL, coincides with *R09D11-CD4-tdTomato* expression (green) in DL lineages and contains one Repo-positive cell (magenta in B). **(C-D)** In early third instar at 72 h AEL, DL1-derived cells are found outside of the DL1 lineage and inside the optic lobe. The axon tract of the DL1 clone coincides exactly with that of *R09D11-CD4-tdTomato* expression (green, green arrowhead in D), which confirms the identity of the lineage. **(E-F)** At 84 hours AEL, more Repo-positive cells have formed and spread laterally inside the optic lobe. The one pH3-positive, dividing glial cell in this clone is shown in green. The migrating glial cells are connected to each other and to the neuronal part of the lineage by cytoplasmic processes (orange arrows in D,F).). Scale bars, 20 µm.

4. 3D reconstruction of the *Drosophila* larval brain at EM resolution reveals embryonically generated type-II NB derived neurons

Nadia Riebli[1], Volker Hartenstein[2], Rick D. Fetter[3], Stephan Saalfeld[4], Stephan Gerhard[5,3]; Albert Cardona[3], Heinrich Reichert[1]

[1]Biozentrum, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland
[2]Department of Molecular, Cell and Developmental Biology, University of California Los Angeles, Los Angeles, California, USA
[3]HHMI Janelia Farm Research Campus, Ashburn, Virginia, USA
[4]Max Plank Institute for Molecular Cell Biology and Genetics, Dresden, Germany
[5]Signal Processing Laboratory 5, Ecole Polytechnique Fédérale de Lausanne Lausanne, Switzerland

Manuscript in preparation

4.1 Summary

Drosophila neural development is subdivided into two temporally separated neurogenic phases. The first neurogenesis takes place during embryonic stages and gives rise to the differentiated "primary" neurons building the functional larval brain. After a period of quiescence around larval hatching, the second neurogenesis takes place during larval stages giving rise to undifferentiated "secondary" neurons, which mature during metamorphosis and thereafter give rise to the adult fly brain. In *Drosophila*, type-II neuroblasts are neural stem cell-like primary progenitors, which amplify the number and diversity of their lineal neural progeny through the generation of intermediate progenitor cells. Recently, insight into type-II neuroblast lineage derived neuronal cell types has been obtained for their postembryonically generated secondary neurons. In contrast, there is currently no information available on the embryonically generated primary neurons in these lineages.

In this report, we use topological guideposts from light microscopic data of type-II NB lineages to identify their embryonically born neurons in the EM data stack of the first larval instar CNS. 3D reconstruction in the serial section EM data stack reveal that a subset of central complex primordium forming cells (CCPaC's) are generated during the embryo. Furthermore, albeit undifferentiated and devoid of synapses, these cells already form the highly organized central complex primordium shortly after larval hatching. Lineage affiliation of the embryonic born CCPaC's revealed further undifferentiated as well as differentiated primary neurons in type-II NB lineages of the first larval instar. 3D reconstruction of the embryonically born cells derived from the type-II NB lineage DPMm1 exposed neuron morphology as well as synaptic identity on a single cell basis. DPMm1 derived primary neurons have widespread innervations in many parts of the larval brain and underline the complexity of the type-II NB lineages reported for postembryonic stages.

Concluding, these findings reveal the diversity of embryonic born neurons of differentiated and undifferentiated identity, which are derived by type-II NB lineages. Furthermore, this report gives insight into the embryonic origin of the central complex primordium and shows the involvement of undifferentiated neurons derived by type-II NB lineages in the formation of this structure. Thus, we here characterize the type-II derived embryonically born undifferentiated neurons, which are not classifiable within the current distinction between primary and secondary neurons. Our work represents a significant first step towards the identification and incorporation of all of the neuronal progeny of type-II NB lineages into a comprehensive connectome of the larval *Drosophila* brain.

4.2 Introduction

The early first instar larval brain of *Drosophila* is formed by 1500 differentiated and functional nerve cells (Larsen et al., 2009). In developmental terms, these neurons correspond to a relatively small number of about 100 genetically and structurally unique neuronal lineages, each of which derive from a stem-cell like neuronal progenitor, the neuroblast (NB) (Hartenstein et al., 2008; Truman and Bate, 1988). These NB lineages as a total represent both structural and developmental modules of the *Drosophila* brain.

During embryogenesis, neuroblasts generate the neurons, which later build up the larval brain. These larval functional neurons, born during embryogenesis, are often referred to as the primary neurons and are fully differentiated at larval hatching (Technau et al., 2006; Hartenstein et al., 2008; Younossi-Hartenstein et al., 2006; Nassif et al., 1998). At the end of embryogenesis, most brain neuroblasts cease their proliferative activity and enter a state of quiescence thus ending the first (embryonic) phase of neurogenesis (Egger et al., 2008; Tsuji et al., 2008). During the late first /early second larval instar stage, the neuroblasts resume proliferation therefore initiating a second (postembryonic) phase of neurogenesis in which the majority of the neurons that are integrated into the adult fly brain circuits are generated. These adult-specific neurons, also called secondary neurons, remain undifferentiated throughout larval stages but then differentiate and mature during metamorphosis (Hartenstein et al., 2008; Truman and Bate, 1988; Prokop and Technau, 1991; Truman et al., 2004; Izergina et al., 2009). However, some embryonic born neurons were reported which do not differentiate until metamorphosis (Consoulas et al., 2002; Zhou et al., 2009). Due to the anatomical interrelationship of the neuroblast and its progeny cells within a lineage, early born neuron somata are generally found distal to the neuroblast and close the neuropil since they are displaced by later born neurons which remain more closely to the NB (Hartenstein et al., 2008; Brody and Odenwald, 2002).

Previous studies have shown that lineally related neurons from the same neuroblast form neurites that fasciculate in a cohesive lineage-specific bundle and arborize in distinct compartments within the neuropil. Within a NB lineage, the axon tract made by primary neurons (PAT) as well as the axon tract made by secondary neurons (SAT) project towards the neuropil within the same neurite bundle (Nassif et al., 1998; Ito and Hotta, 1992; Hartenstein et al., 2008). This unique neurite bundle formed by every individual NB lineage is recognizable from late embryonic stages onwards as well as in the adult brain. The reproducible localization of this axon fascicle as well as the lineage's unique projection pattern within the neuropil and with respect to its neighboring lineages enables the identification of a given lineage (Pereanu

and Hartenstein, 2006; Ito et al., 2013; Yu et al., 2013). Taken together, the NB lineage tracts represent invariant, easily recognizable landmarks in the brain onto which single neurons can be assigned to due to their anatomical association with the corresponding neurite tract (Spindler and Hartenstein, 2010; Larsen et al., 2009).

Recent work has shown that two types of proliferating neuroblasts are present in the central brain of *Drosophila*. The majority of the neuroblasts, referred to as type-I NBs, proliferate through repeated cell divisions in which they self-renew and give rise to a daughter cell called a ganglion mother cell (GMC). This GMC divides once more to generate two postmitotic neurons (Skeath and Thor, 2003; Doe, 2008; Knoblich, 2008). In addition, eight neuroblasts in each hemisphere, called type-II NBs, amplify proliferation through intermediate neural progenitors (INPs). These type-II NBs self-renew and produce INPs, each of which is also capable of a limited number of self-renewing divisions that generate GMCs. Each GMC then divides once giving rise to two neural progeny cells. Due to this amplifying proliferation pattern, type-II NBs create especially large lineages comprising an unusually high diversity of neuronal as well as glial cell types (Bello et al., 2008; Boone and Doe 2008; Bowman 2008; Izergina et al., 2009; Viktorin et al., 2011).

Some insight into type-II NB lineage derived neuronal cell types has been obtained for their secondary neurons through clonal labeling studies. Thus, clonal labeling demonstrates that postembryonically generated type-II NB lineages contain neurons, which contribute to an adult-specific midline neuropil called the central complex (CC) (Izergina et al., 2009; Bayraktar et al., 2010; Yang et al., 2013). The central complex is involved in multimodal information processing and memory as well as in coordination of motor control in locomotor behaviours (Strauss and Heisenberg, 1993; Strauss, 2002; Liu et al., 2006). Like other adult-specific neuropils, the mature CC only fully develops during metamorphosis when postembryonically generated neurons differentiate dendritic as well as axonal terminals and form synapses (Young and Armstrong, 2010b). In addition, a specific type-II NB derived subpopulation of early born neurons has recently been shown to form a central complex primordium readily visible at the third larval instar stage (Riebli et al., 2013). Importantly, in addition to the neuronal subset that innervates the CC, numerous secondary neurons in all type-II NB lineages project widely to diverse other neuropil compartments of the adult brain (Yu et al., 2013; Yang et al., 2013).

Thus, based on MARCM labeling, we currently have information about many of the postembryonically generated neurons in these type-II NB lineages and, in the case of some of the first postembryonic born neurons of the DPMm1 lineage, information is even available at

the single cell level (Izergina et al., 2009; Wang et al., 2014). In contrast, there is currently no information available on primary neurons generated in type-II NB lineages. Indeed, it is not known if type-II NB lineages give rise to any primary neurons during embryogenesis. This is due to the limitations of the clonal genetic methods available, which make it difficult and in many cases impossible to identify and characterize the primary neurons of identified NB lineages in the developing brain. To overcome these difficulties and obtain neuroanatomical access to the embryonically generated neurons in type-II NB lineages, a different type of experimental approach is needed.

Since embryonic born (primary) neurons contribute to the functional larval brain, they have--by definition--differentiated in the first larval instar brain. Furthermore, as NB's are still in quiescence and hence the second wave of neurogenesis has not yet started, there are no secondary cells present at this developmental stage. Thus, if they exist, all of the embryonically generated neurons of the type-II NB lineages should be present in the early first larval instar brain. How might it be possible to identify such neurons? Recently, a complete set of serial sections of the 4h post-hatching first instar larval brain was generated using serial section transmission electron microscope (ssTEM) data (Cardona et al., unpub.). This data set contains 4840 sections at a 3.8x3.8x50 nm resolution. Taking advantage of this complete EM neuroanatomical data set it should be possible to use known anatomical information from light microscopic investigations to identify embryonically generated type-II NB derived neurons.

In this report, we use specific neuroanatomical features of the CC primordium-forming cells obtained from L3 light microscopic analysis to identify the type-II NB lineage fascicles of three identified type II NB lineages, DPMm1, DPMpm1 and DPMpm2, in the L1 ssTEM dataset. 3D reconstructions revealed that the central complex primordium has an embryonic origin. Our analysis shows that the cells of the CC primordium are already present in the early first instar larval brain, albeit in an undifferentiated state. Indeed, this analysis uncovers an additional, hitherto unidentified, population of embryonic born undifferentiated neurons generated by the type-II NB lineages in the early larval brain. In addition, this analysis reveals a remarkably large and diverse set of differentiated primary neurons in type-II NB lineages in the early larval brain. Moreover, in DPMm1, the most anterior dorso-medial type-II NB lineage, these novel primary neurons as well as the undifferentiated neurons are identified at the single cell level. Our work represents a first step towards an incorporation of all of the neuronal progeny of type-II NB lineages into a comprehensive connectome of the larval *Drosophila* brain.

4.3 Materials and Methods

4.3.1 Fly strains and genetics

Unless indicated otherwise, fly stocks were obtained from the Bloomington Drosophila Stock Centre (Indiana University, Bloomington, IN, USA) and maintained on standard cornmeal medium at 25°C. For visualizing type-II NB lineages, flies with *UAS-dcr*^{2}; *wor-GAL4*, *ase-Gal80*; *UAS-mCD8-GFP* (Neumüller et al., 2011) were used. To generate wild type MARCM clones (Lee and Luo, 1999), we mated female *y, w, hs-Flp*^{1}*; tubP-Gal4, UAS-mCD8::GFP*^{LL5}*/CyO,actin-gfp*^{JMR1}*; FRT82B, tub-Gal80*^{LL3} (Bello et al., 2003) to *gcm-lacZ*^{rA87}*/CyO, actin-gfp*^{JMR1}; *FRT82B* males. Eggs were collected for 2 to 4 h, grown to first larval instar (22 to 30 h after egg laying), then heat shocked in a 37°C water bath (GFL 1083, Burgwedel, Germany) for 5 minutes. Larvae were then grown to late wandering third instar. The *Gal4*$^{14\text{-}94}$ line (Zhu et al, 2011) was kindly provided by the Jan lab (University of California, San Francisco, CA, USA). The *R45F08-Gal4* line is the *P{GMR45F08}attP2* enhancer-Gal4 line from Janelia Farm (Ashburn, VA, USA) (Jenett et al., 2012). For *Gal4*$^{14\text{-}94}$ driven flip-out clones, *y, w, hs-Flp; UAS-FRT>CD2,y*$^{+}$*>mCD8::GFP* (G. Struhl provided flies for publication in (Wong et al., 2002)) were crossed to the *Gal4*$^{14\text{-}94}$ flies. Eggs were collected for 2 h and then heat shocked 2.5 to 4.5 h after egg laying in a 34°C water bath for 15 minutes. Then, larvae were grown to late wandering third instar.

4.3.2 Immunohistochemistry

Larval brains were fixed and immunostained as described previously (Viktorin et al., 2011). For larval staining, the primary antibody chicken anti-GFP 1:1,000 (ab13970, Abcam, Cambridge, UK) was incubated overnight at 4°C and the Alexa-conjugated secondary antibody goat anti-chicken 488 was used 1:300 (A11039, Molecular Probes, Eugene, OR, USA); preparations were incubated for 3 h at room temperature.

4.3.3 Confocal Microscopy and image processing

All fluorescent images were recorded using a Leica TCS SP5 confocal microscope (Leica microsystems GmbH, Wetzlar, Germany). Optical sections ranged from 0.76 to 1 µm with a picture size of 1,024 × 1,024 pixels. Collected images were arranged and processed using Fiji (Schindelin, 2008). All adjustments were linear and were performed on whole images.

4.3.4 Acquiring EM data and image processing

The methods for acquiring the complete first larval instar brain by ssTEM (4840 sections at a 3.8x3.8x50 nm resolution) will be published elsewhere (Cardona et al., unpub). The whole post-processing pipeline was packed into the open source TrakEM2 software (Cardona et al., 2012), which is based on ImageJ (Wayne Rasband). Due to sectioning, electron beam heating and counterstaining with heavy metals, each section presented some unique deformations for which corrections were needed. The TrakEM2 software corrected for alignment and deformation in the ssTEM images and subsequently stitched overlapping image tiles into a single serial section. TrakEM2 then aligned across sections and later allowed to navigate through the resulting stack of sections (Cardona et al., 2010a+b, 2012, 2013). For reconstruction and annotation of the resulting multidimensional L1 brain image data set, a web companion to TrakEM2, called CATMAID (collaborative annotation toolkit for massive amounts of image data), was used (Saalfeld et al., 2009,2010; 2012; Gerhard et al, in prep.). CATMAID is a decentralized web interface that allows management, registration, analysis and seamless navigation of large image stacks. The 3D reconstruction of individual neurons was performed by computer-assisted manual labeling of neuronal skeletons (Helmstaedter et al., 2011, Briggman et al., 2011, Bock et al., 2011) in CATMAID. The 3D reconstructions of neurons within this data set reveal small neural processes as well as single synapses permitted unraveling of the morphology and synaptical interconnections of the embryonically generated neurons in the L1 brain. Traced neurons of interest could be color-coded and illustrated individually or in groups in the 3D tool and rotated as well as zoomed in.

4.4 Results

4.4.1 Key neuroanatomical features of type-II NB lineages as derived from light microscopic analysis

A number of neuroanatomical features of type-II NB lineages are known from light microscopic analysis, which facilitate their identification in the EM serial sections of the larval brain (Ito et al., 2013; Yu et al. 2013; Yang et al., 2013; Wang et al., 2014; Pereanu and Hartenstein, 2006; Izergina et al., 2009; Viktorin et al., 2011; Riebli et al., 2013). Here we review some of the most salient of these morphological features.

Previous work has used various Gal4 drivers to label specific subsets of the postembryonically derived neurons by the type-II NB lineages (Jenett et al., 2012; Pfeiffer et al., 2008; Bayraktar et

al., 2010; Izergina et al., 2009; Zhu et al., 2011). One of these comprises a combination of the pan-neuroblast driver *worniu-Gal4* together with the *asense-Gal80* repressor which prevents Gal4 expression in all type-I NB lineages (Yasugi et al., 2014). In the third instar larval brain, this driver specifically reveals the 8 dorsomedially located type-II NBs and their latest born progeny, which include INPs and GMCs as well as some neurons. The strong expression of *wor-Gal4, ase-Gal80* in the 6 drosomedially located type-II NB lineages reveals the lineage-specific secondary axon tract of each individual lineage, which comprises the neurites of the neurons belonging to the given lineage (Figure 4.1A). Each of these 6 type-II NB lineages has been individually identified and named based on their specific spatial and anatomical location as DPMm1 (most dorsal/rostral), DPMpm1; DPMpm2, CM4, CM3, CM1 (most ventral) (Bello et al., 2008; Pereanu and Hartenstein 2006, for an annotation table see Riebli et al., 2013).

Wildtype tubulin-MARCM (mosaic analysis with repressible cell marker; Lee and Luo, 1999) clones induced at larval hatching show the postembryonically generated type-II NB lineage complexity at the end of larval development. Moreover, in contrast to the *wor-Gal4,ase-Gal80* driver, these MARCM clones reveal all of the secondary neurons within the lineage. Whereas a typical postembryonic type-I NB lineage projects only a single axon tract into the neuropil, MARCM clones of postembryonic type-II NB lineages show much more diverse axonal projections that cross the midline at various locations and send multiple projections into diverse ipsilateral neuropil compartments. Nevertheless, all of their neurons initially project into a single stereotyped secondary axon tract, and it is only deeper within the neuropil that this tract starts to subdivide into distinct smaller bundles that project to diverse neuropil compartments (Figure 4.1B).

Further analysis of amplifying NB lineages in the larva using the type-II NB lineage specific Gal4 driver $Gal4^{14\text{-}94}$ which corresponds to the P1 isoform of the Ets transcriptionfactor Pointed (Zhu et al., 2011) reveals a special subset of neurons in the dorsomedially located type-II NB lineages that give rise to a primordial structure at the commissural midline of the larval brain (Figure 4.1C). Based on their spatial position and flip-out experiments, these cells were considered to be early born cells of the type-II NB lineages that were displaced towards the neuropil by their later-born lineal sibling cells. The lineage affiliation of these primordium associated cells can be seen in isolation for individual type-II NB lineages with flip-out experiments which reveal that most, if not all, of these cells are derived from type-II NBs (Figure 4.1D) (see Riebli et al., 2013).

These midline primordium associated neurons can be labeled in a highly specific and exclusive manner using a particular enhancer fragment Gal4-line derived from the *pointed* gene, called

R45F08-Gal4 (Jenett et al., 2012). Expression analysis with this Gal4-line at the third larval instar stage reveals that all of the labeled cells together form the primordial barshaped structure across the midline. On each side of the midline, the primordial structure displays a four-fold symmetry according to the 4 type-II NB lineages that contribute to the structure (DPMm1, DPMpm1, DPMpm2 and CM1) (Riebli et al., 2013). Remarkably, axons from these four lineages enter the commissural structure in a topologically organized manner. Thus, the DPMm1 axons enter the primordium at the most medial location (closest to the midline) followed by DPMpm1 axons, which are laterally adjacent to DPMm1 axons and medially adjacent to DPMpm2 axons, while CM1 axons enter into the structure most laterally (Figure 4.1E). It is known from lineage tracing work that these primordium cells later become give rise to different substructures of the mature central complex (Riebli et al., 2013). These midline primordium cells are therefore de facto central complex primordium associated cells (hereafter referred to as CCPaC's) and the structure they produce corresponds to the central complex primordium (hereafter referred to as CCpr).

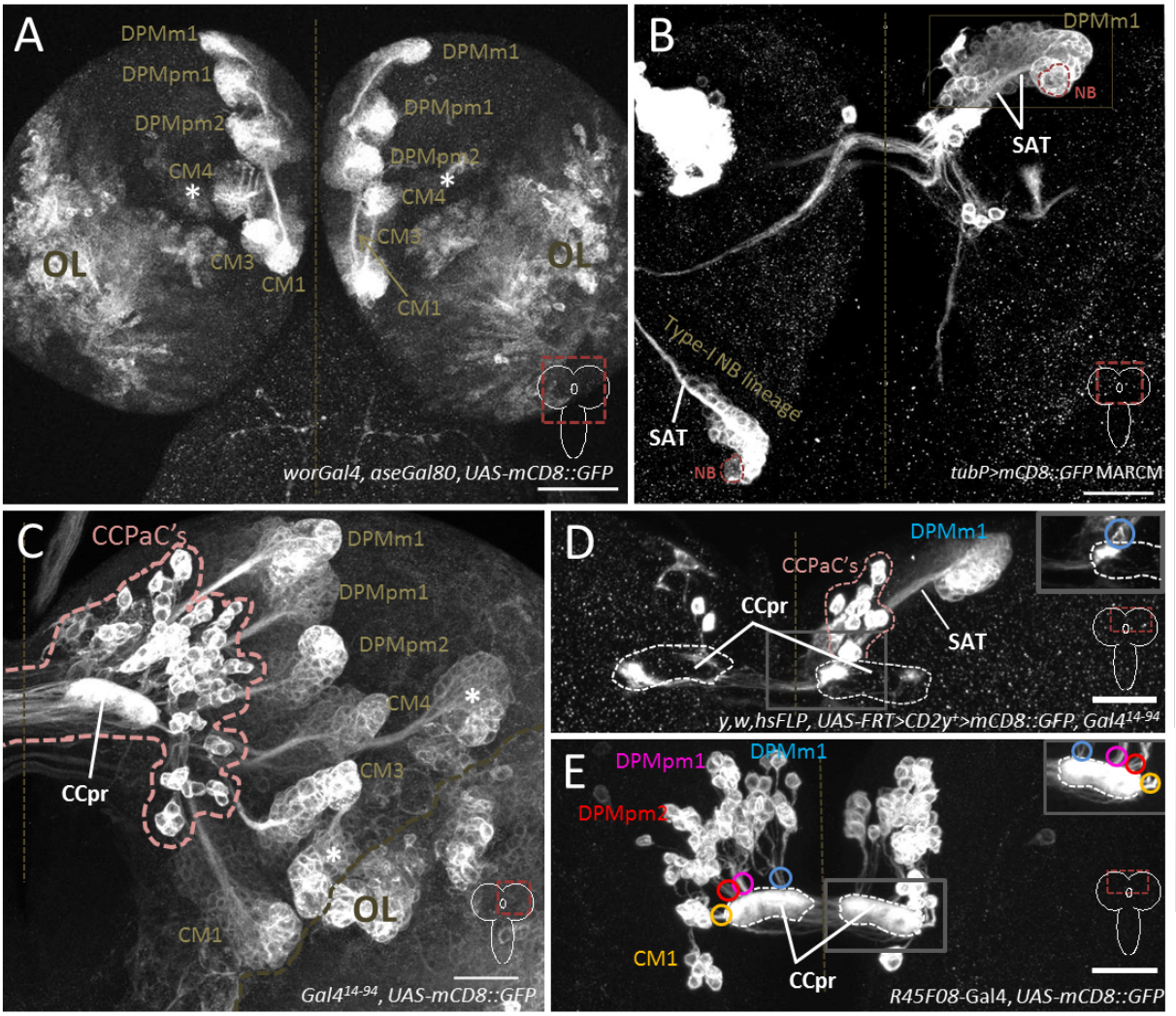


Figure 4.1 Light microscopic data reveal type-II NB lineages and central complex primordium forming cells (CCPaC's) as well as the central complex primordium (CCpr) in the late larval brain.
(A-E) Z-projection of multiple optical sections. **(A)** *worGal4,aseGal80* driven labeling of eight type-II NB lineages shows lineage-specific neurite fascicles. **(B)** postembryonically induced, *tubulin-Gal4* driven MARCM-based labeling of the type-II lineage DPMm1 and a type-I NB lineage shows lineage-specific secondary axon tract (SAT). The SAT of the type-II NB lineage DPMm1 subdivides into multiple axonal fascicles which cross the midline and also project ipsilaterally, whereas the SAT of the type-I NB lineage only shows a single projection into the neuropil. NB, neuroblast. **(C)** *Pntp1-Gal4* driven labeling of the eight type-II NB lineages and the central complex primordium associated cells (CCPaC's) as well as the central complex primordium (CCpr) in one hemisphere. **(D)** embryonically induced *PntP1-Gal4* driven flip-out clone of the type-II NB DPMm1 lineage shows that central complex primordium associated cells (CCPaC's) are lineal descendants of the DPMm1 NB that innervate the central complex primordium (CCpr) on both sides of the midline. **(E)** *R45F08-Gal4* exclusively labels the CCPaC's of the four type-II NB lineages and reveals the lineage dependant topological organization with which the neurites enter the central complex primordium (CCpr). **(D+E)** Different colors are assigned to different type-II NB lineages and color-coded circles reveal the lineage-specific entry point of the axon fascicles into the central complex primordium (CCpr). Insets are maximum intensity projections of multiple adjacent confocal

slices. Dashed line represents the midline. OL, optic lobes; white asterisks, lateral type-II NB lineages. Scale bars, 25µm.

In summary, a good deal of information has been gathered about these amplifying type-II NB lineages in the late larva as well as in the adult. Moreover, various genetic labeling approaches have revealed a specific subpopulation of neurons in these lineages which forms a central complex primordium and later become integrated into different substructures of the mature CC. Importantly, these CCPaC's already display a highly organized topological morphology in the third larval instar brain.

In contrast to this large amount of data about postembryonically generated cells of the type-II NB lineages, there is currently nothing known concerning embryonically generated neurons of these lineages. Indeed, it has been impossible to access embryonically generated neurons of type-II NB lineages using clonal MARCM labeling, flip-out techniques or various lineage specific Gal4 drivers. Due to this lack of data in embryonic or early larval stages, the identity, developmental origin and possible complexity of embryonically generated type-II NB derived neurons remain elusive. This poses a major gap in the current understanding of type-II NB lineages. Given these genetic methodological limitations, we adopted an alternative method to identify and characterize the postulated embryonically generated type-II NB derived neurons. This approach is based on an ultrastructural analysis which takes advantage of the high resolution given by the serial section transmission electron microscope (ssTEM) data generated for the first larval instar brain by the HHMI Janelia Farm Fly EM Project team (Figure 4.2B).

4.4.2 EM serial sections reveal NB's as well as undifferentiated and differentiated neurons

The EM data set used in our analysis comprises the entire first instar larval brain in registered and aligned sections which make it possible to reconstruct complete neurons on a single cell level. Given the high resolution of this dataset, different types of somata can be distinguished in the L1 brain based on their morphology. For example, the somata of the mushroom body neuroblasts (MB NB’s) are unequivocally identified as the biggest and most prominent cell bodies located dorsolaterally at the brain surface with a diameter of about 10µm (Katharina Eichler, personal communication) (white border in Figure 4.2A). These MB NB's contain a large number of mitochondria in the cytoplasm indicative of their high energy consumption due to ongoing mitotic activity. (Mushroom body NB's do not undergo a period of quiescence (Prokop and Technau 1991; Ito et al., 1992). Numerous slightly smaller cell bodies (5-7 µm diameter) that display a smaller nucleus, an electron dense and less expanded cytoplasm as well as more

condensed chromatin compared to the MB NB's can be observed at various locations in the central brain. These cells correspond to NBs, which are still in quiescence (yellow borders in Figure 4.2A and 4.2C). Of about the same size as these quiescent NBs are most of the cell bodies found in the larval brain cortex that represent differentiated neurons; their chromatin is less dense and their cytoplasm considerably lighter in the sections. Several of these cells are indicated with red asterisks in Figure 4.2A. Additionally, cortex glia cells as well as neuropil glia cells can be identified due to their broad cell membrane processes, which extend throughout brain compartments both in the cortex and around the neuropil. Glia cells also have a very electron dense cytosol and condensed chromatin (blue arrows in Fig. 4.2A). Finally, scattered throughout the brain but with an accumulation in the dorsomedial area are small cell bodies (3-4 µm diameter) that have a small dark cytoplasm and clumpy condensed chromatin, comparable to that seen in quiescent NBs, suggesting these are inactive, undifferentiated neuronal cells (pink borders in Figure 4.2A and 4.2C).

Beneath the cell cortex layer, a prominent neuropil is located in which the processes of differentiated neurons are interconnected via synapses. Given the high resolution of the EM data, neuronal interconnections within the neuropil can be reconstructed by determining morphologically which neuronal processes are synaptically interconnected, thus, allowing a comprehensive connectome to be established (Cardona et al., 2010). Sectioned synaptic zones are 0.15-0.3µm in size and can be identified due to an accumulation of synaptic vesicles and the presence of an electron dense T-bar on the presynaptic side as well as to the characteristic synaptic densities on the postsynaptic side (Figure 4.2C') (Cardona et al., 2010; Feeney et al., 1998; Kittel et al 2006, Prokop and Meinertzhagen 2006).

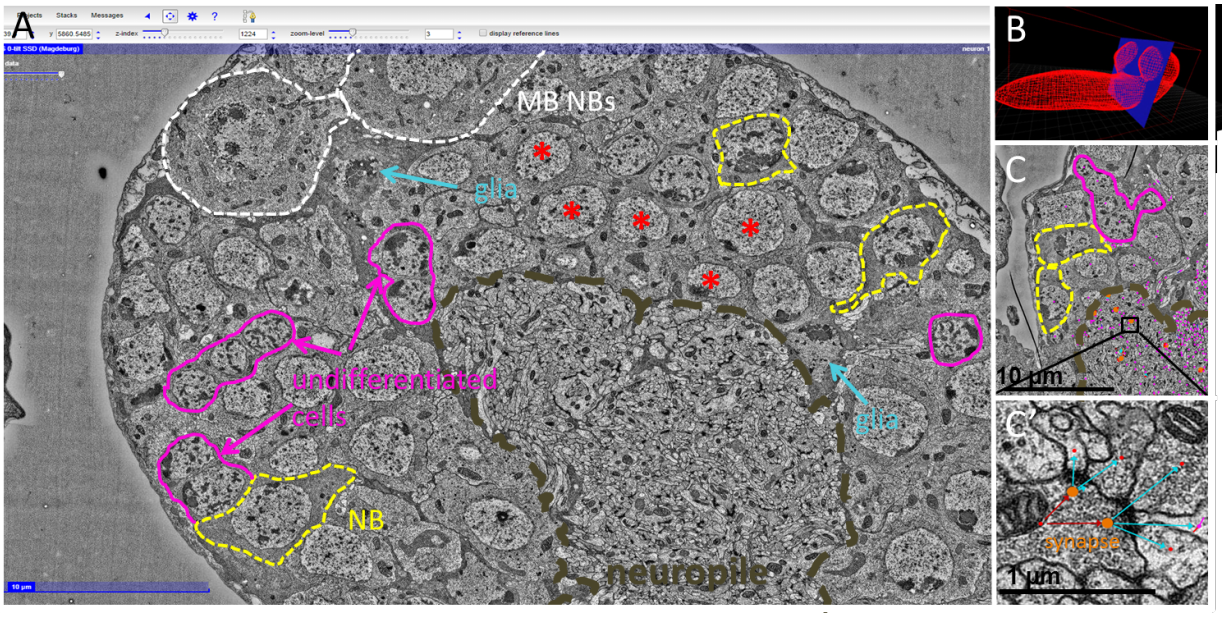


Figure 4.2 Serial section transmission electron microscope (ssTEM) data of the early first instar larval brain reveal morphologically distinguishable cell bodies and synapses.
(A) Z-projection of a single optical section of the ssTEM data set in CATMAID showing the brain cortex and the neuropil of the right brain hemisphere. Mushroom body neuroblasts (MB NBs, white circles (10 µm)) show a large nucleus and a cytoplasm packed with mitochondria. Glia cells (blue arrow) show an electron dense cytosol and condensed chromatin in the nucleus and membrane processes that spread throughout the cortex and around the neuropil. Differentiated cells (a selection thereof indicated with red asterisks) show light cytoplasm and chromatin is uncondensed. **(A+C)** Quiescent neuroblasts (NBs, yellow circles (5-7 µm)) show an electron dense cytoplasm and condensed chromatin in the nucleus. Undifferentiated cells (pink circles (3-4 µm)) show a small dark cytoplasm and condensed chromatin in the nucleus. The neuropil is located in the centre of the brain (brown dotted line). **(B)** Scaffold of the first larval instar brain as reveald by CATMAID. Blue plane represents the z-position of the single optical section seen in (A). **(C)** Right brain hemisphere showing traced neuronal skeletons in the neuropil (pink dots). **(C')** is a close-up of the black box in (C) showing two electron dense synapses with orange nodes representing the presynaptic side of each synapse and the blue arrows heading into the postsynaptic cells.

By definition, the neurons of the first larval instar brain, and correspondingly all the neurons represented by the ssTEM data, are generated in the embryo. Thus, if type-II NBs generate embryonically born neurons, these neurons should also be present in the first larval instar brain. We therefore aimed to identify type-II NB lineage derived neurons among the total neurons of all the NB lineages in the EM serial sections of the L1 brain. For identification of putative type-II NB derived neurons in the EM sections, we used information on the neuroanatomical features of the type-II NB lineage derived neurons as revealed by previous light microscopic analyses of larval brains (see above). Since previous work has shown that CCPaC's are early born neurons generated by the type-II NBs, we assumed that at least a subset of these cells might be of embryonic origin and, in consequence, hypothesised that a corresponding central complex

primordium structure might already be found in the first larval instar. To determine if this is the case, we made use of a series of highly stereotyped neuroanatomical features of the larval CCPaC's to search for corresponding cells in the EM serial sections.

First, in view of the CCPaC's morphology in late larval stages, we searched the EM sections for 4 lineages that project into a dense commissural structure in a characteristic four-fold symmetrical manner on each side of the commissure. Second, since the central complex primordium at late larval stages is surrounded by neuropil glia, we also expected the commissural fascicles of the putative embryonic born CCPaC's to be surrounded by glia as they enter the commissure. Third, given that the primordium at mid L2 is reduced in size and cell number as compared to later larval stages, we expected that the putative CCPaC's in L1 would also be reduced in number and give rise to a relatively small midline primordium. Fourth, since the CCPaC's at L3 do not display any synaptic markers such as Bruchpilot (nc82) and are therefore likely to be undifferentiated, we expected that this would also be the case for the putative embryonic born CCPaC's (Riebli et al., 2013).

4.4.3 Identification of undifferentiated CCPAC's and the central complex primordium in EM serial sections of the L1 brain

Based on these expected anatomical features, we identified two, bilaterally symmetrical axon fascicles in the EM sections of the L1 brain which likely correspond to projections of CCPaC's. These two fascicles are associated with the brain commissure and each of them comprises 45-49 processes (Figure 4.3A). Moreover, each of the two fascicles is surrounded by a glial sheath that is generated by a single large glia cell located adjacent to the fascicle (Figure 4.3A and 4.3A').

To verify that these two neuronal fascicles do indeed correspond to the projections of embryonically generated CCPaC's, we reconstructed all of the neuronal cells that project processes into these fascicles. For this, we traced each of the neurites present in the fascicles back to its cell body of origin (retrograde trace) as well as across the commissure and into the contralateral neuropil (anterograde trace). This revealed the complete morphology of all of the neurons that projected processes into the fascicles. Remarkably, when the reconstructions of all of these neurons and their processes are viewed together in 3D (via digital superimposition), an excellent representation of the expected type-II NB lineage-derived central complex primordial structure at the first larval instar is attained at EM resolution (white cells in Figure 4.3B, B' and D and compare to Figure 4.3C). (In this Figure and all to follow, the entire arborization for each

neuron is shown as reconstructed while the cell body is represented in a simplified form by a sphere).

The morphology of this reconstructed primordial structure in L1 resembles that of the previously characterized CCPaC-derived primordium in L3 in the following respects. First, the neurons that contribute to the midline primordium structure all lack differentiated axonal or dendritical arbors (Figure 4.3B, B' and D). Second, once the axons of these neurons have projected across the midline into the contralateral hemisphere they end abruptly without forming branch points or bifurcations (Figure 4.4A). Third, the specific arrangement of the cell bodies and their neurites resembles that of the CCPaC's at third larval instar, even though cell numbers are considerably lower at the L1 stage as compared to L3 (about 25 cells per hemisphere in L1 compared to 90 cells per hemisphere at L3; Riebli et al., 2013) (compare Figure 4.3C with 4.3D). Fourth, taken together as a population, these cells manifest the expected four-fold modularity of tracts that enter the primordium neuropil ipsilateral to the midline (compare colour coded circles in Figure 4.3C with 4.3D).

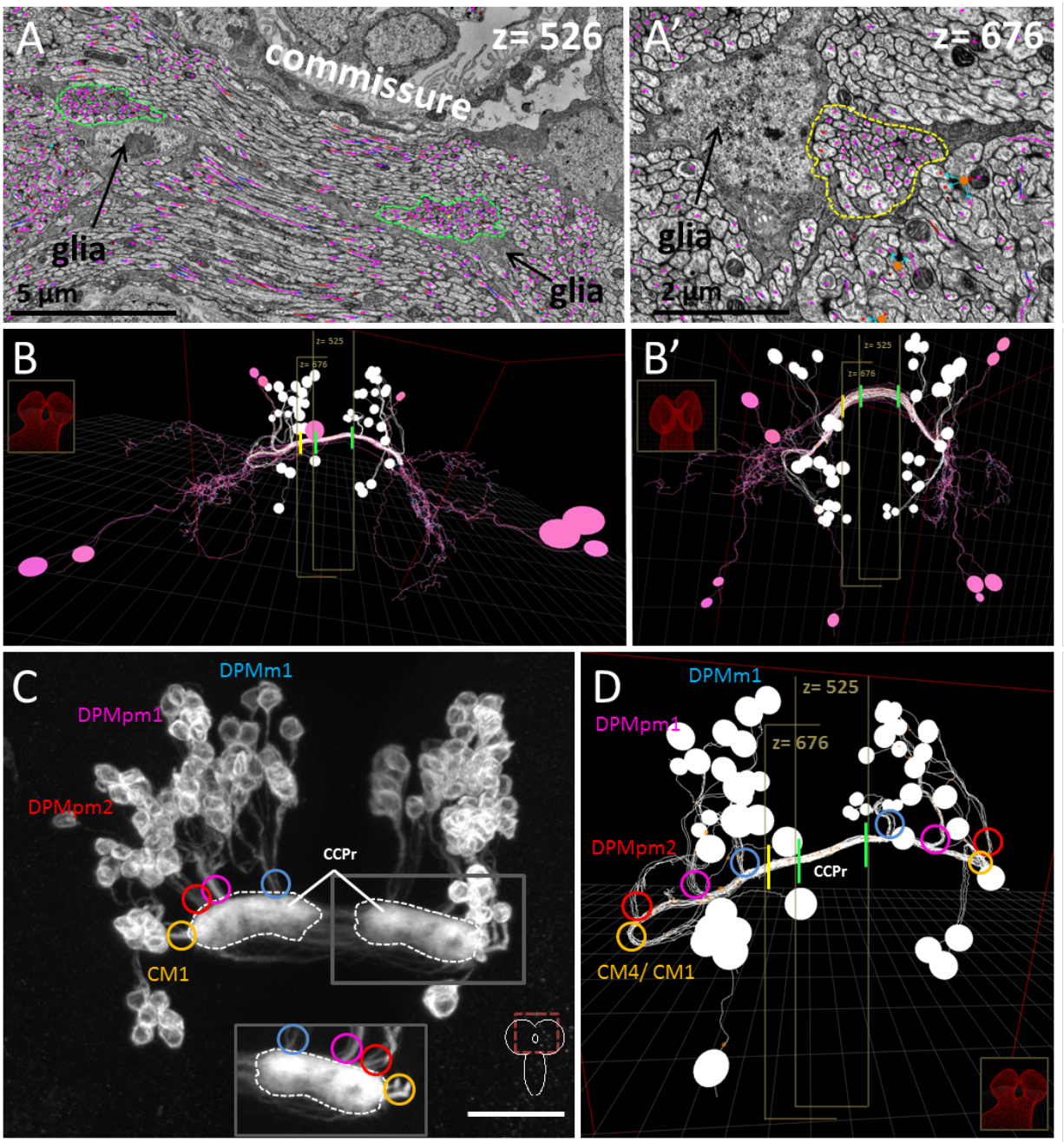


Figure 4.3 3D reconstruction of a distinct commissural fascicle in the ssTEM data stack reveals embryonic origin of the central complex primordium associated cells (CCPaC's) and of the central complex primordium (CCPr).

(A) Single optical section in the ssTEM data stack of the commissural area shows a neurite fascicle entering the commissure from both brain hemispheres. The cross sections of the fascicle (green lines) are surrounded by glial cells on either side of the commissure. **(A')** Cell body of the glia ensheathing the fascicle (yellow lines) entering from the right brain hemisphere is shown on a more posterior z-plane. These two z-planes are indicated as green and yellow bars in (B+D). **(B+B')** 3D reconstruction of all the cells contributing to the fascicle crossing the midline reveals a central complex primordium made up by the central complex primordium associated cells (cells in white), as well as 4-5 additional cells that are

differentiated and /or located outside the dorso-posterior medial area of the central brain (cells in pink). **(C)** *R45F08-Gal4* driver labeling the CCPaC's at third larval instar. Z-projection of multiple optical sections. Inset in (C) is maximum intensity projections of multiple adjacent confocal slices. **(C+D)** Comparison of the topological features of the CCPaC's and the CCPr in the light microscopic data at third larval instar (C) with the reconstructed CCPaC's and the CCPr of the early first larval instar at EM resolution (D). Different colors are assigned to different type-II NB lineages and color-coded circles reveal the lineage-specific entry point of the axon fascicles into the central complex primordium (CCpr). Scale bar 10 µm.

Given this excellent agreement in neuroanatomical terms of the L1 primordium as reconstructed from EM data with that of the L3 primordium as visualized by light microscopy, we conclude that the reconstructed cells of the L1 primordium correspond to the postulated embryonically generated CCPaC's. Thus, embryonically generated undifferentiated CCPaC's form a central complex primordium with a four-fold modularity, which is already present at the onset of postembryonic development at the early first larval instar stage. Given that CCPaC's are generated by type-II NB lineages, the reconstructed CCPaC's of the L1 primordium represent the first example of embryonically generated type-II NB lineage derived neurons. Based on their entry point into the commissural primordium, we tentatively assign subgroups of the CCPaC's to the type-II NB lineages DPMm1, DPMpm1, DPMpm2, and CM4/CM1 (Figure 4.3 D).

4.4.4 Identification and comprehensive reconstruction of the embryonically born neurons in the L1 DPMm1 lineage reveal undifferentiated neurons and differentiated neurons

It is unlikely that the CCPaC's of the L1 brain are the only neurons in the type-II NB lineages that are generated during embryogenesis. To identify the full complement of embryonically generated type-II NB derived neurons we took advantage of the fact that all of the neurons of a given NB lineage initially project their neurites into a single fascicle that exits the cortical cell body layer at a lineage-specific location (see Figure 4.1 A, B, C and D). Thus, by identifying this initial fascicle for each type-II NB lineage and by reconstructing all of the neurons that project neurites into this fascicle, it should be possible to identify all of the neurons generated by a given type-II NB. Since identification of the initial fascicle of each type-II NB lineage in the EM serial sections of the L1 brain was possible by following the axons of the CCPa neurons towards their cell bodies, subsequent reconstruction of all of the neurons that project their neurites into this fascicle should reveal the full set of embryonically generated neurons in the corresponding type-II NB lineages.

To carry out this type of comprehensive type-II NB lineage reconstruction of embryonically generated neurons, we focussed on the DPMm1 lineage in the EM serial sections of the L1 brain

hemisphere. Identification of the main fascicle of this lineage was possible by following the most medial of the four-fold CCPaC axon tracts back to the cell body layer. Full reconstruction of the neurons, that projected neurites into this initial fascicle of the DPMm1 lineage revealed 41 neurons (Figure 4.4C). To elucidate the degree of contralateral lineage homology and also to control for errors in reconstruction, both the left and the right DPMm1 lineages were reconstructed and analysed. In all of the respects mentioned below, both of these reconstructions were highly similar (Figure 4.5B, 4.6B, 4.6D, 4.6F, 4.7B, 4.7D, 4.7F).

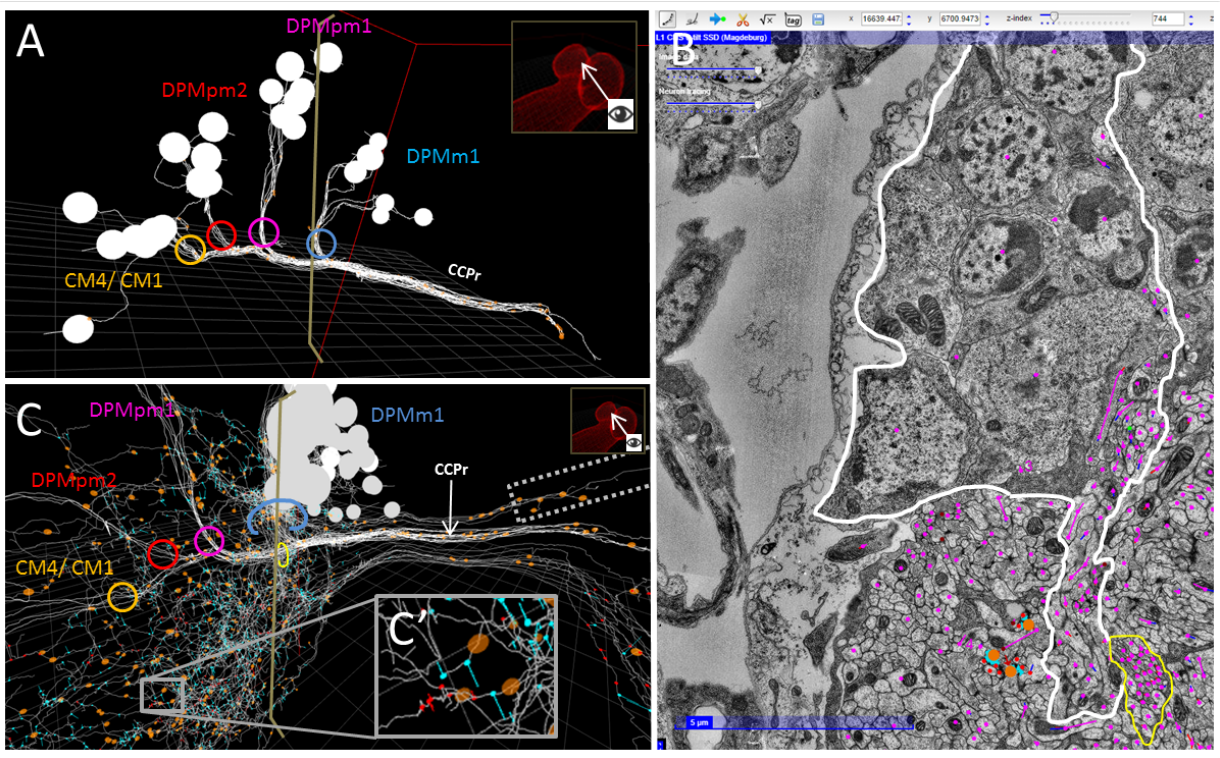


Figure 4.4 The axon fascicles of the CCPaC's enable the identification of the lineage specific main axon fascicle of DPMm1 and thus reveal all the DPMm1 NB derived neurons at L1.
(A+C) CCPaC's in white, beige bar indicates the z-plane as shown in the EM data stack in (B). Different colors are assigned to different type-II NB lineages and color-coded circles reveal the lineage-specific entry point of the axon fascicles into the central complex primordium (CCpr). **(A)** Embryonically derived CCPaC's of the right brain hemisphere do not show any axon branching and /or synapses and their trajectories end abruptly after crossing the commissure. **(B)** Z-plane showing the area of the DPMm1 fascicle tract as revealed by the CCPaC's in (A). Cells and axon tracts belonging to the DPMm1 lineage of the right hemisphere are surrounded in white in the EM data stack. The closely associated CCPr is surrounded in yellow (compare to yellow circle in C). **(C)** 3D reconstruction of all the cells belonging to the DPMm1 fascicle in (B) reveals a total of 41 neurons that contain undifferentiated neurons who's axons cross the commissure dorsal to the CCPr and then end abruptly (white dotted rectangle) as well as differentiated neurons that cross the commissure ventral to the CCPr and form synapses on both sides of the midline. Whereas the color-coded circles of DPMpm1, DPMpm2 and CM4/CM1 only show the CCPaC's of these lineages, the blue circle of DPMm1 surrounds the complete neurite bundle of all the DPMm1 derived neurons. **(C')** Blow-up showing presynaptic (red pins) and postsynaptic (blue pins) connections as well as areas of uncertain tracing (brown dots) of differentiated neurons.

In the reconstructed DPMm1 lineage of the right hemisphere, in addition to the 7 CCPaCs, approximately 17 cells have features of undifferentiated neurons; they do not form dendrites and have axons that stop abruptly without forming terminals. One subset of these (5 neurons) projects axons across the commissure and then ends in the contralateral neuropil without differentiating, reminiscent of the axons of the CCPaC's. The commissural axons of these neurons form a fascicle that runs dorsal to the fascicle formed by the axons of the CCPaC's (Fig. 4.4C and 4.5A). A second subset (2 neurons) project axons posteriorly into the ipsilateral neuropil where they end. The remaining 10 neurons are very diverse in their axonal projection patterns. Some project axons towards the ventral ganglia, others have axons that either remain in the initial fascicle or exit the initial fascicle and then immediately terminate (Figure 4.5A). In summary, approximately half of the embryonically generated neurons in the DPMm1 lineage are undifferentiated in the L1 brain. None of these undifferentiated neurons show morphological evidence for pre-, or postsynaptic specialisations in the EM serial sections.

The remaining 17 neurons in the DPMm1 lineage have features of differentiated neurons namely both dendritic arbors and axonal arbors, and all of these neurons manifested morphological evidence for pre-, and postsynaptic specialisations in their EM serial sections. Furthermore, these differentiated neurons show a remarkably large diversity of neuroanatomical types. Many, but not all, of these neurons have commissural projections, and several are present in pairs of similar morphology. Others have specific neuroanatomical features indicating that they are individually identifiable cells. The morphologies of each of the DPMm1 derived differentiated neurons as reconstructed from EM serial sections are described in detail below.

4.4.5 Differentiated neurons in the L1 DPMm1 lineage are highly diverse and innervate multiple neuropil areas of the brain

Within the DPMm1 lineage of the right hemisphere, we found 7 different types of differentiated neurons that varied in their neuroanatomical features. Neurons of the same morphological type projected together, innervated the same neuropil area and had neurites whose neighborhood relationship was maintained over many micrometers. For the majority of neurons identified in one (the right) hemisphere, we found homologous neurons of virtually identical morphology in the contralateral (left) hemisphere (Figure 4.6B, 4.6D, 4.6F and Figure 4.7B, 4.7D, 4.7F).

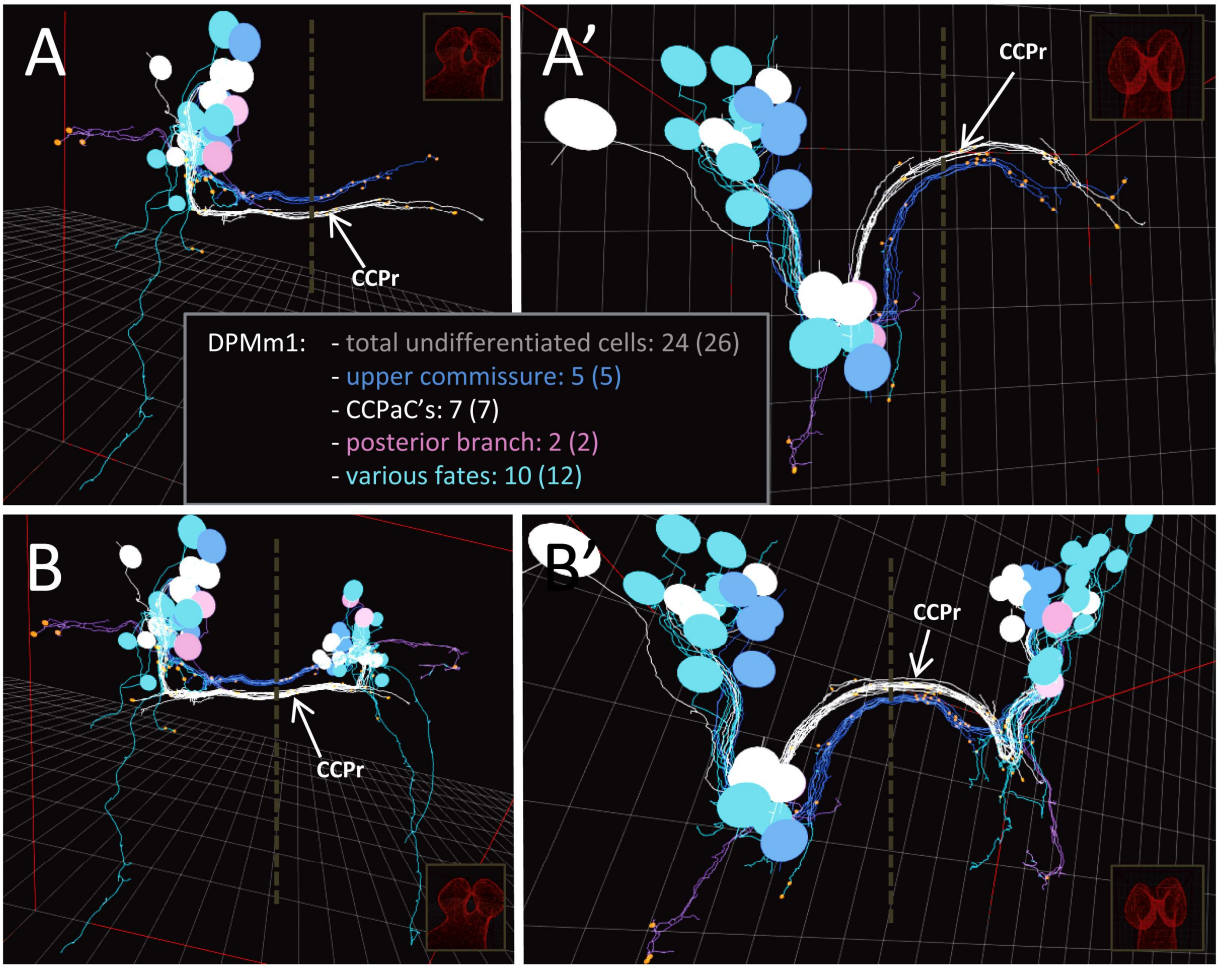


Figure 4.5 DPMm1 derived undifferentiated neurons have various morphological identities at L1.
(A) DPMm1 derived undifferentiated neurons of the right brain hemisphere, color-coded according to their morphological identities and shown from posterior (A) and dorsal (A'). Central complex primordium associated cells (CCPaC's) cross the commissure then halt without branching and /or forming any synapses (white cells). A second identity of undifferentiated cells cross the commissure more dorsal than the CCPaC's and also stop abruptly (dark blue cells). A third identity of undifferentiated cells project posterior and lateral (pink cells). The third identity of undifferentiated cells are mixed and include cells that project ventrally as well as cells that do not exit the main lineage axon tract (light blue cells). The inset shows the different cell identities and their cell numbers as found in the right brain hemisphere and the left hemisphere (numbers in brackets). **(B)** DPMm1 derived undifferentiated neurons of both hemispheres, color-coded according to their morphological identities and shown from posterior (B) and dorsal (B'). Note the perfect overlap of projections derived from both hemispheres when crossing the midline. Dashed line represents the midline.

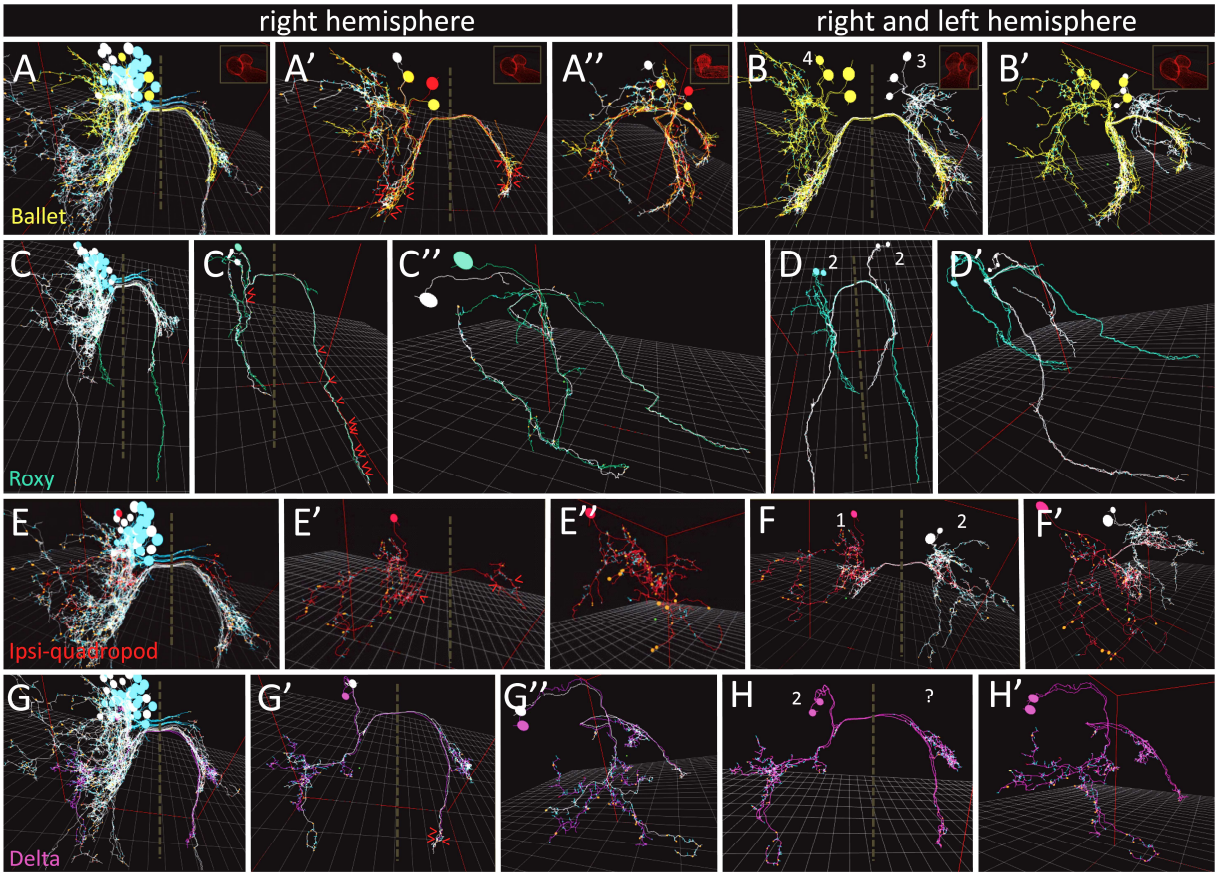


Figure 4.6 DPMm1 derived differentiated neurons with contralateral projections have various morphological identities at L1.

(A) Ballet neurons innervate broadly on the ipsilateral side and locally on the contralateral side. **(B)** 4 Ballet neurons were found on the right hemisphere (yellow cells) and 3 on the left hemisphere (white cells). **(C)** Roxy neurons innervate sparsely on the ipsilateral side and after crossing the commissure descend to innervate the VNC on the contralateral hemisphere. **(D)** 2 Roxy neurons were found in each brain hemisphere. **(E)** The Ipsi-quadropod neuron innervates broadly on the ipsilateral side and sparsely on the contralateral side. **(F)** One Ipsi-quadropod neuron was found on the right hemisphere (red cell) and two on the left hemisphere (white cells). **(G)** Delta neurons innervate broadly on the ipsilateral side and sparsely in two neuropil areas on the contralateral hemisphere. **(H)** Two Delta neurons were found in the right hemisphere (pink cells) but none in the left hemisphere. (A, A', B, C, C', D, E, E', F, G, G' and H) are posterior views. (A'', B', C'', D', E'', F', G'', H') are lateral views. Blue cells in (A, C, E and G) represent the undifferentiated cells of the DPMm1 lineage on the right hemisphere. Grey cells in (A, C, E and G) represent the differentiated cells of the DPMm1 lineage on the right hemisphere. White cells in (B, B', D, D', F and F') represent the cells of the corresponding morphological identity derived from the DPMm1 lineage of the left hemisphere. Red arrowheads in (A', C', E' and G') show areas where in addition to the abundant postsynaptic terminals also presynaptic connections are found. Dendritic terminals are abundantly distributed on the neurite branches of all the neurons described except in the commissural area and the cortex. Dashed line represents the midline.

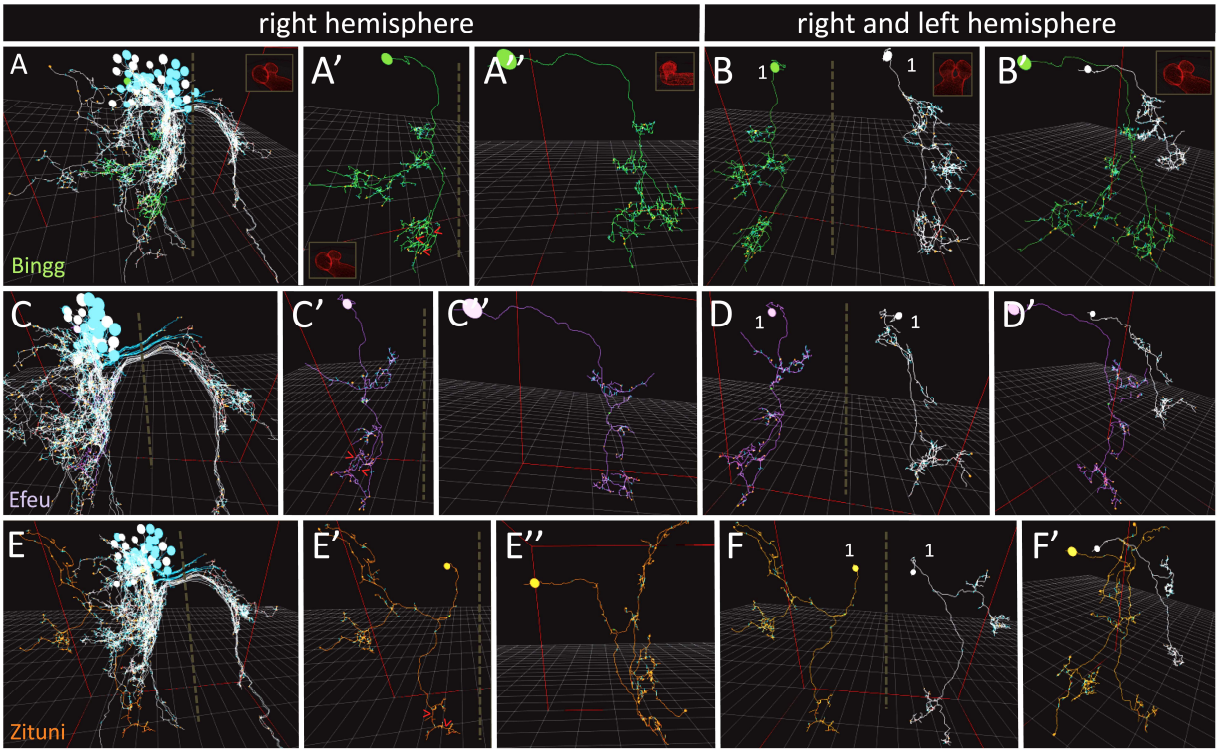


Figure 4.7 DPMm1 derived differentiated neurons with exclusively ipsilateral projections have various morphological identities at L1.
(A) Bingg neurons innervate in three neuropil areas on the ipsilateral hemisphere. **(B)** One Bingg neuron was found in each brain hemisphere. **(C)** The Efeu neuron innervates the neuropil broadly along its main axon tract on the ipsilateral hemisphere. **(D)** One Efeu neuron was found in each brain hemisphere. **(E)** The Zituni neuron first innervates into two distinct neuropil areas of the lateral neuropil and then sends an additional axon tract ventrally within the ipsilateral hemisphere. **(F)** One Zituni neuron was found in each brain hemisphere.
(A, A', B, C, C', D, E, E' and F) are posterior views. (A'', B', C'', D', E'', F') are lateral views. Blue cells in represent the undifferentiated cells of the DPMm1 lineage on the right hemisphere. Grey cells in (A, C and E) represent the differentiated cells of the DPMm1 lineage on the right hemisphere. White cells in (B, B', D, D', F and F') represent the cells of the corresponding morphological identity derived from the DPMm1 lineage of the left hemisphere. Red arrowheads in (A', C' and E') show areas where in addition to the abundant postsynaptic terminals also presynaptic connections are found. Dendritic terminals are abundantly distributed on the neurite branches of all the neurons described except in the commissural area and the cortex. Dashed line represents the midline.

The first type of neuron, which we call Ballet neurons, form extensive arborizations in the hemisphere ipsilateral to their cell body, project a process across the commissure and form regionalized terminals in the contralateral hemisphere. Morphological analysis of synaptic specializations in these neurons indicates that postsynaptic specializations are primarily located on the extensive ipsilateral arbors, whereas most presynaptic specializations are found on the arbors of the contralateral projecting processes. Ballet neurons are the only cells found in more than two replicates per hemisphere (4 on the right hemisphere and 3 on the left hemisphere)

(Figure 4.6A). The second neuronal type, which we call Roxy neurons, are descending neurons that innervate the VNC. This type is represented by a pair of neurons, which may be homologs, in each hemisphere. Roxy neurons form sparse arborizations in the ipsilateral hemisphere, project across the commissure and then ventrally into the contralateral connective towards the VNC. In this cell type, postsynaptic specializations are found predominantly in the ipsilateral arbors and sparse presynaptic specializations are seen in the processes that reach the VNC (Figure 4.6C). The third type of neurons, which we refer to as Ipsi-quadropod neurons, form extensive ipsilateral arborizations, project across the midline and arborize regionalized in the contralateral hemisphere. These neurons have either one or two representatives per hemisphere and their synaptic specializations are predominantly postsynaptic, with some sparse presynaptic specializations located in arbors that flank the midline (Figure 4.7A). The fourth type of neurons, referred to as Delta neurons, are present in two copies in the right hemisphere, however their contralateral representatives remain elusive. Delta neurons form broad arborizations in the ipsilateral hemisphere, project a process across the commissure and into the contralateral hemisphere where they bifurcate and form a lateral and a ventral arborization. These two contralateral arborizations manifest predominantly presynaptic specializations; the ipsilateral arborizations manifest predominantly postsynaptic specializations (Figure 4.7G). The fifth neuronal type, which we refer to as Bingg neurons, are restricted to one hemisphere and branch profusely in 3 different ipsilateral neuropil areas. These neurons have only one representative per hemisphere and their synaptic specializations are mainly postsynaptic with just a small number of presynaptic terminals found on the arbors that innervate the most dorsoventral neuropil compartment (Figure 4.6E). The sixth neuronal type, called Efeu neurons, are local neurons present in one representative per hemisphere; their arbors form diffuse terminal arborizations which are all restricted to the ipsilateral hemisphere. Most of the synaptic specializations in Efeu neurons are postsynaptic, however, some presynaptic specializations are present in the most ventral innervation area (Figure 4.7C). The seventh neuronal type, called Zituni neurons, are present in single copies per hemisphere and have two processes which form two separate arborizations in the ipsilateral hemisphere as well as a third process which projects ipsilaterally towards the subesophageal ganglion where it forms terminal arbors. These terminal arbors have both presynaptic and postsynaptic specializations; the other two arbors projecting laterally have primarily postsynaptic specializations (Figure 4.7E).

The complete set of superimposed differentiated and undifferentiated neurons for both DPMm1 lineages are shown in Figure 4.8, in which each different neuron type is colour coded as in Figure 4.6 and Figure 4.7. Two morphological features of the ensemble of neurons in this lineage are noteworthy. First, within the lineage, embryonically generated neurons of the same

morphological type form their projections in close vicinity to each other. This is most obvious in their commissural projections, where neurons of the same types project in the same fascicles; this is true for differentiated as well as for undifferentiated neurons (CCPa neurons project in a different fascicle than do the other undifferentiated neurons). Second, taken together, the morphologically diverse set of embryonically generated neurons of the DPMm1 lineage extends highly branched processes into a large area of the central brain neuropil. Thus, these embryonically derived type-II NB generated neurons, like their postembryonically generated siblings, are not only diverse in their morphology, but also innervate many different neuropils of the central brain and some cases even project out of the brain to innervate the VNC. Finally, it is noteworthy, that the clustering of all DPMm1 lineage cell bodies (as expected near the dorsal midline of the brain) makes it possible to identify the spatially adjacent neuroblast of the lineage. Analysis of this neuroblast in the EM serial sections shows that this progenitor cell extends cytoplasmic processes along the neurite fascicle of clustered daughter cells (Figure 4.8C). Comparable neuroblast processes have been reported in earlier work in the VNC and are thought to disappear as soon as the NBs exit quiescence and increase in size (Truman and Bate 1988, Tsuji et al., 2008; Chell and Brand 2010).

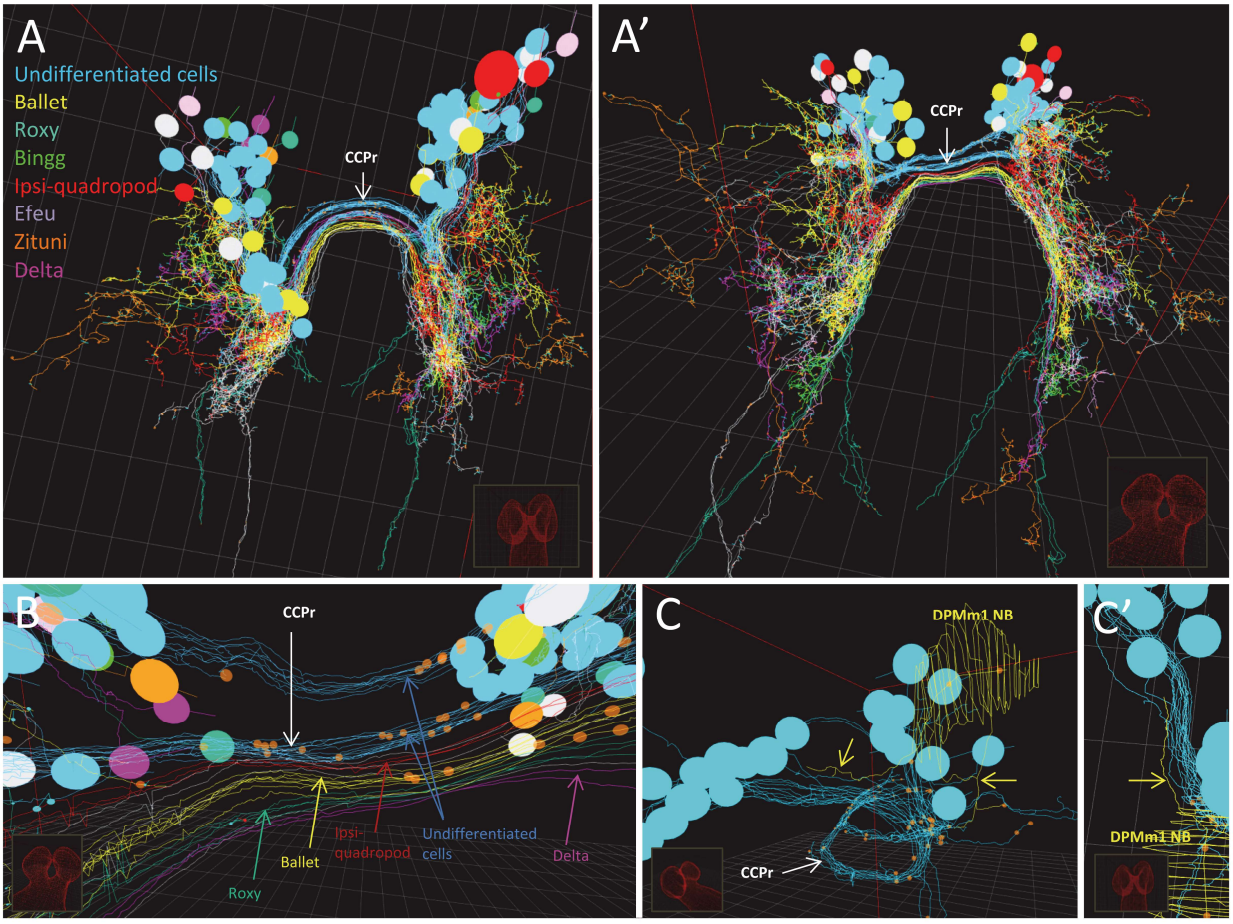


Figure 4.8 The complete type-II NB lineage DPMm1 as reconstructed from serial section EM data.
(A, B) DPMm1 NB lineages of both brain hemispheres shown with neurons color-coded according to their morphological identity reveal broad innervation in the brain neruopil. (A) dorsal view (A') posterior view (B) close up posterior view of the commissural area. Color-code of different cell identities is given in (A). **(B)** DPMm1 NB derived neurons with a given morphological identity cross the commissure in an identity-dependant topologically organised fashion on the dorso-ventral axis. Most ventral of all the DPMm1 NB derived neurons cross the differentiated neurons. Dorsal to the differentiated neurons crosses the central complex primordium (CCPr) made up by undifferentiated neurons and even more dorsal crosses the second commissural fascicle of undifferentiated neurons. **(C)** DPMm1 NB shown next to the DPMm1 NB derived undifferentiated neurons. Note the NB's outgrowing processes (yellow arrows), one of which extends along the neuronal fascicle of undifferentiated neurons (yellow arrow in (C')). **(C')** Dorsal view of DPMm1 NB and undifferentiated cells.

4.5 Discussion

In this report we use topological guideposts from light microscopic analysis of type-II NB lineages to identify their embryonically born neurons in the EM data stack of the first larval instar CNS. We demonstrate that a subset of central complex primordium forming cells are generated during embryogenesis and that these cells, albeit undifferentiated and devoid of synapses, already form the highly organized central complex primordium visible shortly after larval hatching. Furthermore, the here described embryonically born differentiated neurons have widespread innervations in many parts of the larval brain underlining the complexity of the type-II NB lineages reported for postembryonic stages. These findings reveal detailed insights into the embryonic complexity of type-II NB lineages and the embryonic formation of the central complex primordium. In addition, this work uncovers a new cell type of embryonically born undifferentiated neurons of type-II NB lineages, which is not classifiable within the current distinction between primary and secondary neurons.

4.5.1 Embryonic origin of the central complex primordium

Previous studies have shown that the central complex primordium is already present in the third larval instar brain (Riebli et al., 2013; Young and Armstrong, 2010b). The cells giving rise to the central complex primordium (CCPaC's) have been analyzed in detail (at third larval instar stages and into the adult) and shown to be derived from the four type-II NB lineages DPMm1, DPMpm1, DPMpm2 and CM1. Even though these CCPaC's were reported to be early born, previous studies could not reveal whether they were among the first born cells during postembryonic development, or if they were of embryonic origin.

Using single-cell 3D reconstruction of neurons at EM resolution in a 4h L1 brain, we clearly find an embryonically derived central complex primordial structure. Remarkably, the four-fold lineage-specific topological order of projections, as described for third larval instar stages, is already established shortly after larval hatching. However, only a subset subset of the CCPaC's is generated during the embryo. Whereas in late larval stages 90 CCPaC's are found to build the CC primordium in each hemisphere (Riebli et al., 2013), at early L1 only 25 CCPaC's are present. Thus, during postembryonic stages more cells are added and their axons then project along the embryonically generated scaffold of the primordial neuropil structure. This generation of adult specific neurons in the embryo suggests that the conceptual separation of the two neurogenesis phases in *Drosophila* is less strict than presumed.

4.5.2 Type-II NB derived undifferentiated neural cells in the early first instar larval brain

Due to their apparent lack of terminal branches, the CCPaC's have been reported to be of an undifferentiated nature at third larval instar (Riebli et al., 2013). In agreement with this, our ultrastructual data show that the embryonically generated CCPaC's indeed lack synapses as well as neurite arborizations at the first larval instar stage. The discovery of embryonically generated but postembryonically (in larval stages) undifferentiated neurons contrasts with the currently accepted distinction of primary neurons (postembryonically differentiated and functional) and secondary neurons (postembryonically generated and undifferentiated cells that only mature during metamorphosis). Thus, our study introduces a new cell type of embryonic born neurons in type-II NB lineages that remain undifferentiated during postembryonic larval stages and do not mature before metamorphosis. Indeed, differentiation and maturation of all CCPaC's, irrespective of their time of birth, is likely to occur only during metamorphosis.

In addition to the embryonically generated CCPaC's our study uncovers a large variety of other undifferentiated neurons at L1. In some cases, the projections of these undifferentiated cells proceed alongside neurites of differentiated primary neurons (the neurites of some of these undifferentiated neurons project ventrally alongside the Efeu neurons, the Zituni and the Ballet neurons). In other cases, the undifferentiated cells have completely different projection patterns that are unrelated to those of the neurons of the functional larval brain (e.g. the CCpr and the upper commissural bundle, see Figure 4.5). Reconstruction of all the undifferentiated cells of DPMm1, DPMpm1, DPMpm2 and CM4 did not reveal additional specific primordia of individual central complex substructures such as the protocerebral bridge, the ellipsoid body, the fan-shaped body or the noduli (data not shown). Therefore, we hypothesize that the CCPr described in this work is in fact the primordial neuropile structure for the central complex as a whole. We suggest that all the secondary cells contributing to the adult central complex substructures use the scaffold provided by the CCpr as initial guidance substrate for their projections.

To date there is no direct neuroanatomical evidence for the existence of intermediate progenitor cells (INPs) in embryonic type-II NB lineages. Also, our 3D reconstruction of the embryonically derived DPMm1 lineage did not reveal any cell type that had the morphology expected for an intermediate progenitor cell or for a GMC. Both of these cell types would be expected to lack a neuronal process and their spatial localization within the lineage would be close to the neuroblast. With the exception of the neuroblast, all of the cells reconstructed in

the DPMm1 NB lineage of first larval instar brain had at least one outgrowing neuronal process. This suggests that during the quiescent period there are no longer any intermediate progenitor cells present. This in turn implies that all embryonically generated INPs undergo their last mitotic division before larval hatching leaving their lineage of origin populated with numerous young, undifferentiated neurons. It seems likely that the relatively large number and diverse morphological features of the embryonic born neurons in the DPMm1 NB lineage is due to the presence of amplifying INP cells in the embryo. However, definite proof that INPs are indeed present during the embryonic development of the type-II NB lineages remains to be shown.

4.5.3 Type-II NB derived differentiated primary neurons in the early first instar larval brain

Further single cell reconstruction of entire type-II NB lineages in the context of the entire L1 brain connectome should provide the basis for a complete lineage-specific connection map and, hence, reveal insight into information processing that occurs in the embryonically generated differentiated neurons. This should also shed light on one of the intriguing finds of our study, namely that the embryonic born differentiated cells generated by DPMm1 have numerous input synapses (postsynaptic specializations) but remarkably limited output synapses (presynaptic specializations). Furthermore, the identity of the additional cells found to cross the midline through the CC primordial bundle remains to be determined through additional tracing and lineage affiliation. Previous studies have reported up to 15 NB lineages to be involved in the formation of the adult central complex neuropil structures (Yang et al., 2013).

The 3D reconstruction of the DPMm1 NB lineage at EM resolution reveals a remarkably high diversity of differentiated primary neurons that are identifiable on a single cell basis. These functional neurons broadly innervate the ipsilateral brain hemisphere and many of them project across the midline and innervate the contralateral hemisphere, and some even project into the VNC. Interestingly, these cells often come in pairs of two, which poses the question whether they are derived from a GMC clone or whether these cells both derive from two separate INP clones as has been shown to be the case in the first two postembryonic INP clones of the DPMm1 NB lineage (Wang et al., 2014). To investigate this, novel types of clonal analysis for the embryonic stages will be needed.

4.6 Authors' contribution

NR carried out all the immunohistochemical experiments. NR and VH reconstructed most of the neuronal arbors (small contributions of tracing are paid tribute to in the Acknowledgements). RF collected the ssTEM image data. SS and SG contributed the analysis software. HR and AC conceptualized the project. NR and HR analyzed the data and wrote the manuscript.

4.7 Acknowledgements

We would like to thank Javier Valdés Alemán, Ingrid Andrade, Philipp Schlegel, Jennifer Lovick, Haluk Lacin, and Katharina Eichler for contributing to this work with tracing neurons or fragments thereof. Furthermore, we thank Gudrun Viktorin and Yanrui Jiang, Casey Schneider-Mizell and Jose Botella for interesting discussion as well as Tom Kazimiers, and Susanne Flister for discussion and technical help. This work was supported by the SNF.

5. The first nervous system

Nadia Riebli[1], Heinrich Reichert[1]

[1]Biozentrum, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland

Manuscript under minor revision

Handbook of Evolutionary Neuroscience, edited by Stephen V. Shepherd
expected publication 2016

published by Wiley-Blackwell, John Wiley and Sons Ltd.

5.1 Introduction

The origin of the first nervous system is an intriguing enigma. Stated in its simplest form, a nervous system can be defined as a set of interconnected neural cells that process information via electrical and/or chemical signals. In consequence, by definition, the first nervous system evolved after the evolutionary transition from unicellular to multicellular life forms. Since nervous systems allow integration of sensory input and coordination of motor output in a behaviorally relevant manner, there are obviously significant selective advantages in evolving more sophisticated and complex nervous systems. In animal evolution this has led to the emergence of centralized nervous systems which comprise distinct agglomerations of functionally specialized neurons, that may be subdivided into separate parts (ganglia), are interconnected by axon tracts (neuropil) and connect to the periphery via nerves (Arendt *et al.*, 2008). Moreover, in most extant bilaterian animals, nervous system centralization combined with cephalization has resulted in the appearance of brains which are prominent anterior ganglia that receive major input from sense organs located on the head and send descending motor output to the somatic effector apparatus in the remaining body via nerve cords. In this review, we focus on the evolution of complex nervous systems from simple neural origins and consider evidence from comparative, developmental and molecular genetic studies that shed light on this fascinating evolutionary process.

5.2 The ambiguity of nervous system origins

A phylogenetic assessment of the origin of nervous systems based on currently available paleontological data is both enlightening and disappointing. On one hand, there is clear fossil evidence for the existence of complex nervous systems, including brains, in bilaterian animals that date back to at least 530-540 Mya (million years ago). Thus, the fossil record for arthropod-like trilobites and agnatiha-like stem vertebrates indicates that both groups already had brains and central nervous systems with features typical of extant arthropods, which are members of the protostome supergroup, and of extant vertebrates, which are members of the deuterostome supergroup (Fortey, 2000; Holland and Chen, 2001). This implies that centralized nervous systems with brains evolved before the protostome-deuterstome split in the urbilaterian ancestor of both major bilaterian supergroups. Centralization of nervous systems must have occurred earlier, probably after the split between bilaterians and radiate animals such as cnidarians which is dated at 600 – 630 Mya (Peterson *et al.*, 2004). However, fossil evidence for nervous systems from this precambrian period is scarce and difficult to interpret.

Hence, although evidence for the existence of central nervous systems in the early Cambrium is solid, we are left with little information on the origin of the first nervous system from paleontology.

A phylogenetic evaluation of the origin of nervous systems based on comparative neuroanatomical analyses of extant animals is also ambiguous, albeit for different reasons.
First, the nervous systems of all extant animals are by definition modern in that they have had the same amount of time to evolve (hundreds of millions of years). Furthermore, this evolutionary process can lead to both increase and reduction of nervous system complexity. Thus, even when a nervous system appears to be rather simple and "primitive" in neuroanatomical respects, this simple morphology can be due to a secondary loss of more complex structures due to the environmental features that the animal has adjusted to and due to the requirements of its ecological niche. Hence, it is a priori unclear which, if any, of the living animals have nervous systems that reflect the original, primitive nervous system in the precambrian ancestor of bilaterian and radiate animals.
Second, animal phylogeny is currently in flux and a source of considerable controversy mainly due to the interpretation of new data from genetic and genomic analysis. As a result it is often unclear which group of extant animals is basal and, thus, most likely to have a "primitive" type of nervous system. This is exemplified by the recent dramatic changes in "flatworm" phylogeny and their implications for brain evolution in bilaterians. Flatworms are classically considered to represent the simplest organizational form of all living bilaterians with a true central nervous system and based on their simple body plans have been traditionally grouped together in a single phylum at the base of the bilaterians (Hyman, 1940; Bullock and Horridge, 1965). However, subsequent molecular phylogenetic analyses have removed the flatworms from this basal position and placed the entire flatworm phylum within the Lophotrochozoa, one of the two protostome superclades (Adoutte *et al.*, 1999, 2000). From this molecular phylogenetic viewpoint, there is no reason to assume that the flatworm central nervous system is any more basal than that of the other lophotrochozoan animals. Current molecular phylogenomic studies have now actually split the flatworms into two widely separated clades, the platyhelminth flatworms which remain embedded among the lophotrochozoan phyla and the acoelomorph flatworms which are placed either at the base of the bilaterians or associated with the deuterostomes either as basal deuterostomes or the as the sister group of hemichordates and echinoderms (Figure 5.1) (Philippe *et al.*, 2007, 2011; Hejnol *et al.*, 2009; Mwinyi *et al.*, 2010). Thus, depending on their precise phylogenetic position, the acoel (but not the platyhelminth) flatworms and their supposedly "primitive" nervous systems are either basal to all bilaterians or

basal only to the deuterostomes or highly derived and related to hemichordate nervous systems.
Third, it has been found to be very difficult to consider the nervous systems of extant animal groups as primitive even when they are located on a very basal position within the tree of live. It is widely, but not universally, agreed that Porifera, Cnidaria, Ctenophora and Placozoa are basal animal groups. Since sponges and placozoans neither have nervous systems nor neurons, they are of limited help in defining the first nervous system. Neurons and nervous systems are present in Cnidaria and Ctenophora, as well as in all other eumetazoan animals, and therefore it has been hypothesized that the first nervous system evolved after the evolutionary separation of the Porifera from the Radiata (reviewed in Lichtneckert and Reichert, 2007). Since this implies that the Cnidaria might be the most basally branching phylum of the Eumetazoa manifesting a nervous system, the nervous organization of these animals has been studied in some detail. These studies show that cnidarian nervous systems are remarkably diverse ranging from diffuse nerve nets, in which there is little central integration and the sensory input and motor output are processed locally, to clearly centralized nervous systems with ganglion-like nervous centers that are associated with sophisticated sensory organs such as lens eyes (Satterlie, 2011). It is largely arbitrary to consider any one of the diverse nervous system types to be basal and hence “primitive” in this phylum, since even the diffuse nerve-net like nervous system type might represent the secondary loss of a previously present centralized nervous system.

In view of the problems in elucidating the origin of the nervous system based on classical comparative neuroanatomical analysis, a number of investigations in the last two decades have explored a novel approach to nervous system evolution that combines comparative studies with developmental and molecular genetic analysis. This new integrated approach has revealed considerable insight into the evolutionary origin of the brain and central nervous system of bilaterian animals. Moreover, it has provided new insight into the origin of centralized nervous systems that may also be relevant for understanding the origin of the first metazoan nervous system.

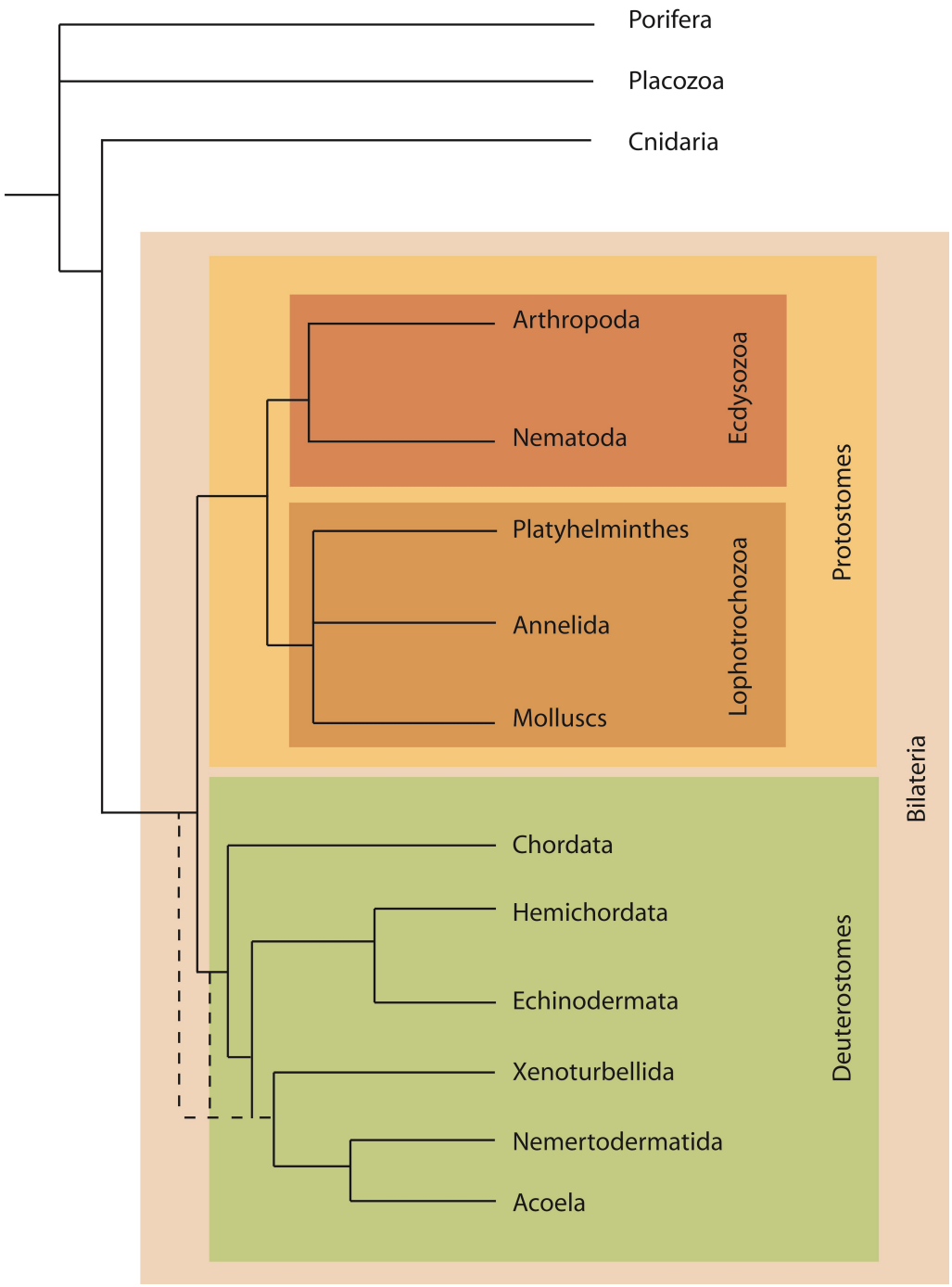


Figure 5.1 Summary scheme of the metazoan phylogeny.
It is widely agreed that the cnidarians are the sister clade to the bilaterian animals. Note that the former flatworm group has been split into the "Acoela" and the "Platyhelmithes" (Philippe *et al.*, 2007). Whereas the Platyhelminthes remained embedded within the Lophotrochozoans, the phylogenetic position of the Acoela is still a matter of debate. The newest studies either place the Xenoturbellida, the Nemertodermatida and the Acoela at the base of the Bilateria as a sister group to all other bilaterian animals (Hejnol *et al.*, 2009), at the base of the Deuterostomes, or within the Deuterostomes (Philippe *et al.*, 2011).

5.3 The first bilaterian nervous system

5.3.1 Diversity of bilaterian nervous systems

Many different morphological types and shapes of nervous systems are found in extant bilaterians. All deuterostomes investigated have central nervous systems and peripheral nervous systems. The peripheral nervous systems can be highly variable in structure ranging from the nerve-net type of organization seen in the vertebrate enteric nervous system to the ordered ganglionic organization exemplified by the vertebrate autonomic nervous system. The central nervous system of deuterostome chordates is in general less variable in structure. In the chordates, which include the vertebrates, the central nervous system comprises an anterior brain, subdivided into multiple compartment-like substructures, that is associated with sensory organs and is connected to a dorsally located nerve cord which links the brain to the peripheral body parts. In the urochordate tunicates, this type of central nervous system organization is only present in the larva and is radically reduced after metamorphosis in the sedentary adult form. In cephalochordates, the subdivisions in the brain and nerve cord are cryptic but can be revealed with molecular markers (Nieuwenhuys, 2002).

The central nervous systems of the remaining deuterostome phyla are more diverse. Hemichordates, which have been thought to possess only a net-like peripheral nervous system, are now known to have a fully formed central nervous system comprising dorsal as well as ventral nerve cords (Lowe *et al.*, 2003; Nomaksteinsky *et al.*, 2009; reviewed in Benito-Gutierrez and Arendt, 2009). Echinoderms also possess central nervous systems, which are, however, clearly divergent from those of other deuterostomes due to the secondary acquisition of radial symmetry in these animals (Nieuwenhuys, 2002). Acoel flatworms (whether they are bona-fide deuterostomes or not, and probably the flatworm-like xenoturbellids) have a central nervous system comprising an anterior ganglion and multiple nerve cords (Bullock and Horridge, 1965; Semmler *et al.*, 2010).

Most protostomes also have both central nervous systems and peripheral nervous systems. Prominent among the central nervous systems are the complex multiganglionic brains and nerve cords of most free-living arthropods, annelids and molluscs culminating in the remarkably complex brain of cephalopods. These complex nervous systems can be markedly reduced or absent in sedentary or parasitic forms in these and other protostome phyla. Central nervous systems with a somewhat more simple organization consisting of an anterior ganglion and

associated nerve cords are seen in free-living members of phyla as diverse as platyhelminth flatworms, ribbon worms, tardigrades, chaaetognathes, sipunculids, rotifers, ectoprocts, and nematodes (Bullock and Horridge, 1965, Kotikova and Raiikova, 2008). As in deuterostomes, the peripheral nervous systems of protostomes can be highly variable in structure ranging from a diffuse nerve-net type seen in molluscs to the ganglionic organization of some of the components of the arthropod peripheral nervous system.

Taken together, these data support the notion implied by current paleontological findings that centralized nervous systems were present in ancestral bilaterians before the protostome-deuterostome split. Moreover, based on comparative neuroanatomical data, these ancestral bilaterian central nervous systems likely consisted of an anterior brain-like ganglion ("protobrain") connected to descending nerve cord-like structures ("protocords") which may or may not have had ganglionic features (Ghysen, 2003). What is not at all obvious from these data is whether the diverse central nervous systems of extant bilaterians evolved separately or if they all had a common urbilaterian origin.

In its ontogeny, the bilaterian central nervous system is a complex three-dimensional structure that develops from a two-dimensional embryonic neuroepithelium. Since this neuroepithelium is located dorsally in most deuterostomes and ventrally in most protostomes, an independent evolutionary origin of the central nervous system in these two animal groups has been postulated (gastroneuralia-notoneuralia concept; *e.g*. Brusca and Brusca 1990). However, more recently (and supporting earlier ideas) the notion that the central nervous systems of protostomes and deuterostomes are homologous and derive from a common ancestral (urbilaterian) brain has been put forward (Arendt and Nübler Jung, 1994; Ghysen, 1992; Reichert and Simeone, 2001). This notion has received considerable support from the astounding conservation of developmental mechanisms that pattern the anteroposterior and dorsoventral axes of the central nervous system in several vertebrate and invertebrate model systems, as well as from the remarkable similarities in developmental origins of neuronal cell types and complex circuitry in bilaterian central nervous systems. Taken together, these recent comparative developmental genetic data indicate that similar mechanisms operate in many major stages of central nervous system formation in vertebrates and invertebrates, implying a monophyletic origin of the centralized bilaterian nervous system (Reichert, 2009; Arendt *et al.*, 2008; Hirth, 2010).

5.3.2 Conserved mechanisms for anteroposterior pattering of the bilaterian central nervous system

During early development, the two-dimensional neuroepithelium that gives rise to the neurons of the bilaterian central nervous system is subdivided into compartment-like domains along both its axes by the regionalized expression of patterning genes. These genes and their respective patterns of expression are comparable in vertebrates and invertebrates. Patterning along the anteroposterior axis involves the cephalic gap genes which are expressed in the anterior brain, the (homeotic) Hox genes which are expressed in the posterior brain and nerve cord, and a set of other genes which delimit specific compartment interfaces in the central nervous system (Figure 5.2).

Cephalic gap genes such as *orthodenticle* (*otd*)/*Otx* and *empty spiracles* (*ems*)/*Emx* encode transcription factors that were originally identified in *Drosophila* embryogenesis as key patterning elements for anterior cephalic domains (Dalton *et al.*, 1989; Finkelstein and Perrimon, 1990; Cohen *et al.*, 1990). In addition to their role in head development, these genes are expressed in the anterior neuroectoderm of vertebrates and invertebrates and play key, evolutionarily conserved roles in central nervous system pattering (reviewed in Lichtneckert and Reichert, 2008). The most prominent of these is exemplified by *otd*/*Otx* which is expressed in the anterior brain ("protobrain") of bilaterians as diverse as planarians, nematodes, annelids, molluscs, arthropods, urochordates, cephalochordates and vertebrates including mammals (Wada *et al.*, 1998; Hirth and Reichert, 1999; Finkelstein *et al.*, 1990 b; Nederbragt *et al.*, 2002; Bruce and Shankland, 1998; Arendt *et al.*, 2001; Tomsa and Langeland, 1999; Lanjuin *et al.*, 2003; Umesono *et al.*, 1999; Acampora *et al.*, 2001a; Schilling and Knight, 2001).

Functional studies carried out in *Drosophila* and mouse show that *otd*/*Otx* genes are required for formation and regionalization of the anterior neuroectoderm in both animals. Mutation of *otd* in *Drosophila* results in defective anterior neuroectoderm specification and failure in formation of stem cell-like neuroblasts in this region (Hirth *et al.*, 1995; Younossi-Hartenstein *et al.*, 1997). Mutation of *Otx2*, one of two *otd* homologs in mouse, results in lack of anterior brain structures due to an impairment in the specification of the anterior neuroectoderm (Acampora *et al.*, 1995). The evolutionary conservation of expression and function of *otd*/*Otx* genes in anterior brain specification is underscored by cross-phylum transgenetic experiments in which the mammalian *Otx* genes were expressed in fly *otd* mutants and, inversely, in which *Drosophila otd* was expressed in mouse *Otx* mutants (Leuzinger *et al.*, 1998; Acampora *et al.*, 1998, 2001a, 2001b). In both cases the transgene was able to effect a cross-phylum rescue of brain

development. Comparable cross-phylum rescue experiments carried out for the *ems*/*Emx* genes, which are also regionally expressed in anterior brain regions of vertebrate and invertebrate bilaterians, showed that murine *Emx1* can rescue brain defects in fly *ems* mutants (Hirth *et al.*, 1995; Hartmann *et al.*, 2000). Interestingly, an *Emx* transgene from a non-bilaterian cnidarian (*Acropora*) was not able to rescue the *ems* mutant brain defects of *Drosophila*, although it did rescue head patterning defects in the fly mutant (Hartmann *et al.*, 2010).

Hox genes encode a set of evolutionarily conserved homeodomain transcription factors that are involved the specification of regionalized identity during development (Carroll, 1995); their role in anteroposterior regionalization is thought to have evolved early in metazoan history (Finnerty, 2003). They are generally expressed along the developing anteroposterior body axis in the same order as their arrangement on chromosomes ("co-linearity"). Hox gene expression is especially prominent in the developing central nervous system, which may be the ancestral site of Hox gene action in bilaterians (Hirth and Reichert, 2007). Hox genes are expressed in an ordered set of domains in the developing central nervous system of bilaterians as divers as acoels, nematodes, annelids, molluscs, arthropods, urochordates, cephalochordates and vertebrates including zebra fish, chicken, mouse, and human (Kourakis *et al.*, 1997; Hirth and Reichert, 1999; Hughes and Kaufman, 2002; Irvine and Martindale, 2000; Steinmetz *et al.*, 2011; Kenyon *et al.*, 1997; Wada *et al.*, 1999; Ikuta *et al.*, 2004; Lee *et al.*, 2003; Lumsden and Krumlauf, 1996; Vieille-Grosjean *et al.*, 1997; Carpenter, 2002; Moens and Prince, 2002, Hejnol and Martindale, 2009, Wilkinson *et al.*, 1989, Hunt *et al.*, 1991).

Mutant analyses of Hox gene action in central nervous system development of fly and mouse reveal a comparable function in specification of regional identity. In *Drosophila*, Hox genes are required for the specification of regionalized neuronal identity in the posterior brain (Hirth *et al.*, 1998). In mouse, Hox genes are involved in specifying the rhombomeres of the developing hindbrain (Studer *et al.*, 1996; Studer *et al.*, 1998; Gavalas *et al.*, 1998). This evolutionary conservation of Hox gene action in central nervous system development is emphasized by the fact that cis-regulatory regions driving the specific spatiotemporal expression of Hox genes are interchangeable between insects and mammals (Malicki *et al.*, 1992; Popperl *et al.*, 1995). Together, these data imply that expression, function and regulation of Hox gene action in central nervous system development are conserved features of this developmental control gene family.

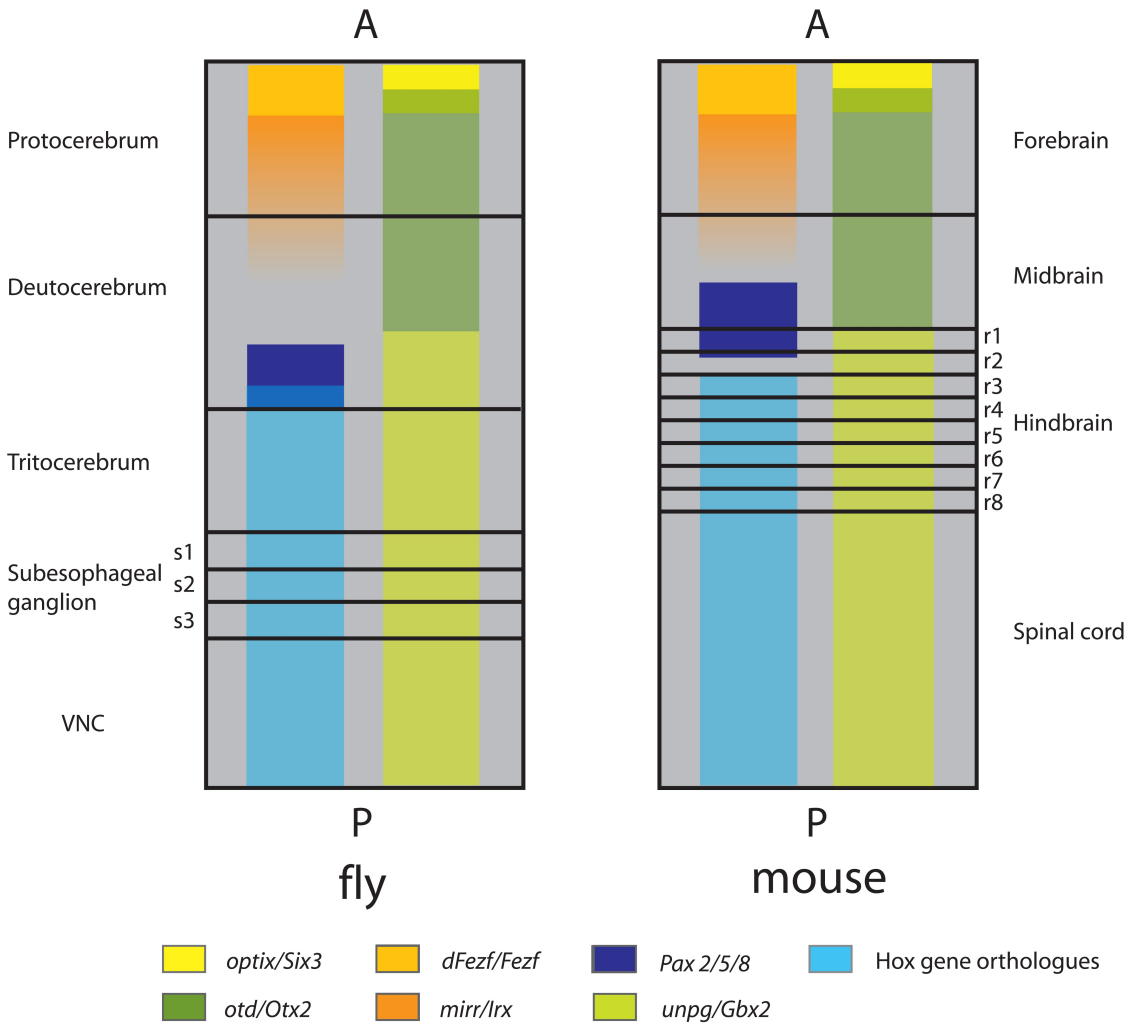


Figure 5.2 Simplified summary scheme of the anteroposterior order of conserved gene expression in embryonic CNS development of bilaterians.

Dorsoventral patterning is not indicated. Schematic diagram shows the expression of the patterning genes *optix/Six3*, *otd/Otx2*, *dFezf/Fezf*, *mirr/Irx*, *Pax 2/5/8*, *unpg/Gbx2* and Hox gene orthologues in the developing CNS of *Drosophila* and mouse. Expression domains are color-coded. (left) Gene expression in *Drosophila* CNS of embryonic stage 14. Borders of the protocerebral, deutocerebral, tritocerebral, mandibular (s1), maxillary (s2), labial (s3), and ventral nerve cord (VNC) neuromeres are indicated by horizontal lines. (right) Gene expression in mouse CNS of embryonic day 9.5- 12.5. Borders of the forebrain, midbrain and the hindbrain and its rhombomeres (r1-r8) are indicated by horizontal lines. In both *Drosophila* and mouse, an *optix*/*Six3* expression domain patterns the most anterior CNS region and overlaps with the *otd/Otx2* expression pattern (Steinmetz *et al.*, 2010) which is anterior to the abutting *unpg/Gbx2* expression (Urbach, 2007, Wassarman *et al.*, 1997, Bouillet *et al.*, 1995). In both animals, a *Pax2/5/8*- expression domain is positioned close to the interface between the anterior *otd/Otx2* and the posteriorly abutting *unpg/Gbx2* expression domains (Hirth *et al.*, 2003; Rowitch and Mcmahon, 1995; Asano and Gruss 1992). Hox genes orthologues expression follows posteriorly to the *Pax2/5/8* expression domain in both animals (Hirth *et al.*, 1998; Davenne *et al.*, 1999; Lichtneckert and Reichert 2007). Furthermore, the interface of the relative expression of *dFezf/Fezf* and *mirr/Irx* was reported to be conserved between fly and mouse (Oliver *et al.*, 1995; Irima *et al.*, 2010). Inspired by Lichtneckert and Reichert, 2007

While Hox genes are expressed in the posterior brain and nerve cord of bilaterians, they are excluded from the region of *otd*/*Otx2* and *ems*/*Emx* gene expression in the anterior brain. In vertebrates, a marked boundary region in the developing brain called the midbrain-hindbrain boundary (MHB) is located anterior to the expression domain of the Hox genes, and this region has an essential organizer function in patterning the midbrain and anterior hindbrain (Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). In vertebrates the developing MHB is delimited by the interface of the posterior *Otx2* expression domain and an abutting *Gbx2* expression domain, and it is also characterized by the expression of *Pax2/5/8* encoding genes. In *Drosophila*, a comparable boundary region is found in the developing brain anterior to the Hox expression domain; this region is also delimited by the interface of the posterior *otd*/*Otx2* domain and the abutting *unplugged(unpg)*/*Gbx2* expression domain, and is similarly characterized by the expression of *Pax2/5/8* (Hirth *et al.*, 2003; Urbach *et al.*, 2007). Comparable expression patterns of homologs of these genes are found anterior to the Hox expression domains in the developing brains of several other deuterostome and protostome taxa (Wada *et al.*, 1998; Wada and Satoh, 2001; Irimia *et al.*, 2010; Steinmetz *et al.*, 2011; Holland, 2009). Hence, a defined boundary region between the anterior (*otd*/*Otx2*-expressing) and the posterior (Hox-expressing) parts of the brain, which together have been considered to be representative of a tripartite organization of the ancestral chordate brain, appears to be evolutionarily conserved in bilaterians.

In vertebrate brains, a second region with organizer function is found at the *zona limitans intrathalamica* (ZLI) which develops within the diencephalon at the boundary between the expression domains of the *Fezf* and the *Irx* genes (Irimia *et al.*, 2010). Comparable patterns of abutting gene expression define a ZLI-like boundary zone in the anterior brain of the basal chordate *Amphioxus* implying that a ZLI-like structure is a conserved feature of chordate brains. Remarkably, a boundary of expression of the homologous insect genes is found in the anterior brain of *Drosophila* where expression of *dFezf*, which is restricted to the anterior part of the brain, and expression of *mirr*, the earliest expressed fly *Irx* gene, adjoin to form a gene expression boundary (Irimia *et al.*, 2010). The conserved nature of this ZLI-like interface of gene expression domains provides further support for the conserved nature of brain development in bilaterians. Additional support for this notion is provided by the conserved expression of *optix*/*Six3* genes in a comparable domain at the most anterior tip of the central nervous system neuroectoderm in animals as diverse as vertebrates, insects, and annelids (Steinmetz *et al.*, 2010; Oliver *et al.*, 1995). Thus the *Six3-Otx2* brain patterning system, like the *Fezf-Irx* and *Otx2-Gbx* patterning systems, may also be universal to central nervous system development in bilaterians.

It is noteworthy, that comparable anteroposterior patterns of expression of a set of homologous genes are found in the net-like peripheral nervous system of the hemichordate *Saccoglossus* (Lowe *et al.*, 2003). Whether this is also true for the developing central nervous system of this hemichordate is currently not known (Nomaksteinsky *et al.*, 2009).

5.3.3 Conserved mechanisms for dorsoventral pattering of the bilaterian central nervous system

In addition to its anteroposterior axis, the neuroectoderm also has a second axis which can be considered either as its medio-lateral or as its dorsoventral axis since the plate-like neuroectoderm can extend in a dorsal direction or can give rise to a neural tube through invagination. A second set of patterning genes subdivides the neuroectoderm along this dorsoventral axis in a manner that is conserved in vertebrates and invertebrates. Key among these is a set of homeobox genes referred to as the columnar genes which control the formation of longitudinal domains in the neuroectoderm. Moreover, the induction of the neuroectoderm that gives rise to the central nervous system appears to rely on conserved dorsoventral patterning mechanisms that determine the dorsoventral body axis itself as well as the location of the neuroectoderm along that axis (Figure 5.3).

A set of conserved interacting signaling molecules play key roles in the establishment of dorsoventral polarity during embryogenesis. Central among these are morphogen-like signaling molecules of the transforming growth factor *β* (TGF*β*) family represented by BMP4 in vertebrates and its homolog Dpp in *Drosophila* (De Robertis, 2008; De Robertis and Sasai, 1996). This BMP signalling pathway appears to be conserved in dorsoventral polarity formation in bilaterian animals such as insects, spiders, vertebrates, amphioxus and annelids, with the exceptions of nematodes and tunicates, which both have a modified type of development (Akiyama-Oda and Oda, 2006; Suzuki *et al.*, 1999; Little and Mullins, 2006; Sasai *et al.*, 1995; Miya *et al.*, 1996; Lowe *et al.*, 2006; Levine and Brivanlou, 2007; Denes *et al.*, 2007; Mizutani *et al.*, 2005, 2006; Yu *et al.*, 2007). The polarizing action of BMP4/Dpp is antagonized in a spatially restricted manner by a second group of conserved extracellular signaling molecules which include Chordin in vertebrates and its homolog Sog in *Drosophila* (Holley *et al.*, 1995). The interacting Chordin/Sog and BMP4/Dpp signaling molecules act from opposing dorsoventral poles, and these poles are inverted in vertebrates versus invertebrates such as arthropods and annelids. (This provides strong support for the “dorsoventral inversion” hypothesis brought forward by Geoffroy Saint-Hilaire (1822) which states that the dorsoventral axis of vertebrates and invertebrates are equivalent but inverted; see Arendt and Nübler-Jung 1994; De Robertis

and Sasai 1996). In addition to its polarizing function, the BMP4/Dpp morphogen suppresses development of the neuroectoderm and this suppressive function is inhibited by Chordin/Sog acting along the induced dorsoventral axis. Hence in both vertebrate and invertebrate bilaterians, the region of the embryo that forms the neuroectoderm (dorsal in vertebrates, ventral in invertebrates) is the one in which Chordin/Sog is expressed and inhibits invading BMP4/Dpp. Indeed, whenever a central nervous system develops in vertebrates, insects, annelids and cephalochordates it derives from a neuroectoderm on the non-BMP body side (Sasai *et al.*, 1995; Mizutani *et al.*, 2005, 2006; Levine and Brivanlou, 2007; Yu *et al.*, 2007; Denes *et al.*, 2007). This suggests that the functional conservation of the Chordin/Sog and the BMP4/Dpp morphogens in CNS neuroectoderm induction represents a conserved dorsoventral patterning mechanism that was already present in the urbilaterian ancestor of vertebrates and invertebrates.

Following early neuroectoderm induction, a conserved set of homeodomain proteins encoded by the *vnd*/*Nkx2.2*, *ind/Gsh* and *msh/Msx1* genes act in further dorsoventral regionalization of the developing CNS (Chan and Jan, 1999; Cornell and Von Ohlen, 2000). All three genes are expressed in specific, non-overlapping longitudinal columnar domains along the dorsoventral (mediolateral) axis of the central nervous system. In *Drosophila*, *vnd* is expressed in a ventral column, *ind* is expressed in an intermediate column, and *msh* is expressed in a dorsal column of the ventral neuroectoderm; in the mouse, *Nkx2.2* is expressed in a ventral column, *Gsh* is expressed in an intermediate column, and *Msx1* is expressed in a dorsal column of the neural tube (Qui *et al.*, 1998; Pera and Kessel, 1998, Pabst *et al.*, 1998; Shimamura *et al.*, 1995; Wang *et al.*, 1996, Valerius *et al.*, 1995; Hsieh-Li *et al.*, 1995; Briscoe *et al.*, 1999; Sussel *et al.*, 1999; Isshiki *et al.*, 1997; McDonald *et al.*, 1998; Chu *et al.*, 1998; Weiss *et al.*, 1998). In both animals these so-called columnar genes control the formation of corresponding columnar dorsoventral identity domains and act in neurogenesis at their site of action. These findings suggest that the role of the columnar genes in dorsoventral patterning of the central nervous system might be conserved throughout bilaterians (reviewed in Arendt and Nübler-Jung, 1999; Reichert and Simeone, 2001; Lichtneckert and Reichert, 2007, Urbach and Technau, 2008). In support of this, comparable longitudinal domains of expression of homologous columnar genes are observed in the neuroectoderm of the lophotrochozoan annelid *Platynereis*. Moreover, even more extensive similarities in putative dorsoventral patterning genes are seen in the annelid versus vertebrate neuroectoderm, in that a columnar *Pax6* expression domain as well as a columnar lateral *Pax3/7* expression domain is apparent in both animals (Ericson *et al.*, 1997; Briscoe *et al.*, 2000; Kriks *et al.*, 2005, Denes *et al.*, 2007). (*Pax3/7* is also expressed in the developing central nervous system of *Drosophila* albeit in a strictly segmented fashion; Kammermeier *et al.*, 2001; Davis *et al.*,

2005). In all three bilaterian superphyla (Deuterostomes, Ecdysozoa and Lophptrochozoa), the expression of these patterning genes is sensitive to BMP4 which specifically regulates their expression in a threshold-dependent manner (Mizutani *et al.*, 2006; Denes *et al.*, 2007). Interestingly, BMP4 may also play an additional, conserved role in promoting sensory over motor neuron fate at later developmental stages (Mizutani *et al.*, 2006; Rusten *et al.*, 2002; Schlosser and Ahrens 2004; Lowe *et al.*, 2006; Denes *et al.*, 2007; reviewed in Arendt *et al.*, 2008; Mieko Mizutani and Bier; 2008).

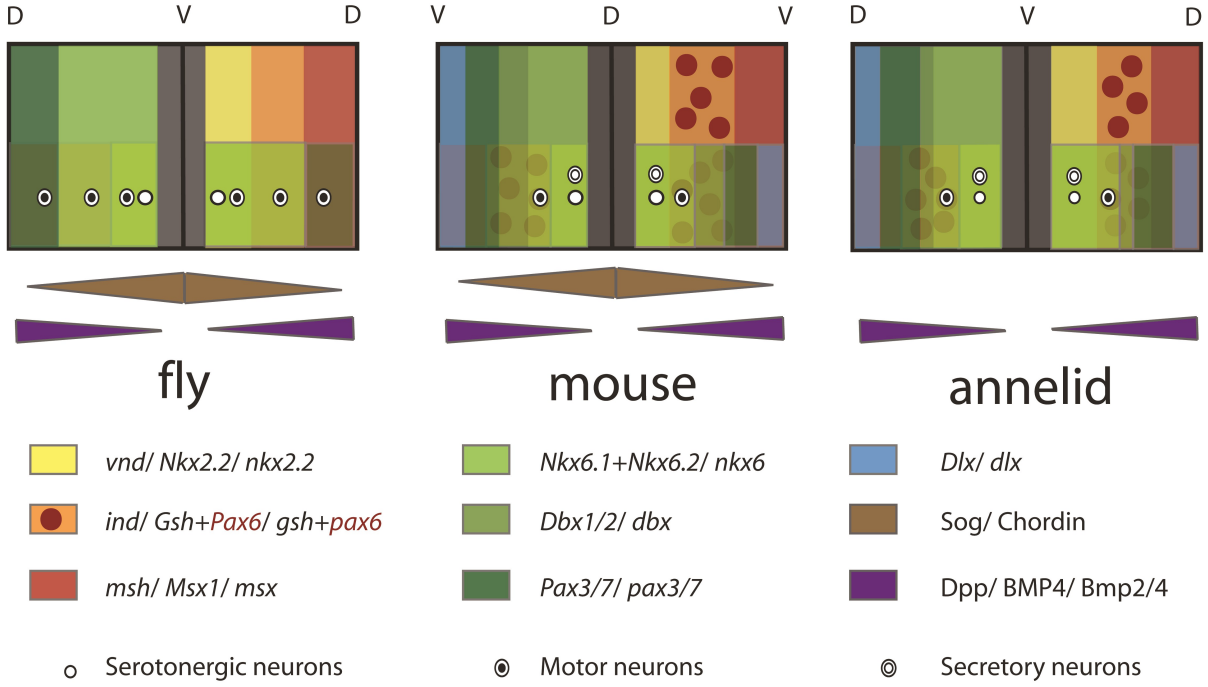


Figure 5.3 Schematic representation of examples of conserved dorsoventral genetic expression boundaries in a segmental part of the neuroectoderm in arthropods (left), vertebrates (middle) and annelids (right).
The vertebrate neuroectoderm is shown before folding. Anteroposterior patterning is not indicated. The neurogenic region is patterned in a dorsoventral fashion by a set of conserved patterning genes in all three animals, here indicated by color code. Note that the neuroectoderm of each animal is subdivided in two parts at its midline by a black vertical line enabling to show normally overlapping gene expression domains more clearly. At the bottom of the bars the overlap is shown for better comprehension. Within this overlay conserved neuron cell types emerging from this particular region are indicated by different circles (Denes *et al.*, 2007; Arendt *et al.*, 2008; Mieko Mizutani and Bier, 2008). The homologous proteins Dpp/BMP4/Bmp2/4 (violet) form a dorsoventrally inverted gradient in vertebrates with respect to *Drosophila melanogaster* and *Platynereis dumerilii*. In *Drosophila* and vertebrates, another homologous protein pair, namely Sog/Chordin (brown) forms an opposing gradient with respect to the Dpp/BMP4 pattern, where it inhibits Dpp/BMP4 and therefore enables induction of neurogenesis and with different gradients gives identity to different subdomains of the neuroectoderm (Lichtneckert and Reichert, 2005). The dorsoventral columnar patterning genes are highly conserved between the bilaterian animals (see comparable relative expression domains of *vnd/ Nkx2.2/ nkx2.2* (yellow), *ind/ Gsh/ gsh* (orange), *msh/ Msx1/ msx* (red), *Nkx6.1+Nkx6.2/ nkx6* (light green) in *Drosophila*, mouse and *Platynereis*) (Lichtneckert

and Reichert 2007; Seibert *et al.*, 2009). In the annelid and the mouse neuroectoderm even more similarities compared to *Drosophila* are apparent, such as the additional *Dbx1/2/ dbx* and *Dlx/ dlx* expression domains, the columnar medial *Pax6* expression (red dots) domain (Mieko Mizutani and Bier, 2008), as well as the *Pax3/7* expression which in *Drosophila* is expressed in a strictly segmented fashion (dark green) (Denes *et al.*, 2007).

Given the remarkable degree of conserved mechanisms for patterning the neuroectoderm, it is conceivable that some of the neural cell types that derive from the compartment like domains of the neuroectoderm might also be conserved in vertebrate and invertebrate bilaterians. Evidence for a conservation of neuron types comes from recent comparative studies of annelid versus vertebrate central nervous system development. Thus, serotonergic projection neurons in the vertebrate hindbrain and serotonergic projection neurons in the *Platynereis* both emerge from the *nkx2.2/nkx6* column, and cholinergic motoneurons with a comparable transcription factor signature emerge from a similar columnar *nkx6/pax6* domain in both vertebrates and annelids (Arendt *et al.*, 2008; Arendt and Nübler-Jung 1999; Ericson *et al.*, 1997; Briscoe *et al.*, 1999; Pattyn *et al.*, 2003; Denes *et al.*, 2007). Similarly, early differentiating neurosecretory cells that produce the conserved neuropeptide arg-vasotocin/neurophysin develop in the anterior n*k2.2* domain of the central nervous system in *Platynereis* and mouse (Arendt *et al.*, 2004). If these observations are indications of a more general conservation of neuronal cell types in bilaterians, then an explanation of these striking similarities based on evolutionary convergence (Moroz, 2009) becomes more and more unlikely, and we are left with the notion of a common, monophyletic origin of the bilaterian central nervous system (see Reichert and Simeone, 2001; Lichtneckert and Reichert, 2005; Mieko Mizutani and Bier, 2008). Indeed, there is increasing evidence that even rather complex central neural circuitries might have a common urbilaterian origin.

5.3.4 Common patterning mechanisms for complex brain circuitry?

There are obvious differences in the olfactory sense organs of vertebrates and insects; the vertebrate olfactory epithelium is in the nasal cavity while the insect olfactory sensilla are on the antenna. Furthermore, the olfactory receptor molecules are evolutionarily distinct (Benton *et al.*, 2006; Wistrand *et al.*, 2006) and also differ somewhat in terms of expression control and activation mechanism in the two clades (Imai *et al.*, 2010). Nevertheless, the circuit organization of the olfactory system in insects and vertebrates is remarkably similar in several respects (Hildebrand and Shepherd, 1997; Kay and Stopfer, 2006). First, a given olfactory sensory neuron in both flies and vertebrates expresses only a single olfactory receptor out of a large repertoire of olfactory receptor genes. Second, the axons of the olfactory sensory neurons that express a

given receptor converge onto the same glomerulus in the primary olfactory center of the brain (vertebrate olfactory bulb, insect antennal lobe). Third, in the glomeruli the sensory neuron axons make synaptic connections with local interneurons and output interneurons (vertebrate mitral/tufted cells, insect projection neurons). Moreover, the development of the olfactory circuitry is similar in several respects. For example, in both animal groups, the *ems*/*Emx* genes are required for olfactory system development (Simeone 1992; Mallamaci *et al.*, 1998; Bishop *et al.*, 2003; Lichtneckert *et al.*, 2008; Das *et al.*, 2008; Sen *et al.*, 2010). Furthermore, although the molecules involved are often different, gradients of axon guidance molecules and axon-axon interactions are important for topographic map formation of olfactory receptor neuron (ORN) projections in the olfactory bulb in mouse and in the antennal lobe in flies (Komiyama *et al.*, 2007; Lattemann *et al.*, 2007; Sweeney *et al.*, 2007; Zhu *et al.*, 2006; Hattori *et al.*, 2007; Luo and Flanagan, 2007; Imai *et al.*, 2009). While it is possible that these strikingly similar organizational and developmental features are all the result of convergent evolution, it is equally possible that they are evolutionarily conserved features which reflect the existence of "primitive" olfactory circuitry in the brain of the urbilaterian ancestors of insects and vertebrates (Figure 5.4).

As in olfaction, there are also obvious differences in the sense organs for vision in vertebrates and insects; vertebrates possess single-lens eyes that contain ciliary-type photoreceptors and insects have compound eyes that comprise rhabdomeric-type photoreceptors. Despite these differences, there are surprising similarities in the structural and functional organization of the two visual systems (Sanes and Zipursky, 2010). At the circuit level, both fly and vertebrate visual systems comprise a few basic neural cell types that diversify into a high number of subtypes. Moreover, synaptic interconnections among these cells take place in sequentially arranged parallel laminar layers which are linked by orthogonal pathways that originate in the photoreceptors and terminate in higher visual centers of the brain. Indeed, these similarities prompted Cajal and Sanchez (1915) to conclude that the "essential plan was maintained with small variations and re-touches of adaptation" in the two apparently different types of visual systems. This notion is supported by more recent studies which indicate that comparable control genes operate in visual system development in insects and mammals, and is exemplified by the comparable role of the *otd*/*Otx* cephalic gap genes in the development of the peripheral and central visual systems in flies and mice (Acampora *et al.*, 1999; Vandendries *et al.*, 1996; Hirth *et al.*, 1995; Acampora 1998; Finckelstein *et al.*, 1990). As in the case of the olfactory system, the shared organizational and developmental features might be due to convergent evolution. However, they might also be due to evolutionary conservation of the "essential plan" of an ancestral visual system that was already present in the urbilaterian brain.

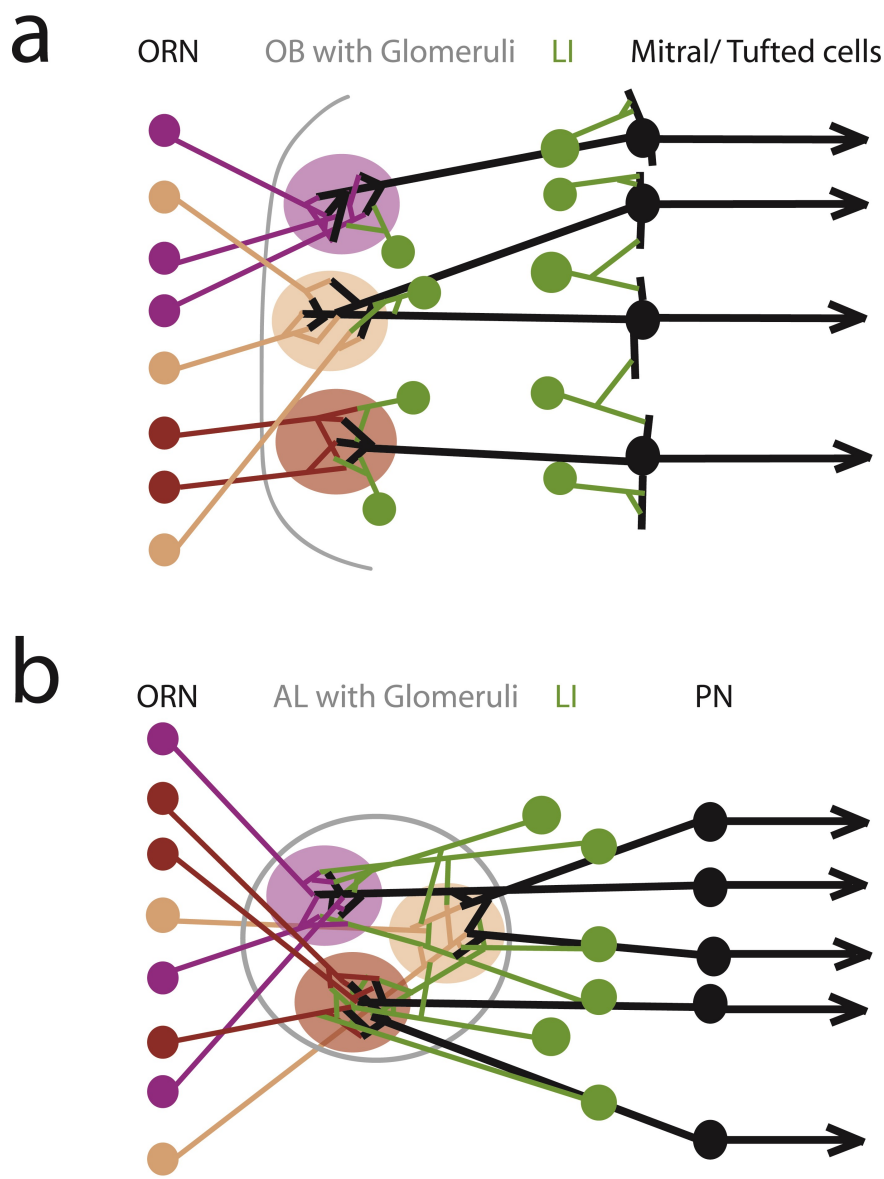


Figure 5.4 General similarities of olfactory circuit organization in mammals (a) and insects (b). ORN expressing the same olfactory receptor project to the same glomerulus in both animals (expressed olfactory receptor type in neurons is indicated by differently colored neurons). In the glomeruli the ORN connect to the dendrites of the mitral/tufted cells in the mammals **(a)** or PN in insects **(b)**. In both animals, the sensory information is then transmitted by the mitral/tufted cells or the PN into higher brain centres. Different LI interconnect the information from the various glomeruli and process this olfactory information in fly and mouse. AL, antennal lobe; ORN, olfactory receptor neurons; OB, olfactory bulb; LI, local interneurons; PN, projection neurons. Inspired by Kay and Stopfer, 2006

Remarkably, recent evidence suggests that evolutionarily related higher brain centers might also have been present in the urbilaterian ancestor of vertebrates and invertebrates. In higher invertebrates such as annelids and arthropods, the mushroom body of the protocerebrum represents a high-order associative brain center involved in learning and memory. In

vertebrates, comparable associative learning and memory functions are carried out by the cerebral cortex and hippocampus which are developmental derivatives of the pallium. When the expression of a suite of conserved developmental control genes is compared in the developing mushroom body of the annelid *Platynereis* and in the developing pallium of the mouse, similar spatial patterns of expression are observed (Tomer *et al.*, 2010). Based on these results, and in support of earlier findings, it has been proposed that the two higher brain centers are in fact homologous and that the urbilaterian ancestor might already have possessed a "high-order" associative brain center from which the extant mushroom body and pallium evolved (Tomer *et al.*, 2010; Seweeney and Liqun Luo, 2010; Strausfeld *et al.*, 1998).

In all three cases (olfaction, vision, learning/memory) the similarities in organization and development of the corresponding complex neural systems in vertebrates and invertebrates might have evolved independently through convergent evolution. Alternatively, they could be due to evolution of the extant neural systems from the same ancestral system that was already present in the urbilaterian brain. While further comparative studies are needed to resolve this issue, the increasing evidence for a urbilaterian animal that possessed a centralized brain with surprisingly complex sensory and associative brain centers similar to those of higher vertebrates and invertebrates provides further support for a monophyletic origin of the bilaterian central nervous system.

5.4 The first metazoan nervous system: insights from cnidarians

Since comparative and developmental genetic data imply that the last bilaterian common ancestor already possessed a complex centralized nervous system, when in animal evolution did centralization of nervous systems take place? As mentioned above, centralized nervous systems are found in cnidarians, which are one of the most basally branching animal phyla that have a nervous system. (While nervous systems are also present in ctenophorans, there is relatively little data on their organization, function and development, and they will not be considered further in this review.) Hence, the question arises of whether the radial nervous systems of Cnidaria and the bilateral nervous system of bilaterians are evolutionarily related (monophyletic origin of metazoan nervous systems) or not (polyphyletic origin of cnidarian versus bilaterian nervous systems).

Cnidarian neurons are found in the ectodermal and the endodermal cell layers, and in terms of cell types correspond to sensory cells, motoneurons and ganglionic interneurons (Watanabe *et al.*, 2009; Galliot *et al.*, 2009). In terms of their neuroanatomy, the nervous systems of cnidarians show a great deal of variability among species and even among single animals between their life cycle stages as sessile polyps or swimming medusae (Bullock and Horridge, 1965; Mackie, 2004). Polyps of cnidarians generally have diffuse epithelial nerve nets, but they also display regionalized concentrations of morphologically and neurocytochemically distinct neuronal subsets (Grimmelikhuijzen and Graff, 1985; Koizumi *et al.*, 1992; Grimmelikhuijzen *et al.*, 1996; Marlow *et al.*, 2009; Piraino *et al.*, 2011). In medusoid cnidarians such as the swimming jellyfish, an even more complex, radial nervous system organization is apparent. In addition to the peripheral nerve net, these nervous systems have single or double nerve rings that contain multiple neural cell types in circuitry for coordinated movement control and for processing of sensory information from sense organs such as statocysts, ocelli, and lens eyes (Figure 5.5) (Mackie 2004; Koizumi, 2007; Garm *et al.*, 2007, 2006; Parkefelt and Ekström, 2009; Piraino *et al.*, 2011). Moreover, the rhopalia of some cubomedusan cnidarians represent a ganglionic-like centralization of multiple sensory cells together with a premotor pacemaker neuropil of neural processes and synapses (Satterlie, 2010). Remarkably, even the larval forms of some cnidarians can have surprisingly complex regionalized nervous systems. The “crawling” planula larva of the hydrozoan *Clava multicornis* manifests a concentration of different neural cell types at the anterior pole of the animal that form a neural plexus in association with large number of sensory cells arranged in the area at the anterior tip of the animal (Piraino *et al.*, 2011).

Thus, both in terms of morphology and in terms of function, the nervous systems of cnidarians present a remarkable degree of centralization supporting the notion that the cnidarian nervous systems might be representative of the first integrating concentrations of nervous tissue in metazoan evolution (Bullock and Horridge, 1965; Arendt *et al.*, 2008; Satterlie, 2011). However, due to their intrinsic radial organization, the nervous systems of cnidarians manifest a different morphological Bauplan from that of bilaterian nervous systems. This makes it difficult to assess the degree of evolutionary relationship between cnidarian and bilaterian nervous systems based on anatomy alone. Might a comparative developmental genetic analysis provide additional insight?

Many of the developmental control genes involved in axial patterning in insects and vertebrates are known to be conserved in cnidarians implying that that a substantial number of these genes were already present in the last common ancestor of bilaterians and cnidarians (Finnerty *et al.*,

2004; Ball *et al.*, 2004; Finnerty, 2003; Galliot, 2000 Kusserow *et al.*, 2005; Jacobs *et al.*, 2007, Boero *et al.*, 2007, Technau *et al.*, 2005). Furthermore, there is now increasing evidence for a conservation of developmental mechanisms of patterning in cnidarians and bilaterians. For example, the BMP4/Dpp and Chordin/Sog morphogen system appears to be present in cnidarians (Saina *et al.*, 2009; Finnerty *et al.*, 2004; Rentzsch *et al.*, 2007; Technau and Steele, 2011). However, in contrast to the situation in bilaterians, the expression of both signaling components is found on the same side of the secondary ("directive") body axis rather than forming opposing gradients, and no morphological regionalization of the nervous system along the secondary axis of polyps has been observed. Regionalized expression in the developing cnidarian nervous system has been reported for the homologs of the *Emx* genes, *Msx* genes and *Gsx* genes, and for the latter a functional role in nerve net development has been established (de Jong *et al.*, 2006; Miljkovic-Licina *et al.*, 2004, 2007; Galliot *et al.*, 2009). *Otx* and Hox genes have also been identified in cnidarians, however, their expression patterns vary greatly among different species, and these genes do not seem to be involved in regionalized neuronal versus non-neuronal determination or, in the case of *Otx*, in head development (Ryan *et al.*, 2007; Chiori *et al.*, 2009; Quiquand *et al.*, 2009; Yanze *et al.*, 2001; Finnerty *et al.*, 2004; Smith *et al.*, 1999; Müller *et al.*, 1999, Technau and Steele 2011).

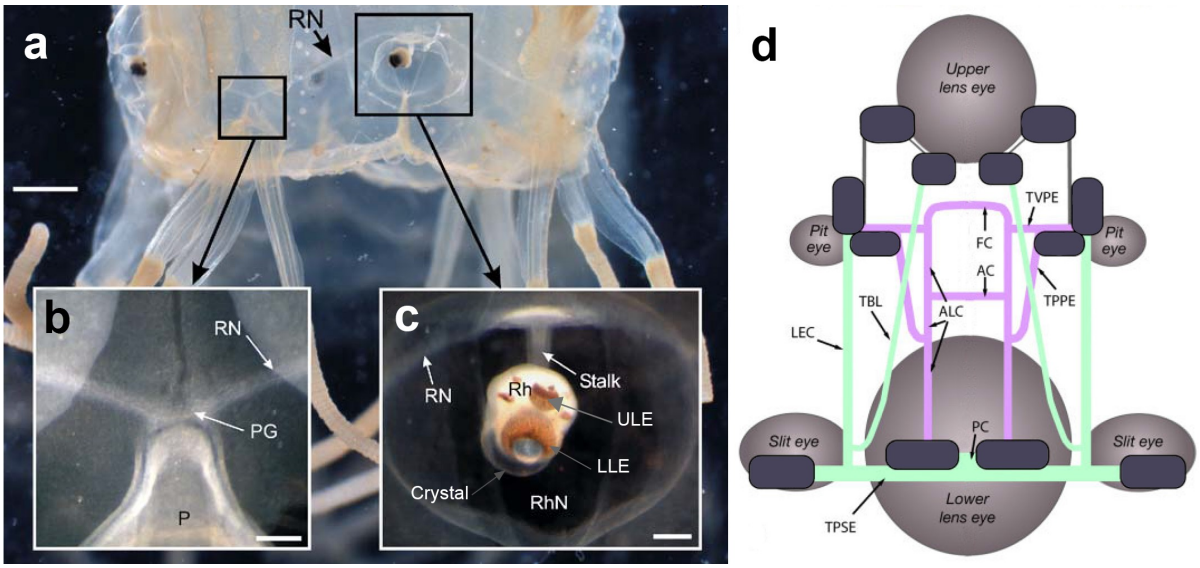


Figure 5.5 Complexity of the CNS of the cubozoan jellyfish *Tripedalia cystophora*.
(a) The ring nerve RN connects the pedalial ganglion PG **(b)** with the rhopalia Rh **(c)** in the central nervous system. **(c)** the Rh constitute the main sensory structures of cubomedusae. Rh hang within the RhN on a stalk and carry six eyes (indicated are just the two lens eyes ULE and LLE). **(d)** Schematic overview of commissural connections (light green and violet) between the different neuronal cell groups (dark blue) of the 6 distinct eyes (grey circles) in the rhopalium, indicating the remarkable complexity of this visual and integrating structure. Rh, rhopalium; RhN, rhopalial niche; LLE, large lens eye; ULE, upper lens eye; RN, ring nerve; PG, pedalial ganglion; P, pedalium; AC, anterior commissure; ALC, apical lateral

connective; FC, frontal commissure; LEC, lateral exe connective; PC, posterior commissure; TBL, basal lateral tract; TPPE, posterior pit eye tract; TPSE, posterior slit eye tract; TVPE, vertical pit eye tract. Bars indicate 1mm (a), 100µm (b, c). (a, b, c) Modified and reprinted from Garm, A., Ekstrom, P., Boudes, M. & Nilsson, D. E. (2006). Rhopalia are integrated parts of the central nervous system in box jellyfish. *Cell Tissue Res* 325(2), 333-43. Figure 1. a, c, d. With kind permission from Springer Science+Business Media. (d) Modified and reprinted from Parkefelt, L., Skogh, C., Nilsson, D. E. & Ekstrom, P. (2005). Bilateral symmetric organization of neural elements in the visual system of a coelenterate, Tripedalia cystophora (Cubozoa). J Comp Neurol 492(3), 251-62. Figure 1. d. With kind permission of John Wiley & Sons, Ltd.

Currently, it is difficult to interpret this gene expression data in cnidarians in terms of mechanisms for nervous system development. It is even more difficult to draw conclusions on the possible conservation of these largely uncharted mechanisms in cnidarian nervous system development as compared to those that are known to operate in bilaterian nervous system development. Such considerations must wait until further experimental insight is obtained into the developmental genetic origin of the cnidarian nervous system, and meaningful molecular and mechanistic comparisons with bilaterian nervous system development can be carried out. However, the currently established findings do at least suggest that the genetic toolkit, which is used to generate the nervous systems of bilaterians, is also largely present in these radialy symmetric animals. Hence, this genetic tool kit was probably already present in the last ancestor of cnidarians and bilaterians. Whether or not this toolkit was used in this common eumetazoan ancestor to build “the first nervous system” remains an intriguing enigma.

Acknowledgements: Supported by the SNF.

6. Discussion

6.1 The central complex primordium as formed in the embryo by type-II NB lineage derived undifferentiated neurons

In this report, we introduced the primordium of the central complex neuropil (CCpr) and described its embryonic origin. At early first larval instar, embryonically born central complex associated cells (CCPaC's), albeit undifferentiated and devoid of synapses, form a topologically organized scaffold of the CCpr. During postembryonic stages, more CCPaC's are generated and contribute to the primordial neuropil. However, until late larval stages, the embryonically as well as the postembryonically generated CCPaC's remain undifferentiated. During metamorphosis, the CCPaC's start to differentiate and form functional interconnections in various substructures of the central complex (CC), namely the protocerebral bridge (PB), the fan-shaped body (FB) and the ellipsoid body (EB) as well as in the central complex input areas, the bulbs (BU). These findings give new insights into the embryonic origin of the central complex primordium, as well as the subsequent integration of the CCPaC's into adult specific circuitry. Furthermore, this work introduces an intermediate cell identity in type-II NB lineages which does not correspond to either the primary or the secondary neurons. These cells represent embryonic born but postembryonic undifferentiated neurons, for which we suggest the term "embryonic secondary neurons". In fact, the CCpr is made from a subset of the embryonic secondary neurons revealed in the first larval instar brain. In the following we interpret the data revealed by this work in the light of general brain development.

6.1.1 The ambiguity of a larval functional central complex

Detailed anatomical studies revealed remarkable similarities in the structure of the adult central complex in various hemimetabolous as well as holometabolous insects (Williams, 1975, Strausfeld, 1976, Hanesch et al., 1989). Data about the development of this neuropil structure in *Drosophila* has been accumulating in the last few years but mainly focused on postembryonic stages (Young and Armstrong, 2010b; Yang et al., 2013; Bayraktar et al., 2010; Jiang and Reichert 2012). This work reveals the embryonic origin of a central complex primordium in the holometabolous fly *Drosophila melanogaster*. During metamorphosis, this primordial structure matures and differentiates into the adult central complex and most of its substructures.

Whereas the structural precursor of the adult central complex has been described in detail in this work, there is no conclusive evidence for the existence of a functional neuropil structure involved in locomotor behavior of the larval central brain. As the adult central complex is involved in many adult specific behaviors such as complex locomotion, visual pattern memory, courtship behavior and spatial orientation memory (Liu et al., 2006; Pan et al., 2009; Popov et al., 2005; Neuser et al., 2008; Poeck et al., 2008), this implies a larval equivalent CC to be highly reduced if not absent. In contrast to the adult, the larval locomotion and sensory circuits are comparatively rudimentary and straightforward (Stocker, 2008; Gerber and Stocker, 2007; Keene and Sprecher, 2012; Kohsaka et al., 2012). The limited behavioral repertoire of the legless *Drosophila* larva even led to the hypothesis that an elaborate locomotion integration center such as the central complex is not needed in larval stages (Hanesch et al., 1989). Nevertheless, larvae exhibit various rhythmic and highly stereotypic patterns of locomotion including feeding, peristalsis, bending and turning (Green et al., 1993; Kane et al., 2013; Keene and Sprecher, 2012). So far, larval locomotion is thought to be produced by segmentally organized networks in the larval VNC called central pattern generators (CPGs). Whereas the motor neurons and the sensory feedback neurons were studied in some detail, data about the upstream interneurons of these circuits is scarce und their identity unknown (Song et al., 2007; Kohsaka et al., 2012). In recent years some evidence accumulated for the involvement of central brain neurons in larval locomotion (Silva et al., 2014; Scantlebury et al., 2010; Rodriguez and Campos, 2009). Furthermore, one study reported that CC affecting mutants which were isolated for their adult CC abnormalities also showed locomotor defects in larvae. Because basic locomotor functions were unaffected in these mutants, the mutational effect was suggested to occur in a higher control center (Varnam et al., 1996, for review see Strauss, 2002). However, a specific larval neuropil structure responsible for these phenotypes was not shown. These data do suggest but not proove the existence of a larval central complex. Nevertheless, it is possible that the localized direct circuits in the VNC (CPGs) are sufficient to coordinate larval behavior in *Drosophila* independent of a higher integration.

In *Drosophila*, the same lineages involved in the generation of a larval neuropil were shown to also give rise to the corresponding neuropil structure in the adult, whenever present (see for the antennal lobe; Das et al., 2013; for the MB; Kunz et al., 2012). Thus, if a larval central complex is present we expect the structure to be made out of the same 15 lineages that were reported to give rise to the adult central complex, seven of which are type-II NB lineages (Yang et al., 2013). 3D reconstruction of the differentiated DPMm1 derived neurons did not reveal any larval central complex candidate neuropil structure. We assume that the full reconstruction of

the differentiated neurons generated by the 15 NB lineages contributing to the adult central complex will reveal larval central complex, if such a structure is present.

6.1.2 The CCpr represents a primordium for all the adult specific central complex substructures

It was previously suggested that small precursors of most of the adult specific neuropil compartments already exist in the early larva (Younossi-Hartenstein 2003). Our data confirms the so far unknown existence of an embryonically generated central complex primordium. In our first study we referred to this primordium as a fan-shaped body primordium (FBpr) because the first structure which develops out of the primordium is the fan-shaped body. Since we were relying on two *pnt*-Gal4 driver lines in the experiments presented, it could not be ruled out that primordia of the other substructures exist at third larval instar but were not labeled by these driver lines. In this case, the outgrowing CCPaC's would then follow the guidance of these presumptive primordial structures to innervate the ellipsoid body and the protocerebral bridge. One way of addressing this issue is the ablation of the CCPaC's using *R45F08*-Gal4 to drive *hid,rpr* expression in these cells which leads to the induction of programmed cell death (Goyal et al., 2000). This would reveal whether the whole central complex would be missing in the adult or just one substructure. Due to the broad and unspecific expression of this driver line in early larval stages, such experiments were unsuccessful and led to larval death. Subsequent introduction of a temperature-sensitive construct did also lead to flies that were not viable. Another approach is to search for such primordia in the serial section EM dataset of the L1 brain. However, in our 3D reconstructions no obvious signs of primordial CC substructures could be identified by tracing embryonic secondary cells derived from type-II NB lineages and type-I NB lineages throughout the central brain (data not shown). Since these data give the complete set of undifferentiated embryonic secondary cells present and do not rely on the expression of driver lines, we suggest, that the CCpr reported here is the primordium out of which all the future CC substructures will develop. However, presumptive individual primordia of these CC substructures might not be as easily identifiable as the midline crossing primordium shown here. Since neuropil structures of the larva and the adult were reported to be generated by the same NB lineages (Das et al., 2013, Kunz et al., 2012), type-I NB lineages with elaborate innovation of the according CC substructures in the adult might give rise to such unidentified CC substructure primordia. To address this question, we would suggest to first identify, then reconstruct the embryonic secondary neurons of the ellipsoid-body ring-neurons generating lineage EBa1 (which we believe corresponds DALv2 in Wong et al., 2013), and the protocerebral bridge innervating NB lineage PBp1 (Yang et al., 2013). 3D reconstruction of undifferentiated

neurons derived by these two lineages could reveal the EB and the PB primordium in the L1 brain, if present at all. Until such primordia of central complex substructures are unambiguously identified, we consider the CCpr described here as the sole precursor of all the adult CC neuropils.

6.1.3 Other neuropil primordia reported in the *Drosophila* CNS

The antennal lobe of the larva and the adult are both generated by the same five NB lineages, which generate both projection neurons and interneurons. Even though the larval olfactory system lacks the cellular complexity of the adult, it has all the major functional components of the olfactory processing circuitry seen in the adult fly (Jhaveri and Rodrigues, 2002; Stocker, 2008; Tissot et al., 1997). Whereas the larval antennal lobe consists only of 21 glomeruli, this number gets doubled during metamorphosis generating an adult antennal lobe of 50 glomeruli (Das et al., 2013; Heimbeck et al., 1999; Stocker, 2008; Masse et al., 2009). The mechanisms underlying this reorganization of neuropil however remain elusive. During embryonic stages, axon ingrowth of olfactory receptor neurons pioneers the development of the larval olfactory lobe neuropil. To ensure appropriate patterning of connectivity in the larval antennal lobe, synaptic interactions among the receptor neurons and the ingrowing projection neurons is required (Prieto-Godino et al., 2012). During metamorphosis, the adult-specific antennal lobe starts to form adjacent and dorsolateral to the larval-specific antennal lobe (Jefferis et al., 2004). The embryonically generated olfactory receptor neurons degenerate during metamorphosis, but the projection neurons survive and get integrated into the adult olfactory circuit. The development of the adult olfactory circuits is initiated by the remodeling of the projection neuron dendrites in glomerular-sized neuropil areas independent of and before the arrival of the adult-specific olfactory receptor neurons (Sweeney et al., 2011; Ray and Rodrigues, 1995; Jefferis et al., 2001, Marin et al., 2005). The proper innervation of the projection neurons during metamorphosis depends on signals provided by the embryonically born degenerating olfactory receptor neurons (Sweeney et al., 2011). It is after the innervation of the lobe by projection neurons that olfactory receptor neurons innervate the olfactory lobe and the protoglomeruli are formed (Luo and Flanagan, 2007; Jefferis et al., 2004; Oland and Tolbert, 1996, 2003; Jhaveri and Rodrigues, 2002). Olfactory receptor neurons begin to differentiate only after the olfactory glomeruli have formed (Clyne et al., 1999). In conclusion, the embryonically generated olfactory receptor neurons together with the projection neurons play a significant role in the patterning of the adult olfactory circuits.

Unlike all the other NB lineages of the *Drosophila* brain, the four mushroom body (MB) NBs do not undergo a period of quiescence and divide throughout development until late pupal stages. Therefore, a comparison of the development of the mushroom body neuropil to other neuropil structures is of interest but the interpretations come with certain reservations. The mushroom body neuropil and its three-lobed architecture are made in the late embryo and their overall morphology is maintained throughout metamorphosis (Ito et al., 1997; Kurusu et al., 2002). The larval mushroom body neuropil consists of intrinsic γ neurons which survive into adulthood and get integrated into adult MB circuits (Boulanger et al., 2011; Lee et al., 1999; Kunz et al., 2012). These neurons have been shown to be responsible for some aspects of locomotion as well as appetitive olfactory learning in L1, suggesting that the underlying neuronal circuits of the adult MB are already established during embryonic stages (Pauls et al., 2010). MB intrinsic neurons generated postembryonically grow along the embryonic pioneer γ neurons. However, these postembryonically generated neurons commence to build MB lobes of slightly different identities (α/β and α'/β' lobes). Concluding, in the MB too there are embryonically born neurons which guide the innervation of later born neurons and get integrated into adult circuits.

In the adult *Drosophila* brain, the optic lobes comprise around half of the fly brain. Visual input from the compound eyes is received in the optic lobe neuropils where it gets processed in four major synaptic ganglia, which are oriented in perpendicular layers. The four optic lobe neuropils are -from distal to proximal- called the lamina, the medulla, the lobular plate and the lobula. In contrast to the adult, the larval optic neuropil is quite rudimentary. The larval eye is called the Bolwigs organ and is made out of 12 photoreceptor neurons (as compared to roughly 6400 photoreceptor neurons in the adult) which terminate in the larval optical neuropil (LON) (Figure 6.1). Interestingly, the LON is later incorporated into the adult accessory medulla during metamorphosis (Sprecher et al., 2011). During embryonic development, two epithelial neurectodermal placodes are generated that represent the inner optic anlage (IOA) and the outer optic anlage (OOA), out of which the adult specific optic neuropils will develop during larval stages. The IOA will give rise to the lobula neuropil whereas the OOA will give rise to the lamina and the medulla neuropil. The cell populations of the IOA and the OOA arise in close proximity to the Bolwigs organ and the delaminating precursor cells of the eye-antennal imaginal disc (Sprecher et al., 2011; Younossi-Hartenstein et al., 2003). None of the medulla, lobula or lamina neuropile primordia is present at embryonic stages. However, a cluster of three optic lobe pioneers (OLPs) develops in close proximity to the OOA near the insertion of the stalk (Tix et al., 1989; Chang et al., 2003). OLPs innervate the LON from embryogenesis onwards and are maintained into the adult fly. Photoreceptor axons coming from the Bolwigs nerve seem to use the OLP axons as a guide whilst they enter the LON from laterally (Boschert et l., 1990; Tix et

al., 1989; Campos et al., 1995; Nassif et al., 1998). The dendrites of the OLP within the LON were reported to get input from the photoreceptor neurons from the Bolwigs organ and they are believed to project the visual information into the central brain (Sprecher et al., 2011). The axon tract made up by the optic lobe pioneers was reported to even cross the commissure and have a forerunner function for an adult specific fascicle tract (Nassif et al., 1998, 2003). During larval stages, cell bodies of the OLPs come to lie in the medulla cortex, accordingly, their terminal dendrites become part of the adult medulla. Nonetheless, the functional and developmental relevance of these OLPs during development is not yet clear (Sprecher et al., 2011). During the early third instar larva, the epithelial cells within the OOA convert into dividing neuroblasts on the medial and lateral edge of the OOA. The progeny cells of these neuroblasts then start to give rise to the primordia of the medulla and the lamina which then mature during metamorphosis (Meinertzhagen and Hanson, 1993; Nassif et al., 2003).

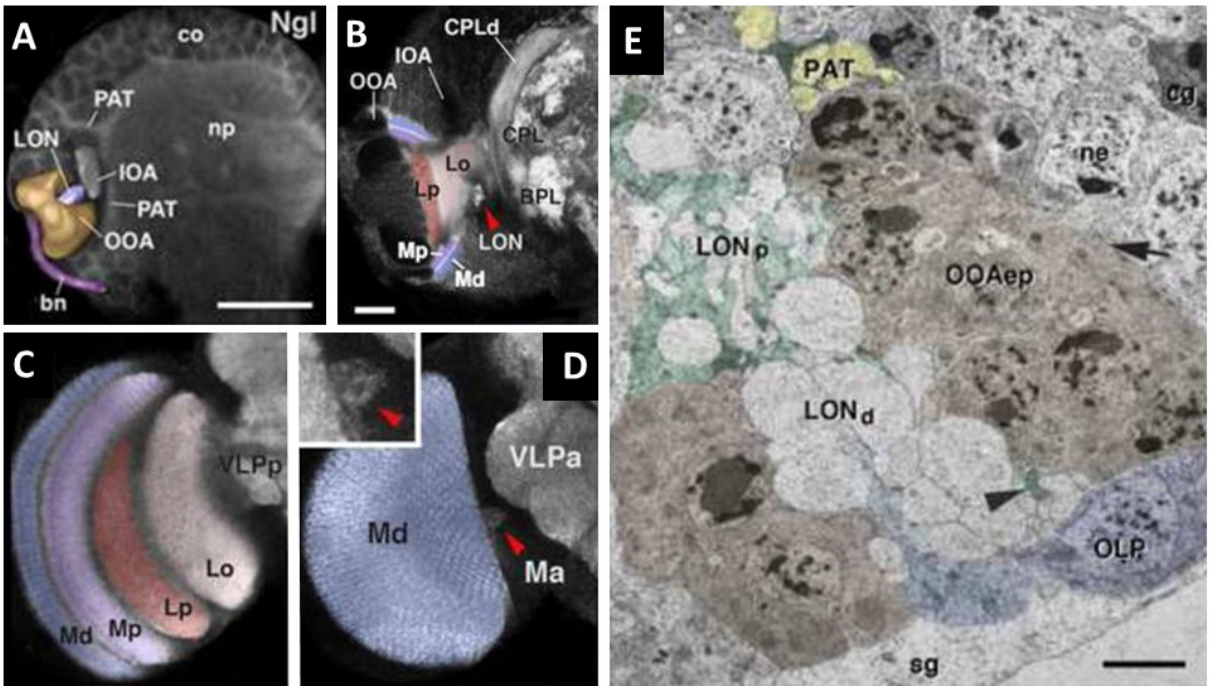


Figure 6.1 Development of the optic lobe neuropil.
(A-D) Section of one Drosophila brain hemisphere as revealed by confocal microscopy. Lateral to the left, dorsal up. **(A)** Labeling with Neuroglian (Ngl), showing neuronal cell bodies in the cortex (co) and their neuronal processes which form the central neuropil (np). Z-projection of a confocal stack (3 µm). Added to the section is a 3D digital model (anterior view) of the outer optic anlage (OOA, beige), the inner optic anlage (IOA, gray), the larval optical neuropil (LON, purple) and the Bolwigs nerve (bn, magenta). Scale bar 10 µm **(B-D)** Frontal single confocal section of the late third larval instar (B), the adult (C) and an adult more anterior section (D) (lateral to the left). Different optic neuropils are rendered in different colors (distal medulla, Md, blue; proximal medulla, Mp, purple; lobula, Lo, faint pink; lobula plate, Lp, red.). Neuropils are labeled by anti-DN-cadherin (DNcad, faint gray). Neuropil compartments stained with DNcad: BPL, baso-posterior lateral compartment; CPL, centro-posterior lateral compartment; CPLd centro-posterior lateral dorsal compartment; VLPp, ventrolateral protocerebrum, posterior subdivision;

VLPa ventrolateral protocerebrum, anterior subdivision. **(B)** Neurons are labeled by DNcad. The IOA and the OOA are DNcad negative. Labeling of synapses with anti-nc82 is added to global neuronal DNcad staining, which results in bright signal demarcating larval neuropil compartments containing mature synapses, including the larval optic neuropil (red arrowhead). **(D)** Anterior surface and medial rim of the distal medulla, to which the accessory medulla (Ma), the descendant of the LON, is attached (red arrowhead, enlarged view shown in inset). **(E)** TEM section showing outer optic anlage epithelium (OOAep) and LON (LONd, distal LON; LONp, proximal LON). OOA epithelium is shaded in brown; glial grocesses are in green; optic lobe pioneers (OLP) in blue; primary axon tract (PAT) in yellow. Arrow points at interface between apical membranes of OOA epithelial cells and neurons (ne). Surface glia (sg) surrounds brain surface, including OOA and optic lobe pioneers (OLP). Cortex glial cell (cg), cortex neuron cell body (ne). Figure adapted from Sprecher et al., 2011, with permission from Elsevier.

When comparing the knowledge gained from OL, MB and antennal lobe neuropil development with the formation of the CC as described in this work, many similarities as well as discrepancies are found. First, the postembryonic neurons of these developing neuropils all seem to use neuronal pioneer cells which were born in the embryo as guidelines to innervate into the proper neuropil areas. We believe, in the mushroom bodies, the γ neurons fulfil this task whereas in the antennal lobe the olfactory receptor neurons together with the projection neurons have pioneer function. Much of the evidence presented to date suggests that in the visual system the optic lobe pioneer neurons might be the first to set up the scaffold upon which the larval as well as the adult optic lobes are built. Additionally, the discovery of an embryonic origin of the CCPr as reported in this work further strengthens the theory that all of the adult specific neuropil compartments of *Drosophila* are represented by primordial structures of various degrees of differentiation in the early larva. This means that the ground plan for the adult brain is already made in the embryo and almost no adult specific brain structures arises completely de novo during metamorphosis. Whereas in the MB and the AL, functional primordial structures exist in early larval stages (in form of the larval antennal lobe and the larval mushroom body), in the OL and the CC the primordial structures (CCPr as well as IOA and OOA) are not integrated in larval circuits. This implies that the OL resembles the CC in its development to a certain degree and poses the question whether the OL primordia are made from undifferentiated cells too. The identity of the OL primordial OOA cells will be discussed in following paragraphs. Whether there are in fact embryonic born undifferentiated neurons already building a scaffold of all the adult specific 50 glomerli in the AL at larval stages will be interesting to follow up by further 3D reconstructions in the deutocerebrum of the L1 brain. Interestingly, preliminary studies suggest that no undifferentiated cells are found in the MB NB lineages of the EM data, which further emphasizes the unique developmental character of these lineages.

Putting these insights into an ontogenic context, this implies that the brain development as seen in the holometabolous fly *Drosophila* is much more similar to the straight forward brain

formation seen in hemimetabolous insects than expected. Accumulating data suggests that there exists a remarkably complete constructional blueprint of adult specific brain neuropils in the embryo. We hypothesize that this blueprint exists in the embryo of most -if not all- insects and that the development of the neuropil structures from this blueprint depends on the environmental conditions for which the various insect species adapted. As an example, the holometabolous moth *Manduca sexta* and the beetle *Tenebrio molitor* both develop a larval functional fan-shaped body, most probably because this structure is used to integrate locomotion of their larvae which already posses legs (Panov, 1959; Granger et al., 1989; Wegerhoff and Breidbach, 1992). We therefore hypothesize, that the interruption of NB proliferation during the quiescent phase is more a temporary rest of a continuous developmental process than the gap between two separated neurogenesis periods.

6.1.4 Lineage affiliation and adult morphology of CCPaC's and their later born lineal sibling neurons

Postembryonically generated neurons from four type-II NB lineages were described to give rise to most -if not all- of the small-field neurons contributing to the adult central complex. The lineages DPMm1 (DM1), DPMpm1 (DM2), DPMpm2 (DM3) and CM4 (DM4) all contribute innervation into four equivalent CC subdomains. The innervation of these isomorphic cells into the CC substructures is highly topologically organized according to the relative position of their NB lineage of origin to the central complex (Ito and Awasaki, 2008; Yang et al., 2013). How this specific innervation of the neuropil is ensured however remained unanswered. The four-fold topological organization of the embryonically born CC primordium as described in this work sheds light onto this issue. Neurons born during postembryonic stages probably use this scaffold for orientation and innervation of the primordial structure. Embryonically induced heat-shock flip-out clonal analysis with *pointedP1*-Gal4 revealed that the CCPaC's entering the CCpr structure from medial to lateral are derived by DPMm1, DPMpm1, DPMpm2 and CM1. This stands in contrast to the postembryonically induced heat-shock flip-out MARCM experiments done with *actin>stop>*Gal4 which showed secondary neurons entering the CC from medial to lateral are made from DPMm1, DPMpm1, DPMpm2 and CM4 (compare Figure 2.9 with Figure 6.2). According to previous insights into NB lineage development, one would expect the CCPaC's of a given NB lineage to prewire the primordium for the postembryonic neurons of the same NB lineage to follow (for review see Hartenstein et al., 2008). Analyzing the innervation pattern of postembryonically born CM1 neurons in the adult central complex further adds to this enigma. CM1 derived projections in the fan-shaped body and the noduli are broad, which stands in contrast to the pattern found of matured CM1 derived CCPaC's (Yang et al., 2013). Furthermore,

postembryonically induced CM1 clonal innervation in the protocerebral bridge is shown in only one of the two glomeruli innervated by the CM1 derived CCPaC's. Interestingly, in addition to a broad innervation of the ellipsoid body, postembryonic MARCM clones show an intense innervation in one of the two concentric EB rings also innervated by CM1 CCPaC's in our work. One of the reasons for this discrepancy could be that for some reason the CM4 derived CCPaC's could not be targeted by our *pointedP1*-Gal4 flip-out experiments. This is supported by the fact that flip-out experiments driven by *pointedP1*-Gal4 at L3 did not give all the CCPaC's otherwise shown by direct *pointedP1*-Gal4 expression. Furthermore, *actin>Stop>*Gal4 might not reveal all of the CM1 derived CCPaC's innervations in the adult. 3D reconstruction of the embryonic born CCPaC's contributing to the fascicle entering the CC primordium at the most lateral position did not reveal this issue. Furthermore, a gap in the EM data stack on the right hemisphere at the position of CM4/CM3/CM1 fascicles impeded a doubtless lineage classification. Final lineage relationship of the embryonic born CM1/CM4 CCPaC's will be revealed by more complete tracing and identification of all the type-II NB lineages on both sides, their undifferentiated cells as well as their neighboring NB lineages. Another approach to unravel this enigma would be embryonically induced MARCM clones generated with the recently presented lineage-restricted NB MARCM clonal analysis (Awasaki et al., 2014). However, this new method depends on the existence of a Gal4 driver which is expressed in the NB of the lineage of interest early on during embryogenesis. To date the best candidate drivers to test for such an experiment in type-II NB lineages would be *earmuff*-Gal4 and *pointedP1*-Gal4, as well as *wor*-Gal4, *ase*-Gal80.

A large population of neurons which contribute to the adult fan-shaped body were described on a single cell level (Hanesch et al., 1989; Li et al., 2009, 2013; Chiang 2011; Wolff et al., 2014). It is highly likely that at least some of the CCPaC's were described amongst these neurons. However, single cell analysis of the *R45F08*-Gal4 driver in the adult fly will give the full set of individual morphologies and reveal input and output properties of these neurons. The overall innervation pattern of the CCPaC's suggests that they are small-field neurons (Riebli et al., 2013). Similarly, postembryonic induction of DPMm1, DPMpm1, DPMpm2 and CM4 NB MARCM clones revealed the innervation pattern of adult CC substructures by small-field neurons but did not reveal them on a single-cell level (Yang et al., 2013; Hanesch et al., 1989). The small-field neurons described in Yang et al., 2013 are similar in their innervation of the CC substructures compared to the matured CCPaC's of DPMm1, DPMpm1, DPMpm2 and CM1 described in this work. All the type-II NB clones in both studies show the isomorphic innervation of two distinct glomeruli in the protocerebral bridge and two areas of innervation in the fan-shaped body as well as in two concentric rings in the ellipsoid body. However, in addition to the discrepancy in the lineage affiliation of the neurons contributing to the most lateral fascicle entering the CC, there are two

major differences in the projection patterns described in this report compared to Yang et la., 2013. First, the small-field neurons reported by Yang et al., also innervate the noduli on the contralateral side of the midline, whereas noduli innervation is never seen by mature CCPaC's. Second, there are some differences in the morphology of the two innervation areas of the fan-shaped body. The small-field neurons in Yang et al. of DPMm1 and DPMpm1 have a large innervation area contralaterally and a small one ipsilaterally in the fan-shaped body, the large innervation area for the DPMpm2 and CM4 lineages is ipsilateral and the smaller area is contralateral. In contrast, the adult specific CCPaC's always have a large innervation area on the ispsilateral side and a two-layered innervation contralaterally. Since the cells reported in Yang et al., 2013 and the CCPaC's presented in this work are derived from the same NB lineages (with some reservation for the CM1/CM4 bundle), a subset of the adult neurons described by these two studies might represent the same neurons. However, some additional small-field neurons innervating the noduli are described in Yang et al., whereas some neurons innervating the fan-shaped body on the contralateral side in two distinct layers are only seen in Riebli et al., 2013. The fact that the data described was generated with two distinct driver lines (*R45F08*-Gal4 in Riebli et al., 2013 and *actin>Stop>*Gal4 in Yang et al., 2013) could have led to the labeling of different neurons. Furthermore, the CCPaC clones were generated embryonically, suggesting that the two-layer FB innervating neurons are of embryonic origin and were therefore missed in postembryonic flip-out MARCM clones of Yang et al., 2013. The projection pattern of postembryonically induced DM5 (CM3) MARCM clones showed no innervation in the fan-shaped body and the ellipsoid body but only broad innervation throughout the protocerebral bridge neuropil (Yang et al., 2013). This is in agreement with the observation that this lineage does not give rise to any CCPaC's.

Recently, a subset of postembryonically generated DPMm1 neurons were described by lineage-restricted twin-spot MARCM clonal analysis, which labels sibling clones in two different colors and therefor reveals two-cell or single cell identities within a given NB lineage (Wang et al., 2014; Awasaki et al., 2014). The DPMm1 cells described all derived from the first two INPs after hatching. Single cell analysis of these neurons revealed that around half the cells produced by these INP are central complex intrinsic small-field neurons that potentially represent matured CCPaC's (Figure 6.2 P-S) (Wang et al., 2014). However, the cells shown only represent a subset of CC innervating neurons. Also, whether the same isomorphic CC small-field neurons are produced at similar time points from similar INP clones of different type-II NB lineages will be interesting to see. This issue could be revealed with additional NB lineage-restricted twin-spot MARCM clonal analysis (Awasaki et al., 2014). To reveal the CCPaC's on a single cell level, the recently introduced multicolor flip-out technique (MCFO) would be advisable. This technique

allows the labeling of single neurons labeled by a given Gal4 driver line at high resolution in unique colors (Nern et al., in prep.; Wolff et al., 2014). Understanding the CC innervating population of neurons on a single cell level will facilitate future experiments depicting the mechanisms and genetic factors involved in generating this diversity of neurons.

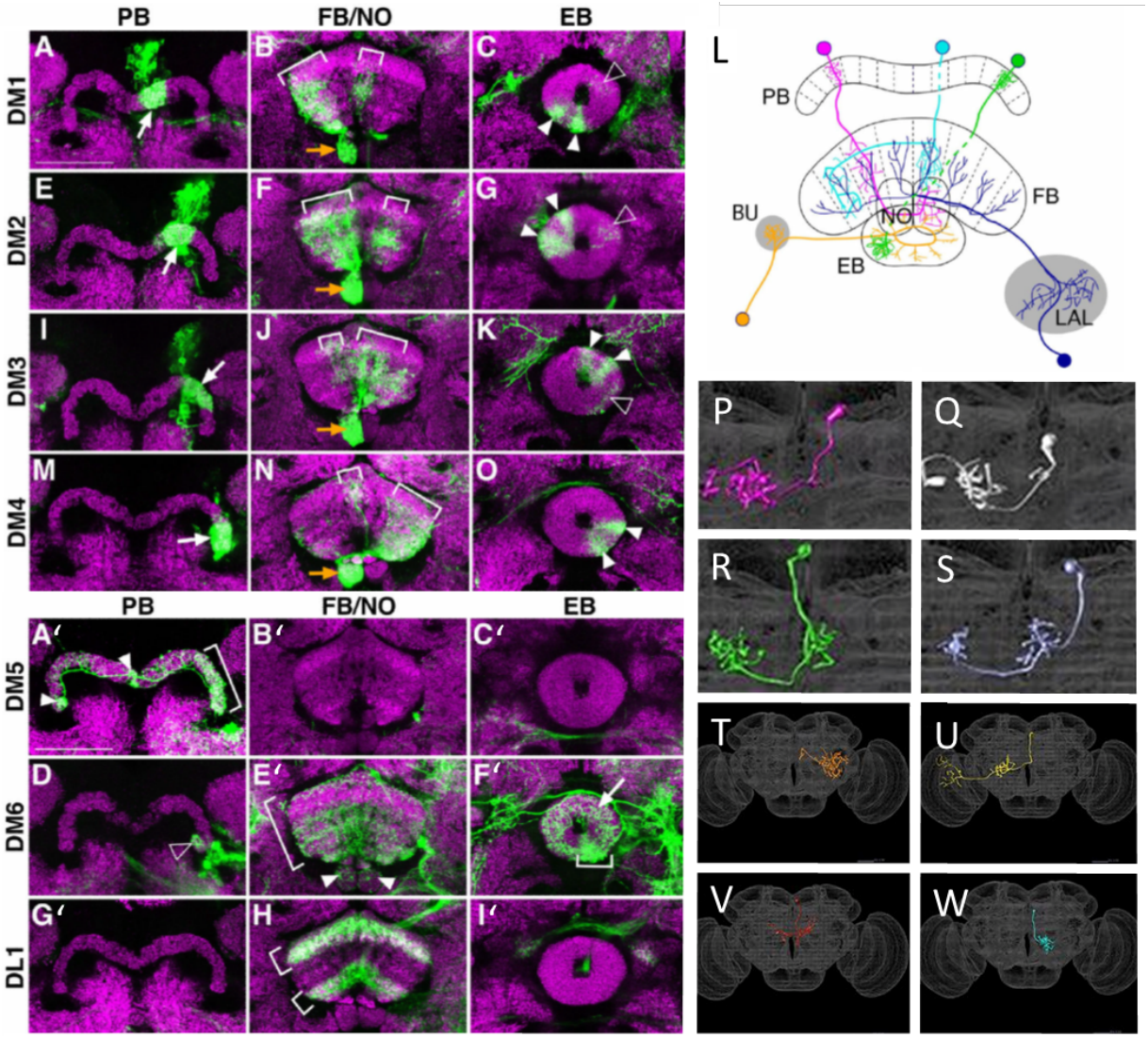


Figure 6.2 Type-II NB lineage derived small-field neurons of the central complex.
(A-O, A'-C', E'-G', I') MARCM NB clones (green) showing central complex innervation of the seven type-II NB lineages DM1 (DPMm1) (A-C), DM2 (DPMpm1) (E-G), DM3 (DPMpm2) (I-K), DM4 (CM4) (M-O), DM5 (CM3) (A'-C'), DM6 (CM1) (D, E'-F'), DL1 (G', H, I'). Their innervation in the substructures of the protocerebral bridge (pb), the fan-shaped and the noduli (NO), and the ellipsoid body (EB) are shown in three separate panels. White arrows, innervation within the PB; brackets, innervation within the FB; orange arrows, innervation within the NO; solid arrowheads, main innervation within the EB; open arrowheads, minor innervation within the EB. Scale bars in A and E are 50µm and apply to all panels. **(L)** Schematic illustration of the CC small-field and large-field neurons. Three types of small-field neurons are shown in magenta (pb-fb-no), cyan (fb-fb) and green (pb-eb). Two types of large-field neurons are shown

in orange (bu-eb) and blue (lal-fb). BU, bulbs; LAL, lateral accessory lobes. **(P-W)** Single-cells generated by the first DPMm1 derived INP clone the after hatching. Cells were registered individually into a preselected adult fly brain template. (P-S) magnified view of the CC neurons showing two small-field neurons innervating fb-bu (P,Q), which are generated by the same GMC clone and two small-field neurons innervating fb intrinsically (R,S) which are generated by two succeeding GMCs of the same INP clone. (T-W) Diverse DPMm1 derived neurons innervating various neuropil areas and are not associated with the CC. (A-O) Figures from Yang et al., 2013, with permission from John Wiley and Sons Ltd. (P-W) Figures from Wang et al., 2014, with permission from the Company of Biologists.

6.1.5 Morphology of embryonic born undifferentiated CCPaC's and cells of the OOA

The cell bodies of the embryonic secondary undifferentiated cells reported in this study all consisted of condensed chromatin and a small cytosol. Previous studies reported chromatin condensation as an indication for either a mitotic cell or an apoptotic cell undergoing programmed cell death (Larsen et al, 2009; Baehrecke, 2002; Boyan et al., 2010). Indeed, programmed cell death was reported to be abundant during embryonic neural development in insects in general (Abrahms et al., 1993; Rogulja-Ortmann et al., 2007; Boyan et al., 2010) as well as during postembryonic stages of *Drosophila* and even in the type-II NB lineages (Larsen et al., 2009; Bello et al., 2003; Kumar et al., 2009; Jiang and Reichert, 2012). In the VNC, postmitotic midline glial cells and neurons were reported to undergo apoptosis upon completion in either building the commissural axon tracts or in pioneering later born neurons during embryogenesis (Sonnenfeld and Jacobs, 1995; Miguel-Aliaga and Thor, 2004). Thus, segment-specific apoptosis occurs in differentiated cells as well as in cells prior to their differentiation in the fly VNC (Rogulja-Ortmann et al., 2007). However, a specific cell undergoing programmed cell death displays an array of morphological signs, such as nuclear breakdown, the presence of pygnotic bodies, membrane fragmentation and glial cells that engulf the dying neuronal processes and the fragmented cell body (White and Steller, 1995; Kurant et al., 2008; Boyan et al., 2010; Watts et al., 2004). We never encountered nuclear breakdown in the undifferentiated cells of the EM reconstructions and did not observe pygnotic bodies nor were neurons or their projections engulfed by glial cells. This asks for an alternative explanation for the condensed chromatin in the undifferentiated embryonic secondary cells described in this line of work. A study in the grasshopper revealed, that only after the axon of a neuron reaches its postsynaptic target does the neurotransmitter accumulate, the soma enlarge and the cell differentiate (Goodmann et al., 1979). This is in agreement with our hypothesis that the embryonic secondary neurons will eventually differentiate and adopt the soma morphology of the differentiated cells as seen in Figure 4.2. Even though light microscopy did not reveal the state of their chromatin, CCpaC's are also undifferentiated during larval stages and do not form

functional synapses. However, the number of CCPaC's found during late larval stages and subsequently in the adult fly is almost the same indicating that these cells do not die after forming the primordium but integrate into the adult circuitry. This is seen in various examples of pioneering neurons, such as the MB γ neurons, the antennal lobe projection neurons and the optic lobe pioneer neurons (Kunz et al., 2012; Marin et al., 2005; Sprecher et al., 2011). Furthermore, this poses the question whether early born cells differ in their developmental program from later born neurons, for whom extensive cell death has been reported during postembryonic stages (Jiang and Reichert, 2012). Additionally, the cells of the epithelial outer optic anlage previously shown in EM stacks of the L1 possess the same morphological identity as do the embryonic secondary cells described in this work (see Figure 6.1E (Sprecher et al., 2011)). These OOA cells were reported to survive until late third instar larval stages and subsequently convert into the optic lobe NBs which produce the medulla and the lamina (Friedrich, 2013). These data further support the notion that in the case of the CCPaC's and the OOA cells, these electron dense nuclei do not in fact indicate apoptotic cells but are a characteristic of cells contributing to neuropil primordia.

Furthermore, the undifferentiated embryonic secondary cells reported by Zhou et al., 2009 also had scant cytoplasm which confers to the morphology found in undifferentiated cells of the first larval instar EM data stack described here. These undifferentiated neurons of the thoracic ganglia were reported to survive larval stages and differentiate during metamorphosis. In accordance, the adult specific but embryonic born MN5 motoneuron was shown to remain in a developmentally arrested state until pupal phases where it starts to form synaptic connections (Consoulas et al., 2002).

Concluding, we suggest that undifferentiated cells endure the period of quiescence by condensing their chromatin. However, staining for apoptotic markers such as TUNEL and acridine orange at first larval instar will proove if these cells are apoptotic or not (Gavrieli et al., 1992; Traganos et al., 1977).

6.2 Embryonic born differentiated neurons of the type-II NB lineage DPMm1 are highly diverse

3D reconstruction of the DPMm1 NB lineage at EM resolution further revealed a high neuronal diversity of two distinct characteristics. First, about half of the cells within this NB lineage are undifferentiated neurons of several identities. Most of these cells project into compartments

which are completely devoid of differentiated neurons of the same NB lineage, thus presumably forming a scaffold for adult specific neuropil compartments (amongst those are the embryonic secondary CCPaC's). The second half of the embryonically generated DPMm1 NB lineage is made of differentiated primary neurons which are highly diverse, innervating various neuropil areas of the brain. 3D reconstruction of the differentiated neurons allowed their identification on a single cell level and revealed synaptic connections of both pre- and postsynaptic identities. These larval functional neurons innervate broadly on the ipsilateral brain hemisphere as well as many of them cross the midline and innervate contralaterally, some of which even into the putative SEG and the VNC. This is in agreement with previous work reporting embryonic born neurons of a dorsomedially located cell cluster to project towards the median bundle and then projecting into the SEG and the VNC (Nassif et al., 1998, 2003).

6.2.1 Different projection patterns of embryonically and postembryonically born DPMm1 neurons

Embryonic born neurons were reported to have a much higher diversity compared to the relative homogeneity of postembryonic born neurons. This was reported in the MB NB lineages and in AL projection neuron and interneuron NB lineages. Whereas the postembryonic born neurons of these lineages are of MB or AL intrinsic character, some primary neurons of these NB lineages were found to be non-intrinsic. In contrast to their postembryonically born sibling neurons, primary neurons of the MB lineages also innervate the VNC and cross the commissure. Similarly, the primary neurons of the AL projection and interneuron NB lineages also innervate outside of the antennal lobe within the SEG (Kunz et al., 2012; Das et al., 2013; Yu et al., 2010). The neurons born in the DPMm1 type-II NB lineage after larval hatching have been studied in great detail and described at late larval stages and in the adult (Izergina et al., 2009; Bayraktar et al., 2010; Riebli et al., 2013; Ito et al., 2013; Yu et al., 2013). NB lineage and single-cell clonal analysis of these postembryonically born DPMm1 neurons revealed a high diversity of neurons which innervate various neuropil areas in both hemispheres of the adult central brain as well as the optic lobes and the subesophageal ganglion (Figure 6.2 I-P in this paragraph from Wang et al., 2014 and Figure 6.3) (Ito et al., 2013; Yu et al., 2013). 3D reconstruction of embryonic born differentiated DPMm1 neurons revealed that these cells are highly diverse too, innervating both hemispheres broadly as well as the putative SEG and the VNC. Whereas postembryonic DPMm1 clones did not reveal VNC innervating neurons, embryonic born neurons of at least two types innervate the early larval VNC. Additionally, postembryonic clones show prominent innervation in the OLs, but none of the embryonic neurons of differentiated or undifferentiated identity

projected towards the OLs. In conclusion, as reported in other NB lineages, we find DPMm1 neurons innervating additional neuropils in the functional larval brain which are not innervated by postemrbyonically DPMm1 derived neurons. Interestingly, we also discovered adult specific neurons in this DPMm1 NB lineage which were not in any form anticipated in the embryo. More complete tracing will enable to identify and assign larval neuropil areas to their adult counterparts. This will shed further light into whether primary neurons innervate the equivalent neuropil compartments in the larva as their postembryonic born sibling cells do so in the adult. Furthermore this approach will reveal a putative larval functional central complex neuropil.

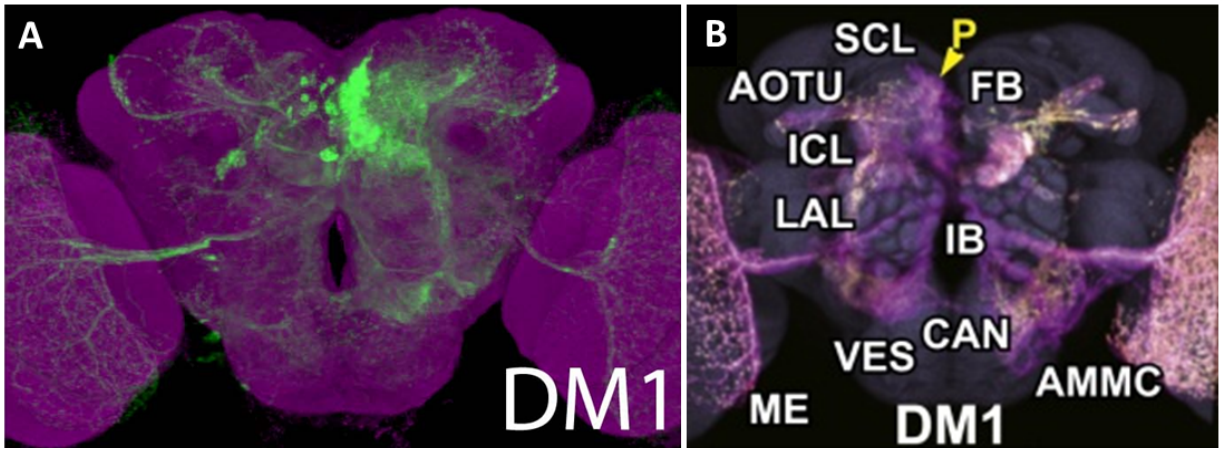


Figure 6.3 Adult DPMm1 (DM1) type-II NB lineage innervates the central brain and the optic lobes.
(A) Actin-flip-out MARCM DPMm1 NB clone (green) shown after warping into an nc82-counterstained adult *Drosophila* brain (magenta). Posterior surface is up. (B) Anterior view of DPMm1 NB elav-MARCM clone. Cell bodies and neuronal fibers (magenta) and the entire neuropil of the template brain (nc82 in gray) are shown. Arrowheads indicate the location of cell-body clusters, with the letter "P" denoting their posterior position. AOTU, anterior optic tubercule; SCL, superior clamp; FB, fan-shaped body; ICL, inferior clamp; LAL, lateral scessory lobe; IB, inferior bridge; VES, vest; CAN, cantle; AMMC, antennal mechanosensory and motor center; ME, medulla. (A) Figure from Yu et al., 2013, with permission from Elsevier. (B) Figure from Ito et al., 2013, with permission from Elsevier.

6.2.2 Integration of embryonic born differentiated neurons into adult circuits

Neurons born in the embryo which control larval behavior become reorganized during metamorphosis and contribute to the adult neuronal circuitry, if they are not removed by programmed cell death (Lee et al., 1999; Marin et al., 2005; Truman et al., 2004; Larsen et al., 2009). Neuronal over proliferation and subsequent cell removal via apoptosis occurs abundantly during *Drosophila* CNS development (Rogulja-Ortmann et al., 2007). During larval development, approximately one-quarter of the neuronal cells derived from the DPMm1 lineage are eliminated by apoptosis before the formation of synaptic connectivity during pupal stages, indicating that these cells represent postembryonically born secondary neurons (Jiang and

Reichert, 2012). Even though dell death is abundant in early larval stages in the central brain, the complete sequence of neurons described from the adPN NB lineage only showed one embryonically born cell destined to die and all the other primary neurons got integrated into adult circuits (Larsen et al., 2009; Yu et al., 2010). Whether the reported differentiated DPMm1 primary neurons survive and get integrated into the adult circuitry or whether these cells undergo apoptosis during metamorphosis remains elusive.

We hypothesize that the larval functional neurons of DPMm1 do not become neurons of the central complex, since these neurons show a completely different identity compared to the CCPaC's due to their commissural crossing on a much more ventral level. However, the postembryonic DPMm1 lineage contains many non CC related neurons, some of which could be of embryonic origin. The adult destiny of the DPMm1 primary neurons is hard to trace, due to the limitations of clonal single cell labeling in the embryo and the tracing of single cells through substantial morphological changes during metamorphosis.

6.2.3 How are such diverse type-II NB derived primary neurons generated during embryogenesis?

To date there is no record of the existence of INPs in embryonic type-II NB lineages. In the EM data stack, cell bodies of INPs and GMCs would be expected to lack any kind of neuronal outgrowth. Since all of the cells reconstructed in the DPMm1 NB lineage of first larval instar EM data stack had at least one outgrowing neuronal process, we could not identify any INPs or GMCs. This suggests that during the period of quiescence, there are no intermediate progenitor cells. Furthermore, this implies that such precursor cells underwent their last mitotic division before larval hatching, leaving the brain with a vast amount of young, undifferentiated neurons. However, our data do not reveal whether any of the embryonic born DPMm1 derived neurons were generated by embryonic INPs. Embryonic induced NB lineage-restricted twin-spot MARCM clonal analysis would reveal embryonic INP clones and their accompanying NB clone (Awasaki et al., 2014). Additionally, antibody staining's for INP specific markers in embryos will give further insight into this issue.

Postembryonic DPMm1 derived neurons generated by a single INP clone were reported to be morphologically distinct with minimal cellular redundancy. The production of these diverse neuronal types is repeated with slight modifications in the sequential INP clone (Wang et al., 2014). The generation of such diverse neurons was shown to depend on temporal fate

patterning genes expressed in the NB as well as in the INP (Bayraktar and Doe, 2013). In the embryo, specific gene expression involved in temporal patterning has already been reported in neurons of an AL projection neuron NB lineage (Kao et al., 2012). 3D reconstructions revealed that the embryonic born differentiated neurons of the DPMm1 lineage are also highly diverse, showing a maximum of four cells of the same morphology. This suggests that INPs are also present in the embryo. Postembryonic INPs were reported to divide 5-6 times, which leads to the production of up to 10 neurons per clone. 3D reconstructions revealed 17 embryonically generated differentiated DPMm1 derived neurons at early larval stages. This leads us to speculate, that these neurons might have derived from 2-3 INP clones. If this were indeed the case, it would be interesting to investigate whether the same temporal cascade of transcription factors is involved in generating this embryonic neuronal diversity as has been reported for the adult INP clones (Bayraktar and Doe 2013).

6.3 The type-II NB lineage DL1 gives rise to a subset of optic lobe glial cells

Clonal analysis revealed that the type-II NB lineage DL1 is in fact a neuroglioblast which generates both central brain interneurons and glial cells of the optic lobe. Shortly after glia cells are born, they migrate into the optic lobe and will later become outer chiasm glia, inner chiasm glia and cortex glia. Other optic lobe glia are also produced outside of the optic lobe in the optic stalk of the larval eye-disc and migrate into the OL where they become surface glia. In addition, there are specialized glial precursor zones located within the optic lobes that contribute to the optic lobe glia population (Edwards and Meinertzhagen, 2010; Hartenstein, 2011; Chotard and Salecker, 2007; Edwards et al., 2012). The DL1 type-II NB lineage is the first evidence of a central brain NB lineage which produces cells contributing to the optic lobe.

The glial cells and neurons derived by DL1 both have very different developmental fates. Whereas the neurons innervate various neuropil areas in the central brain as well as two distinct horizontal layers of the fan-shaped body, DL1 derived glia immediately leave the central brain and migrate into the OL. In contrast to the glia cells produced by the type-II NB lineages which contribute neurons as well as glial cells to the central complex, the DL1 derived glia do not have any neuronal sibling neurons along whose axons they could migrate. How these glial cells orient themselves and target the OL is not known. Studies in the central brain of the *Drosophila* embryo show that glia appear very late during development and after the neurons, suggesting that the spatial information for patterning the neuropil resides within neurons and

not glia. Furthermore glia were reported to migrate alongside pioneer axon tracts of the central brain (Younossi-Hartenstein et al., 2006; Klämbt, 1993). However, glia which contribute to the visual system do not seem to be as dependent on neurons as central brain glia. Subretinal gial cells which originate in the optic stalk during late third larval instar undergo extensive migration into the developing eye disc (Choi and Benzer, 1994; Rangarajan et al., 1999, 2001). In gilgamesh (gish) mutant, glial cells migrate into the eye disc, even before photoreceptor cells have differentiated, indicating that subretinal glia do not require axons to migrate. On the contrary, when glial cells are made to grow ectopically, photoreceptor axons also grow ectopically, indicating that axons follow glia (Hummel et al., 2002). Whether DL1 derived glia migrate independently from axon guidance or whether they follow axons from different NB lineages is currently unknown. If indeed other axons are involved in the guidance process, two possible candidate fiber tracts would be the embryonic born pioneer fiber tract called the "posterior optic tract" as described in Nassif et al., 1998 or the postembryonically born DPMm1 neurons which innervate the optic lobe. Furthermore, the axon guidance and cell adhesion molecules involved in this migratory process would be interesting to study.

Even though our work revealed the developmental origin of some of the optic lobe glia, MARCM analysis and flip-out clones of DL1 never labeled all the inner and outer chiasm glia cells of the optic lobe. This suggests that some glia arise from different NB lineages. The type-II NB lineage CM3 also gives rise to a large amount of glial cells that do not migrate into the commissural area but head laterally towards the optic lobe during third larval instar (Viktorin et al., 2011). MARCM clones of the CM3 NB lineage in the adult show progeny cells within the optic lobe, however, whether these cells are of a glial or neuronal nature remains unclear (Ito et al., 2013; Yu et al., 2013). The pursuit of these CM3 glia throughout development into the adult and subsequent antibody-staining with glial markers would reveal whether DL1 and CM3 together produce a more complete array of optic lobe glial cells.

7. Abbreviations

AEL, after egg laying
AL, antennal lobe
APF, after puparium formation
Bn, Bolwigs nerve
BPL, baso-posterior lateral compartment
BU, bulb
CATMAID, collaborative annotation toolkit for massive amounts of image data
CC, central complex
CCPaC's, central complex primordium associated cells
CCpr, central complex primordium dlrFB, dorsolateral root of fan-shaped body
cg, cortex glia cell
CM1, central-medial lineage 1
CM3, central-medial lineage 3
CM4, central-medial lineage 4
CNS central nervous system
co, cortex
CPG, central pattern generator
CPL, centro-posterior lateral compartment
CPLd, centro-posterior lateral dorsal compartment
CX, calyx
DL1, dorso-lateral lineage 1
DL2, dorso-lateral lineage 2
DM, dorsomedial
DPMm1, dorso-posterior-medial-medial lineage 1
DPMpm1, dorso-posterior-medial-posteriormedial lineage 1
DPMpm2, dorso-posterior-medial-posteriormedial lineage 2
DSHB, developmental studies hybridoma bank
EB, ellipsoid body
EM, electron microscope
FB, fan-shaped body
FBpr, fan-shaped body primordium
GFP, green fluorescent protein
GMC, ganglion mother cell
HHMI, Howard Hughes Medical Institute
INP, intermediate neural progenitor
IPC, inner proliferation center
IOA, inner optic anlage
OOA, outer optic anlage
L1, thirst larval instar
L2, second larval instar
L3, third larval instar
LAL, lateral accessory lobe
lat, lateral type-II NB lineage
LCBR, lateral cell body rind
Lo, lobula
LON, larval optic neuropil
LONd, distal larval optic neuropil
LONp, proximal larval optic neuropil
Lp, lobula plate
lrFB, lateral root of fan-shaped body
Ma, accessory Medulla
MB, mushroom body
Md, distal medulla
MARCM, mosaic analysis with a repressible cell marker
Mp, proximal Medulla
ml, medial lobe
mrFB, medial root of fan-shaped body
NB, neuroblast
Ne, cortex neuron cell body
NO, noduli
np, neuropil
OOAep, outer optic anlage epithelium
OL, optic lobe
OLP, optic lobe pioneers
PAN, Posterior-asense-negative
PAT, primary axon tract
PB, protocerebral bridge
Pnt, pointed
p, peduncle
RFP, red fluorescent protein
SAT, secondary axon tract
SEG, suboesophagealganglion
Sg, surface glia
sp, spur of mushroom body
SPG, supraoesophagealganglion
ssTEM, serial section transmission electron microscope
VCT, ventral commissural tract
VLPp, ventrolateral protocerebrun
VLPa, ventrolateral protocerebrum
VNC, ventral nerve cord

8. References

Abrams, J. M., K. White, et al. (1993). "Programmed cell death during Drosophila embryogenesis." Development **117**(1): 29-43.

Acampora, D., V. Avantaggiato, et al. (1998). "Murine Otx1 and Drosophila otd genes share conserved genetic functions required in invertebrate and vertebrate brain development." Development **125**(9): 1691-1702.

Acampora, D., P. P. Boyl, et al. (2001). "OTD/OTX2 functional equivalence depends on 5' and 3' UTR-mediated control of Otx2 mRNA for nucleo-cytoplasmic export and epiblast-restricted translation." Development **128**(23): 4801-4813.

Acampora, D., M. Gulisano, et al. (2001). "Otx genes in brain morphogenesis." Prog Neurobiol **64**(1): 69-95.

Acampora, D., M. Gulisano, et al. (1999). "Otx genes and the genetic control of brain morphogenesis." Mol Cell Neurosci **13**(1): 1-8.

Acampora, D., S. Mazan, et al. (1995). "Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation." Development **121**(10): 3279-3290.

Adoutte, A., G. Balavoine, et al. (1999). "Animal evolution. The end of the intermediate taxa?" Trends Genet **15**(3): 104-108.

Adoutte, A., G. Balavoine, et al. (2000). "The new animal phylogeny: reliability and implications." Proc Natl Acad Sci U S A **97**(9): 4453-4456.

Akiyama-Oda, Y. and H. Oda (2006). "Axis specification in the spider embryo: dpp is required for radial-to-axial symmetry transformation and sog for ventral patterning." Development **133**(12): 2347-2357.

Albagli, O., A. Klaes, et al. (1996). "Function of ets genes is conserved between vertebrates and Drosophila." Mechanisms of development **59**(1): 29-40.

Alfonso, T. B. and B. W. Jones (2002). "gcm2 promotes glial cell differentiation and is required with glial cells missing for macrophage development in Drosophila." Developmental biology **248**(2): 369-383.

Alvarez, A. D., W. Shi, et al. (2003). "pannier and pointedP2 act sequentially to regulate Drosophila heart development." Development **130**(13): 3015-3026.

Arendt, D., A. S. Denes, et al. (2008). "The evolution of nervous system centralization." Philos Trans R Soc Lond B Biol Sci **363**(1496): 1523-1528.

Arendt, D. and K. Nubler-Jung (1994). "Inversion of dorsoventral axis?" Nature **371**(6492): 26.

Arendt, D. and K. Nubler-Jung (1999). "Comparison of early nerve cord development in insects and vertebrates." Development **126**(11): 2309-2325.

Arendt, D., U. Technau, et al. (2001). "Evolution of the bilaterian larval foregut." Nature **409**(6816): 81-85.

Arendt, D., K. Tessmar-Raible, et al. (2004). "Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain." Science **306**(5697): 869-871.

Asano, M. and P. Gruss (1992). "Pax-5 is expressed at the midbrain-hindbrain boundary during mouse development." Mech Dev **39**(1-2): 29-39.

Awasaki, T., C. F. Kao, et al. (2014). "Making Drosophila lineage-restricted drivers via patterned recombination in neuroblasts." Nature neuroscience **17**(4): 631-637.

Awasaki, T., S. L. Lai, et al. (2008). "Organization and postembryonic development of glial cells in the adult central brain of Drosophila." The Journal of neuroscience : the official journal of the Society for Neuroscience **28**(51): 13742-13753.

Baala, L., S. Briault, et al. (2007). "Homozygous silencing of T-box transcription factor EOMES leads to microcephaly with polymicrogyria and corpus callosum agenesis." Nature genetics **39**(4): 454-456.

Baehrecke, E. H. (2002). "How death shapes life during development." Nature reviews. Molecular cell biology **3**(10): 779-787.

Ball, E. E., D. C. Hayward, et al. (2004). "A simple plan--cnidarians and the origins of developmental mechanisms." Nat Rev Genet **5**(8): 567-577.

Baumgardt, M., D. Karlsson, et al. (2009). "Neuronal subtype specification within a lineage by opposing temporal feed-forward loops." Cell **139**(5): 969-982.

Bayraktar, O. A., J. Q. Boone, et al. (2010). "Drosophila type II neuroblast lineages keep Prospero levels low to generate large clones that contribute to the adult brain central complex." Neural development **5**: 26.

Bayraktar, O. A. and C. Q. Doe (2013). "Combinatorial temporal patterning in progenitors expands neural diversity." Nature **498**(7455): 449-455.

Beckervordersandforth, R. M., C. Rickert, et al. (2008). "Subtypes of glial cells in the Drosophila embryonic ventral nerve cord as related to lineage and gene expression." Mechanisms of development **125**(5-6): 542-557.

Bello, B. C., F. Hirth, et al. (2003). "A pulse of the Drosophila Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis." Neuron **37**(2): 209-219.

Bello, B. C., N. Izergina, et al. (2008). "Amplification of neural stem cell proliferation by intermediate progenitor cells in Drosophila brain development." Neural Dev **3**: 5.

Benito-Gutierrez, E. and D. Arendt (2009). "CNS evolution: new insight from the mud." Curr Biol **19**(15): R640-642.

Benton, R., S. Sachse, et al. (2006). "Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo." PLoS Biol **4**(2): e20.

Betschinger, J. and J. A. Knoblich (2004). "Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates." Current biology : CB **14**(16): R674-685.

Bieber, A. J., P. M. Snow, et al. (1989). "Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1." Cell **59**(3): 447-460.

Birkholz, O., C. Rickert, et al. (2013). "Neuroblast pattern and identity in the Drosophila tail region and role of doublesex in the survival of sex-specific precursors." Development **140**(8): 1830-1842.

Bishop, K. M., S. Garel, et al. (2003). "Emx1 and Emx2 cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical efferents, and thalamocortical pathfinding." J Comp Neurol **457**(4): 345-360.

Bock, D. D., W. C. Lee, et al. (2011). "Network anatomy and in vivo physiology of visual cortical neurons." Nature **471**(7337): 177-182.

Boero, F., B. Schierwater, et al. (2007). "Cnidarian milestones in metazoan evolution." Integr Comp Biol **47**(5): 693-700.

Boone, J. Q. and C. Q. Doe (2008). "Identification of Drosophila type II neuroblast lineages containing transit amplifying ganglion mother cells." Dev Neurobiol **68**(9): 1185-1195.

Boschert, U., R. G. Ramos, et al. (1990). "Genetic and developmental analysis of irreC, a genetic function required for optic chiasm formation in Drosophila." Journal of neurogenetics **6**(3): 153-171.

Bossing, T., G. Udolph, et al. (1996). "The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm." Developmental biology **179**(1): 41-64.

Bouillet, P., C. Chazaud, et al. (1995). "Sequence and expression pattern of the Stra7 (Gbx-2) homeobox-containing gene induced by retinoic acid in P19 embryonal carcinoma cells." Dev Dyn **204**(4): 372-382.

Boulanger, A., C. Clouet-Redt, et al. (2011). "ftz-f1 and Hr39 opposing roles on EcR expression during Drosophila mushroom body neuron remodeling." Nature neuroscience **14**(1): 37-44.

Bowman, S. K., V. Rolland, et al. (2008). "The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in Drosophila." Dev Cell **14**(4): 535-546.

Boyan, G., Z. Herbert, et al. (2010). "Cell death shapes embryonic lineages of the central complex in the grasshopper Schistocerca gregaria." Journal of morphology **271**(8): 949-959.

Boyan, G. and L. Williams (2011). "Embryonic development of the insect central complex: insights from lineages in the grasshopper and Drosophila." Arthropod structure & development **40**(4): 334-348.

Boyan, G. S. and H. Reichert (2011). "Mechanisms for complexity in the brain: generating the insect central complex." Trends in neurosciences **34**(5): 247-257.

Brand, A. H. and F. J. Livesey (2011). "Neural stem cell biology in vertebrates and invertebrates: more alike than different?" Neuron **70**(4): 719-729.

Briggman, K. L. and D. D. Bock (2012). "Volume electron microscopy for neuronal circuit reconstruction." Current opinion in neurobiology **22**(1): 154-161.

Briscoe, J., A. Pierani, et al. (2000). "A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube." Cell **101**(4): 435-445.

Briscoe, J., L. Sussel, et al. (1999). "Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling." Nature **398**(6728): 622-627.

Broadus, J., J. B. Skeath, et al. (1995). "New neuroblast markers and the origin of the aCC/pCC neurons in the Drosophila central nervous system." Mechanisms of development **53**(3): 393-402.

Brody, T. and W. F. Odenwald (2000). "Programmed transformations in neuroblast gene expression during Drosophila CNS lineage development." Developmental biology **226**(1): 34-44.

Brody, T. and W. F. Odenwald (2002). "Cellular diversity in the developing nervous system: a temporal view from Drosophila." Development **129**(16): 3763-3770.

Bruce, A. E. and M. Shankland (1998). "Expression of the head gene Lox22-Otx in the leech Helobdella and the origin of the bilaterian body plan." Dev Biol **201**(1): 101-112.

Brunner, D., K. Ducker, et al. (1994). "The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway." Nature **370**(6488): 386-389.

Brusca, R. C. and Brusca, G. J. (1990). "Invertebrates". Sinauer Associates.

Bullock, T. H. and Horridge, G. A (1965). "Structure and Function in the Nervous Systems of Invertebrates." Vol. 1. W. H. Freeman.

Cabernard, C. and M. Affolter (2005). "Distinct roles for two receptor tyrosine kinases in epithelial branching morphogenesis in Drosophila." Developmental cell **9**(6): 831-842.

Cajal S., Sánchez D. (1915). "Contribución al conocimiento de los centros nerviosos de los Insectos". *Trab. Lab. Inv. Biol.* **13**, 1–68.

Campbell, G., H. Goring, et al. (1994). "RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in Drosophila." Development **120**(10): 2957-2966.

Campos-Ortega, J. A. (1997). "Neurogenesis in Drosophila: an historical perspective and some prospects." Perspectives on developmental neurobiology **4**(4): 267-271.

Campos, A. R., K. J. Lee, et al. (1995). "Establishment of neuronal connectivity during development of the Drosophila larval visual system." Journal of neurobiology **28**(3): 313-329.

Cardona, A. (2013). "Towards semi-automatic reconstruction of neural circuits." Neuroinformatics **11**(1): 31-33.

Cardona, A., S. Saalfeld, et al. (2010). "Identifying neuronal lineages of Drosophila by sequence analysis of axon tracts." The Journal of neuroscience : the official journal of the Society for Neuroscience **30**(22): 7538-7553.

Cardona, A., S. Saalfeld, et al. (2010). "An integrated micro- and macroarchitectural analysis of the Drosophila brain by computer-assisted serial section electron microscopy." PLoS biology **8**(10).

Cardona, A., S. Saalfeld, et al. (2012). "TrakEM2 software for neural circuit reconstruction." PloS one **7**(6): e38011.

Carlson, S. D., J. L. Juang, et al. (2000). "Blood barriers of the insect." Annual review of entomology **45**: 151-174.

Carpenter, E. M. (2002). "Hox genes and spinal cord development." Dev Neurosci **24**(1): 24-34.

Carroll, S. B. (1995). "Homeotic genes and the evolution of arthropods and chordates." Nature **376**(6540): 479-485.

Chan, Y. M. and Y. N. Jan (1999). "Conservation of neurogenic genes and mechanisms." Curr Opin Neurobiol **9**(5): 582-588.

Chang, J., I. O. Kim, et al. (2001). "The CNS midline cells control the spitz class and Egfr signaling genes to establish the proper cell fate of the Drosophila ventral neuroectoderm." The International journal of developmental biology **45**(5-6): 715-724.

Chang, K. C., C. Wang, et al. (2012). "Balancing self-renewal and differentiation by asymmetric division: insights from brain tumor suppressors in Drosophila neural stem cells." BioEssays : news and reviews in molecular, cellular and developmental biology **34**(4): 301-310.

Chang, T., A. Younossi-Hartenstein, et al. (2003). "Development of neural lineages derived from the sine oculis positive eye field of Drosophila." Arthropod structure & development **32**(4): 303-317.

Chell, J. M. and A. H. Brand (2010). "Nutrition-responsive glia control exit of neural stem cells from quiescence." Cell **143**(7): 1161-1173.

Chiori, R., M. Jager, et al. (2009). "Are Hox genes ancestrally involved in axial patterning? Evidence from the hydrozoan Clytia hemisphaerica (Cnidaria)." PLoS One **4**(1): e4231.

Choi, K. W. and S. Benzer (1994). "Migration of glia along photoreceptor axons in the developing Drosophila eye." Neuron **12**(2): 423-431.

Chotard, C. and I. Salecker (2007). "Glial cell development and function in the Drosophila visual system." Neuron glia biology **3**(1): 17-25.

Chu, H., C. Parras, et al. (1998). "Formation and specification of ventral neuroblasts is controlled by vnd in Drosophila neurogenesis." Genes Dev **12**(22): 3613-3624.

Clyne, P. J., S. J. Certel, et al. (1999). "The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POU-domain transcription factor." Neuron **22**(2): 339-347.

Cohen, S. M. and G. Jurgens (1990). "Mediation of Drosophila head development by gap-like segmentation genes." Nature **346**(6283): 482-485.

Consoulas, C., L. L. Restifo, et al., (2002). "Dendritic Remodeling and Growth of Motoneurons during Metamorphosis of *Drosophila melanogaster*. " Journal of Neuroscience **22**(12): 4906-4917

Cornell, R. A. and T. V. Ohlen (2000). "Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning?" Curr Opin Neurobiol **10**(1): 63-71.

Dalton, D., R. Chadwick, et al. (1989). "Expression and embryonic function of empty spiracles: a Drosophila homeo box gene with two patterning functions on the anterior-posterior axis of the embryo." Genes Dev **3**(12A): 1940-1956.

Das, A., T. Gupta, et al. (2013). "Neuroblast lineage-specific origin of the neurons of the Drosophila larval olfactory system." Developmental biology **373**(2): 322-337.

Das, A., S. Sen, et al. (2008). "Drosophila olfactory local interneurons and projection neurons derive from a common neuroblast lineage specified by the empty spiracles gene." Neural Dev **3**: 33.

Davenne, M., M. K. Maconochie, et al. (1999). "Hoxa2 and Hoxb2 control dorsoventral patterns of neuronal development in the rostral hindbrain." Neuron **22**(4): 677-691.

Davis, G. K., J. A. D'Alessio, et al. (2005). "Pax3/7 genes reveal conservation and divergence in the arthropod segmentation hierarchy." Dev Biol **285**(1): 169-184.

de Jong, D. M., N. R. Hislop, et al. (2006). "Components of both major axial patterning systems of the Bilateria are differentially expressed along the primary axis of a 'radiate' animal, the anthozoan cnidarian Acropora millepora." Dev Biol **298**(2): 632-643.

De Robertis, E. M. (2008). "Evo-devo: variations on ancestral themes." Cell **132**(2): 185-195.

De Robertis, E. M. and Y. Sasai (1996). "A common plan for dorsoventral patterning in Bilateria." Nature **380**(6569): 37-40.

Dearborn, R., Jr. and S. Kunes (2004). "An axon scaffold induced by retinal axons directs glia to destinations in the Drosophila optic lobe." Development **131**(10): 2291-2303.

Denes, A. S., G. Jekely, et al. (2007). "Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria." Cell **129**(2): 277-288.

Dickson, B. (1995). "Nuclear factors in sevenless signalling." Trends in genetics : TIG **11**(3): 106-111.

Doe, C. Q. (1992). "Molecular markers for identified neuroblasts and ganglion mother cells in the Drosophila central nervous system." Development **116**(4): 855-863.

Doe, C. Q. (2008). "Neural stem cells: balancing self-renewal with differentiation." Development **135**(9): 1575-1587.

Dumstrei, K., F. Wang, et al. (2003). "Early development of the Drosophila brain: V. Pattern of postembryonic neuronal lineages expressing DE-cadherin." The Journal of comparative neurology **455**(4): 451-462.

Edwards, T. N. and I. A. Meinertzhagen (2010). "The functional organisation of glia in the adult brain of Drosophila and other insects." Progress in neurobiology **90**(4): 471-497.

Edwards, T. N., A. C. Nuschke, et al. (2012). "Organization and metamorphosis of glia in the Drosophila visual system." The Journal of comparative neurology **520**(10): 2067-2085.

Egger, B., J. Q. Boone, et al. (2007). "Regulation of spindle orientation and neural stem cell fate in the Drosophila optic lobe." Neural development **2**: 1.

Egger, B., J. M. Chell, et al. (2008). "Insights into neural stem cell biology from flies." Philosophical transactions of the Royal Society of London. Series B, Biological sciences **363**(1489): 39-56.

Ericson, J., P. Rashbass, et al. (1997). "Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling." Cell **90**(1): 169-180.

Feeney, C. J., S. Karunanithi, et al. (1998). "Motor nerve terminals on abdominal muscles in larval flesh flies, Sarcophaga bullata: comparisons with Drosophila." The Journal of comparative neurology **402**(2): 197-209.

Finkelstein, R. and N. Perrimon (1990). "The orthodenticle gene is regulated by bicoid and torso and specifies Drosophila head development." Nature **346**(6283): 485-488.

Finkelstein, R., D. Smouse, et al. (1990). "The orthodenticle gene encodes a novel homeo domain protein involved in the development of the Drosophila nervous system and ocellar visual structures." Genes Dev **4**(9): 1516-1527.

Finnerty, J. R. (2003). "The origins of axial patterning in the metazoa: how old is bilateral symmetry?" Int J Dev Biol **47**(7-8): 523-529.

Finnerty, J. R., K. Pang, et al. (2004). "Origins of bilateral symmetry: Hox and dpp expression in a sea anemone." Science **304**(5675): 1335-1337.

Fortey, R. A. (2000). "Trilobite: Eyewitness to evolution". Vintage Books.

Friedrich, M. (2013.) "Development and evolution of the *Drosophila* Bolwig's organ: A compound eye relict". Molecular Genetics of Axial Patterning, Growth and Disease in the *Drosophila* Eye, Editors: Amit Singh, Madhuri Kango-Singh. 329-357

Gabay, L., H. Scholz, et al. (1996). "EGF receptor signaling induces pointed P1 transcription and inactivates Yan protein in the Drosophila embryonic ventral ectoderm." Development **122**(11): 3355-3362.

Galliot, B. (2000). "Conserved and divergent genes in apex and axis development of cnidarians." Curr Opin Genet Dev **10**(6): 629-637.

Galliot, B., M. Quiquand, et al. (2009). "Origins of neurogenesis, a cnidarian view." Dev Biol **332**(1): 2-24.

Garm, A., P. Ekstrom, et al. (2006). "Rhopalia are integrated parts of the central nervous system in box jellyfish." Cell Tissue Res **325**(2): 333-343.

Garm, A., Y. Poussart, et al. (2007). "The ring nerve of the box jellyfish Tripedalia cystophora." Cell Tissue Res **329**(1): 147-157.

Gavalas, A., M. Studer, et al. (1998). "Hoxa1 and Hoxb1 synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch." Development **125**(6): 1123-1136.

Gavrieli, Y., Y. Sherman, et al. (1992). "Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation." The Journal of cell biology **119**(3): 493-501.

Geoffroy Saint-Hilaire, E. (1822). "Considérations générales sur la vertèbre". Mem. Mus. Hist. Nat. Paris **9**, 89-119.

Gerber, B. and R. F. Stocker (2007). "The Drosophila larva as a model for studying chemosensation and chemosensory learning: a review." Chemical senses **32**(1): 65-89.

Ghysen, A. (1992). "The developmental biology of neural connectivity." Int J Dev Biol **36**(1): 47-58.

Ghysen, A. (2003). "The origin and evolution of the nervous system." Int J Dev Biol **47**(7-8): 555-562.

Goodman, C. and Doe, Q. (1993). "Embryonic development of the *Drosophila* central nervous system". New York: Cold Spring Harbor Laboratory Press.

Goodman, C. S., M. O'Shea, et al. (1979). "Embryonic development of identified neurons: temporal pattern of morphological and biochemical differentiation." Science **204**(4398): 1219-1222.

Goyal, L., K. McCall, et al. (2000). "Induction of apoptosis by Drosophila reaper, hid and grim through inhibition of IAP function." The EMBO journal **19**(4): 589-597.

Granger, N. A., U. Homberg, et al. (1989). "Serotonin-immunoreactive neurons in the brain of Manduca sexta during larval development and larval-pupal metamorphosis." International journal of developmental neuroscience **7**(1): 55-72.

Green, P., A. Y. Hartenstein, et al. (1993). "The embryonic development of the Drosophila visual system." Cell and tissue research **273**(3): 583-598.

Grimmelikhuijzen, C. J. and D. Graff (1985). "Arg-Phe-amide-like peptides in the primitive nervous systems of coelenterates." Peptides **6 Suppl 3**: 477-483.

Grimmelikhuijzen, C. J., I. Leviev, et al. (1996). "Peptides in the nervous systems of cnidarians: structure, function, and biosynthesis." Int Rev Cytol **167**: 37-89.

Halfon, M. S., A. Carmena, et al. (2000). "Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors." Cell **103**(1): 63-74.

Halter, D. A., J. Urban, et al. (1995). "The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of Drosophila melanogaster." Development **121**(2): 317-332.

Han, C., L. Y. Jan, et al. (2011). "Enhancer-driven membrane markers for analysis of nonautonomous mechanisms reveal neuron-glia interactions in Drosophila." Proceedings of the National Academy of Sciences of the United States of America **108**(23): 9673-9678.

Hanesch, U, Fischbach KF, Heisenberg M (1989). "Neuronal architecture of the central complex in *Drosophila melanogaster*". Cell Tissue Res **257**:343-366

Hartenstein, V. (2011). "Morphological diversity and development of glia in Drosophila." Glia **59**(9): 1237-1252.

Hartenstein, V., C. Nassif, et al. (1998). "Embryonic development of the Drosophila brain. II. Pattern of glial cells." J Comp Neurol **402**(1): 32-47.

Hartenstein, V., S. Spindler, et al. (2008). "The development of the Drosophila larval brain." Advances in experimental medicine and biology **628**: 1-31.

Hartmann, B., F. Hirth, et al. (2000). "Expression, regulation and function of the homeobox gene empty spiracles in brain and ventral nerve cord development of Drosophila." Mech Dev **90**(2): 143-153.

Hartmann, B., M. Muller, et al. "Coral emx-Am can substitute for Drosophila empty spiracles function in head, but not brain development." Dev Biol **340**(1): 125-133.

Hassan, B. A., N. A. Bermingham, et al. (2000). "atonal regulates neurite arborization but does not act as a proneural gene in the Drosophila brain." Neuron **25**(3): 549-561.

Hattori, D., E. Demir, et al. (2007). "Dscam diversity is essential for neuronal wiring and self-recognition." Nature **449**(7159): 223-227.

Heimbeck, G., V. Bugnon, et al. (1999). "Smell and taste perception in Drosophila melanogaster larva: toxin expression studies in chemosensory neurons." The Journal of neuroscience : the official journal of the Society for Neuroscience **19**(15): 6599-6609.

Hejnol, A. and M. Q. Martindale (2009). "Coordinated spatial and temporal expression of Hox genes during embryogenesis in the acoel Convolutriloba longifissura." BMC Biol **7**: 65.

Hejnol, A., M. Obst, et al. (2009). "Assessing the root of bilaterian animals with scalable phylogenomic methods." Proc Biol Sci **276**(1677): 4261-4270.

Helmstaedter, M., K. L. Briggman, et al. (2011). "High-accuracy neurite reconstruction for high-throughput neuroanatomy." Nature neuroscience **14**(8): 1081-1088.

Hidalgo, A. (2003). "Neuron-glia interactions during axon guidance in Drosophila." Biochemical Society transactions **31**(Pt 1): 50-55.

Hildebrand, J. G. and G. M. Shepherd (1997). "Mechanisms of olfactory discrimination: converging evidence for common principles across phyla." Annu Rev Neurosci **20**: 595-631.

Hirth, F. "On the origin and evolution of the tripartite brain." Brain Behav Evol **76**(1): 3-10.

Hirth, F., B. Hartmann, et al. (1998). "Homeotic gene action in embryonic brain development of Drosophila." Development **125**(9): 1579-1589.

Hirth, F., L. Kammermeier, et al. (2003). "An urbilaterian origin of the tripartite brain: developmental genetic insights from Drosophila." Development **130**(11): 2365-2373.

Hirth, F. and H. Reichert (1999). "Conserved genetic programs in insect and mammalian brain development." Bioessays **21**(8): 677-684.

Hirth, F. and Reichert, H. (2007). "Basic Nervous system Types: One or Many?". Evolution of the Nervous System: *History of Ideas, Basic Concepts and Developmental Mechanisms* (Strieder, G. & Rubenstein, J., Eds.) 56-72, Elsevier.

Hirth, F., S. Therianos, et al. (1995). "Developmental defects in brain segmentation caused by mutations of the homeobox genes orthodenticle and empty spiracles in Drosophila." Neuron **15**(4): 769-778.

Hofmeyer, K., D. Kretzschmar, et al. (2008). "Optomotor-blind expression in glial cells is required for correct axonal projection across the Drosophila inner optic chiasm." Developmental biology **315**(1): 28-41.

Holland, L. Z. (2009). "Chordate roots of the vertebrate nervous system: expanding the molecular toolkit." Nat Rev Neurosci **10**(10): 736-746.

Holland, N. D. and J. Chen (2001). "Origin and early evolution of the vertebrates: new insights from advances in molecular biology, anatomy, and palaeontology." Bioessays **23**(2): 142-151.

Holley, S. A., P. D. Jackson, et al. (1995). "A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin." Nature **376**(6537): 249-253.

Homberg, U. and J. G. Hildebrand (1994). "Postembryonic development of gamma-aminobutyric acid-like immunoreactivity in the brain of the sphinx moth Manduca sexta." The Journal of comparative neurology **339**(1): 132-149.

Homem, C. C. and J. A. Knoblich (2012). "Drosophila neuroblasts: a model for stem cell biology." Development **139**(23): 4297-4310.

Homem, C. C., I. Reichardt, et al. (2013). "Long-term live cell imaging and automated 4D analysis of drosophila neuroblast lineages." PloS one **8**(11): e79588.

Hortsch, M., N. H. Patel, et al. (1990). "Drosophila neurotactin, a surface glycoprotein with homology to serine esterases, is dynamically expressed during embryogenesis." Development **110**(4): 1327-1340.

Horvitz, H. R. and I. Herskowitz (1992). "Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question." Cell **68**(2): 237-255.

Hosoya, T., K. Takizawa, et al. (1995). "glial cells missing: a binary switch between neuronal and glial determination in Drosophila." Cell **82**(6): 1025-1036.

Hsieh-Li, H. M., D. P. Witte, et al. (1995). "Gsh-2, a murine homeobox gene expressed in the developing brain." Mech Dev **50**(2-3): 177-186.

Hughes, C. L. and T. C. Kaufman (2002). "Exploring the myriapod body plan: expression patterns of the ten Hox genes in a centipede." Development **129**(5): 1225-1238.

Hummel, T., S. Attix, et al. (2002). "Temporal control of glial cell migration in the Drosophila eye requires gilgamesh, hedgehog, and eye specification genes." Neuron **33**(2): 193-203.

Hunt, P., J. Whiting, et al. (1991). "The branchial Hox code and its implications for gene regulation, patterning of the nervous system and head evolution." Development **Suppl 2**: 63-77.

Hyman, L. H. (1940). "The Invertebrates: Protozoa through Ctenophora". McGraw- Hill.

Ikuta, T., N. Yoshida, et al. (2004). "Ciona intestinalis Hox gene cluster: Its dispersed structure and residual colinear expression in development." Proc Natl Acad Sci U S A **101**(42): 15118-15123.

Imai, T., H. Sakano, et al. "Topographic mapping--the olfactory system." Cold Spring Harb Perspect Biol **2**(8): a001776.

Imai, T., T. Yamazaki, et al. (2009). "Pre-target axon sorting establishes the neural map topography." Science **325**(5940): 585-590.

Irimia, M., C. Pineiro, et al. "Conserved developmental expression of Fezf in chordates and Drosophila and the origin of the Zona Limitans Intrathalamica (ZLI) brain organizer." Evodevo **1**(1): 7.

Irvine, S. Q. and M. Q. Martindale (2000). "Expression patterns of anterior Hox genes in the polychaete Chaetopterus: correlation with morphological boundaries." Dev Biol **217**(2): 333-351.

Isshiki, T., B. Pearson, et al. (2001). "Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny." Cell **106**(4): 511-521.

Isshiki, T., M. Takeichi, et al. (1997). "The role of the msh homeobox gene during Drosophila neurogenesis: implication for the dorsoventral specification of the neuroectoderm." Development **124**(16): 3099-3109.

Ito, K., W. Awano, et al. (1997). "The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells." Development **124**(4): 761-771.

Ito, K. and T. Awasaki (2008). "Clonal unit architecture of the adult fly brain." Advances in experimental medicine and biology **628**: 137-158.

Ito, K. and Y. Hotta (1992). "Proliferation pattern of postembryonic neuroblasts in the brain of Drosophila melanogaster." Developmental biology **149**(1): 134-148.

Ito, M., N. Masuda, et al. (2013). "Systematic analysis of neural projections reveals clonal composition of the Drosophila brain." Current biology : CB **23**(8): 644-655.

Ito, K., Urban J., Technau, GM. (1995). "Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord". Roux's Arch. Dev. Biol **204**:284–307

Izergina, N., J. Balmer, et al. (2009). "Postembryonic development of transit amplifying neuroblast lineages in the Drosophila brain." Neural Dev **4**: 44.

Jacob, J., C. Maurange, et al. (2008). "Temporal control of neuronal diversity: common regulatory principles in insects and vertebrates?" Development **135**(21): 3481-3489.

Jacobs, D. K., N. Nakanishi, et al. (2007). "Evolution of sensory structures in basal metazoa." Integr Comp Biol **47**(5): 712-723.

Jacobs, J. R. and C. S. Goodman (1989). "Embryonic development of axon pathways in the Drosophila CNS. I. A glial scaffold appears before the first growth cones." The Journal of neuroscience : the official journal of the Society for Neuroscience **9**(7): 2402-2411.

Jacobs, J. R. and C. S. Goodman (1989). "Embryonic development of axon pathways in the Drosophila CNS. II. Behavior of pioneer growth cones." The Journal of neuroscience : the official journal of the Society for Neuroscience **9**(7): 2412-2422.

Jacobs, J. R., Y. Hiromi, et al. (1989). "Lineage, migration, and morphogenesis of longitudinal glia in the Drosophila CNS as revealed by a molecular lineage marker." Neuron **2**(6): 1625-1631.

Janssens, D. H., H. Komori, et al. (2014). "Earmuff restricts progenitor cell potential by attenuating the competence to respond to self-renewal factors." Development **141**(5): 1036-1046.

Jefferis, G. S., E. C. Marin, et al. (2001). "Target neuron prespecification in the olfactory map of Drosophila." Nature **414**(6860): 204-208.

Jefferis, G. S., R. M. Vyas, et al. (2004). "Developmental origin of wiring specificity in the olfactory system of Drosophila." Development **131**(1): 117-130.

Jenett, A., G. M. Rubin, et al. (2012). "A GAL4-driver line resource for Drosophila neurobiology." Cell reports **2**(4): 991-1001.

Jhaveri, D. and V. Rodrigues (2002). "Sensory neurons of the Atonal lineage pioneer the formation of glomeruli within the adult Drosophila olfactory lobe." Development **129**(5): 1251-1260.

Jiang, Y. and H. Reichert (2012). "Programmed cell death in type II neuroblast lineages is required for central complex development in the Drosophila brain." Neural development **7**: 3.

Jiang, Y. and H. Reichert (2013). "Analysis of neural stem cell self-renewal and differentiation by transgenic RNAi in Drosophila." Archives of biochemistry and biophysics **534**(1-2): 38-43.

Jiang, Y. and H. Reichert (2014). "Drosophila neural stem cells in brain development and tumor formation." Journal of neurogenetics **28**(3-4): 181-189.

Jones, B. W. (2001). "Glial cell development in the Drosophila embryo." BioEssays : news and reviews in molecular, cellular and developmental biology **23**(10): 877-887.

Jones, B. W., R. D. Fetter, et al. (1995). "glial cells missing: a genetic switch that controls glial versus neuronal fate." Cell **82**(6): 1013-1023.

Kambadur, R., K. Koizumi, et al. (1998). "Regulation of POU genes by castor and hunchback establishes layered compartments in the Drosophila CNS." Genes & development **12**(2): 246-260.

Kammermeier, L. and H. Reichert (2001). "Common developmental genetic mechanisms for patterning invertebrate and vertebrate brains." Brain Res Bull **55**(6): 675-682.

Kane, E. A., M. Gershow, et al. (2013). "Sensorimotor structure of Drosophila larva phototaxis." Proceedings of the National Academy of Sciences of the United States of America **110**(40): E3868-3877.

Kao, C. F., H. H. Yu, et al. (2012). "Hierarchical deployment of factors regulating temporal fate in a diverse neuronal lineage of the Drosophila central brain." Neuron **73**(4): 677-684.

Karcavich, R. and C. Q. Doe (2005). "Drosophila neuroblast 7-3 cell lineage: a model system for studying programmed cell death, Notch/Numb signaling, and sequential specification of ganglion mother cell identity." The Journal of comparative neurology **481**(3): 240-251.

Kay, L. M. and M. Stopfer (2006). "Information processing in the olfactory systems of insects and vertebrates." Semin Cell Dev Biol **17**(4): 433-442.

Keene, A. C. and S. G. Sprecher (2012). "Seeing the light: photobehavior in fruit fly larvae." Trends in neurosciences **35**(2): 104-110.

Kenyon, C. J., J. Austin, et al. (1997). "The dance of the Hox genes: patterning the anteroposterior body axis of Caenorhabditis elegans." Cold Spring Harb Symp Quant Biol **62**: 293-305.

Kittel, R. J., C. Wichmann, et al. (2006). "Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release." Science **312**(5776): 1051-1054.

Klaes, A., T. Menne, et al. (1994). "The Ets transcription factors encoded by the Drosophila gene pointed direct glial cell differentiation in the embryonic CNS." Cell **78**(1): 149-160.

Klambt, C. (1993). "The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells." Development **117**(1): 163-176.

Klambt, C. (2009). "Modes and regulation of glial migration in vertebrates and invertebrates." Nature reviews. Neuroscience **10**(11): 769-779.

Klambt, C. and C. S. Goodman (1991). "The diversity and pattern of glia during axon pathway formation in the Drosophila embryo." Glia **4**(2): 205-213.

Klämbt, C. and C. S. Goodman (1991). "Role of the midline glia and neurons in the formation of the axon commissures in the central nervous system of the Drosophila embryo." Annals of the New York Academy of Sciences **633**: 142-159.

Klämbt, C., J. R. Jacobs, et al. (1991). "The midline of the Drosophila central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance." Cell **64**(4): 801-815.

Knoblich, J. A. (2008). "Mechanisms of asymmetric stem cell division." Cell **132**(4): 583-597.

Kohsaka, H., S. Okusawa, et al. (2012). "Development of larval motor circuits in Drosophila." Development, growth & differentiation **54**(3): 408-419.

Koizumi, O. (2007). "Nerve ring of the hypostome in hydra: is it an origin of the central nervous system of bilaterian animals?" Brain Behav Evol **69**(2): 151-159.

Koizumi, O., M. Itazawa, et al. (1992). "Nerve ring of the hypostome in hydra. I. Its structure, development, and maintenance." J Comp Neurol **326**(1): 7-21.

Komiyama, T., L. B. Sweeney, et al. (2007). "Graded expression of semaphorin-1a cell-autonomously directs dendritic targeting of olfactory projection neurons." Cell **128**(2): 399-410.

Kotikova, E. A. and O. I. Raiikova (2008). "[Architectonics of the central nervous system in Acoela, Plathelminthes, and Rotifera]." Zh Evol Biokhim Fiziol **44**(1): 83-93.

Kourakis, M. J., V. A. Master, et al. (1997). "Conserved anterior boundaries of Hox gene expression in the central nervous system of the leech Helobdella." Dev Biol **190**(2): 284-300.

Kowalczyk, T., A. Pontious, et al. (2009). "Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex." Cerebral cortex **19**(10): 2439-2450.

Kriegstein, A. and A. Alvarez-Buylla (2009). "The glial nature of embryonic and adult neural stem cells." Annual review of neuroscience **32**: 149-184.

Kriks, S., G. M. Lanuza, et al. (2005). "Gsh2 is required for the repression of Ngn1 and specification of dorsal interneuron fate in the spinal cord." Development **132**(13): 2991-3002.

Kuert, P. A., V. Hartenstein, et al. (2014). "Neuroblast lineage identification and lineage-specific Hox gene action during postembryonic development of the subesophageal ganglion in the Drosophila central brain." Developmental biology **390**(2): 102-115.

Kumar, A., B. Bello, et al. (2009). "Lineage-specific cell death in postembryonic brain development of Drosophila." Development **136**(20): 3433-3442.

Kunz, T., K. F. Kraft, et al. (2012). "Origin of Drosophila mushroom body neuroblasts and generation of divergent embryonic lineages." Development **139**(14): 2510-2522.

Kurant, E., S. Axelrod, et al. (2008). "Six-microns-under acts upstream of Draper in the glial phagocytosis of apoptotic neurons." Cell **133**(3): 498-509.

Kurusu, M., T. Awasaki, et al. (2002). "Embryonic and larval development of the Drosophila mushroom bodies: concentric layer subdivisions and the role of fasciclin II." Development **129**(2): 409-419.

Kurusu, M., Y. Maruyama, et al. (2009). "A conserved nuclear receptor, Tailless, is required for efficient proliferation and prolonged maintenance of mushroom body progenitors in the Drosophila brain." Developmental biology **326**(1): 224-236.

Kusserow, A., K. Pang, et al. (2005). "Unexpected complexity of the Wnt gene family in a sea anemone." Nature **433**(7022): 156-160.

Lage, P., Y. N. Jan, et al. (1997). "Requirement for EGF receptor signalling in neural recruitment during formation of Drosophila chordotonal sense organ clusters." Current biology : CB **7**(3): 166-175.

Lanjuin, A., M. K. VanHoven, et al. (2003). "Otx/otd homeobox genes specify distinct sensory neuron identities in C. elegans." Dev Cell **5**(4): 621-633.

Larsen, C., D. Shy, et al. (2009). "Patterns of growth, axonal extension and axonal arborization of neuronal lineages in the developing Drosophila brain." Developmental biology **335**(2): 289-304.

Lattemann, M., A. Zierau, et al. (2007). "Semaphorin-1a controls receptor neuron-specific axonal convergence in the primary olfactory center of Drosophila." Neuron **53**(2): 169-184.

Lee, P. N., P. Callaerts, et al. (2003). "Cephalopod Hox genes and the origin of morphological novelties." Nature **424**(6952): 1061-1065.

Lee, T., A. Lee, et al. (1999). "Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast." Development **126**(18): 4065-4076.

Lee, T. and L. Luo (1999). "Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis." Neuron **22**(3): 451-461.

Lee, T., S. Marticke, et al. (2000). "Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in Drosophila." Neuron **28**(3): 807-818.

Leuzinger, S., F. Hirth, et al. (1998). "Equivalence of the fly orthodenticle gene and the human OTX genes in embryonic brain development of Drosophila." Development **125**(9): 1703-1710.

Levine, A. J. and A. H. Brivanlou (2007). "Proposal of a model of mammalian neural induction." Dev Biol **308**(2): 247-256.

Li, Q., T. S. Ha, et al. (2013). "Combinatorial rules of precursor specification underlying olfactory neuron diversity." Current biology : CB **23**(24): 2481-2490.

Li, W., Y. Pan, et al. (2009). "Morphological characterization of single fan-shaped body neurons in Drosophila melanogaster." Cell and tissue research **336**(3): 509-519.

Lichtneckert, R., B. Bello, et al. (2007). "Cell lineage-specific expression and function of the empty spiracles gene in adult brain development of Drosophila melanogaster." Development **134**(7): 1291-1300.

Lichtneckert, R., L. Nobs, et al. (2008). "Empty spiracles is required for the development of olfactory projection neuron circuitry in Drosophila." Development **135**(14): 2415-2424.

Lichtneckert, R. and H. Reichert (2005). "Insights into the urbilaterian brain: conserved genetic patterning mechanisms in insect and vertebrate brain development." Heredity **94**(5): 465-477.

Lichtneckert, R. and H. Reichert (2008). "Anteroposterior regionalization of the brain: genetic and comparative aspects." Adv Exp Med Biol **628**: 32-41.

Lichtneckert, R. & Reichert, H. (2007). "Origin and Evolution of the First Nervous System". Evolution of the Nervous System: *Evolution of Nervous Systems in Invertebrates* (Strausfeld, N. & Bullock, T., Eds.) 291-315, Elsevier.

Lin, C. Y., C. C. Chuang, et al. (2013). "A comprehensive wiring diagram of the protocerebral bridge for visual information processing in the Drosophila brain." Cell reports **3**(5): 1739-1753.

Lin, S., S. L. Lai, et al. (2010). "Lineage-specific effects of Notch/Numb signaling in post-embryonic development of the Drosophila brain." Development **137**(1): 43-51.

Lin, S. and T. Lee (2012). "Generating neuronal diversity in the Drosophila central nervous system." Developmental dynamics : an official publication of the American Association of Anatomists **241**(1): 57-68.

Little, S. C. and M. C. Mullins (2006). "Extracellular modulation of BMP activity in patterning the dorsoventral axis." Birth Defects Res C Embryo Today **78**(3): 224-242.

Liu, A. and A. L. Joyner (2001). "Early anterior/posterior patterning of the midbrain and cerebellum." Annu Rev Neurosci **24**: 869-896.

Liu, G., H. Seiler, et al. (2006). "Distinct memory traces for two visual features in the Drosophila brain." Nature **439**(7076): 551-556.

Loesel, R., D. R. Nassel, et al. (2002). "Common design in a unique midline neuropil in the brains of arthropods." Arthropod structure & development **31**(1): 77-91.

Lovick, J. K., K. T. Ngo, et al. (2013). "Postembryonic lineages of the Drosophila brain: I. Development of the lineage-associated fiber tracts." Developmental biology **384**(2): 228-257.

Lowe, C. J., M. Terasaki, et al. (2006). "Dorsoventral patterning in hemichordates: insights into early chordate evolution." PLoS Biol **4**(9): e291.

Lowe, C. J., M. Wu, et al. (2003). "Anteroposterior patterning in hemichordates and the origins of the chordate nervous system." Cell **113**(7): 853-865.

Lui, J. H., D. V. Hansen, et al. (2011). "Development and evolution of the human neocortex." Cell **146**(1): 18-36.

Lumsden, A. and R. Krumlauf (1996). "Patterning the vertebrate neuraxis." Science **274**(5290): 1109-1115.

Luo, L. and J. G. Flanagan (2007). "Development of continuous and discrete neural maps." Neuron **56**(2): 284-300.

Mackie, G. O. (2004). "Central neural circuitry in the jellyfish Aglantha: a model 'simple nervous system'." Neurosignals **13**(1-2): 5-19.

Malicki, J., L. C. Cianetti, et al. (1992). "A human HOX4B regulatory element provides head-specific expression in Drosophila embryos." Nature **358**(6384): 345-347.

Mallamaci, A., R. Iannone, et al. (1998). "EMX2 protein in the developing mouse brain and olfactory area." Mech Dev **77**(2): 165-172.

Marin, E. C., R. J. Watts, et al. (2005). "Developmentally programmed remodeling of the Drosophila olfactory circuit." Development **132**(4): 725-737.

Marlow, H. Q., M. Srivastava, et al. (2009). "Anatomy and development of the nervous system of Nematostella vectensis, an anthozoan cnidarian." Dev Neurobiol **69**(4): 235-254.

Martin, J. R., T. Raabe, et al. (1999). "Central complex substructures are required for the maintenance of locomotor activity in Drosophila melanogaster." Journal of comparative physiology. A, Sensory, neural, and behavioral physiology **185**(3): 277-288.

Masse, N. Y., G. C. Turner, et al. (2009). "Olfactory information processing in Drosophila." Current biology : CB **19**(16): R700-713.

Maurange, C. (2012). "Temporal specification of neural stem cells: insights from Drosophila neuroblasts." Current topics in developmental biology **98**: 199-228.

McDonald, J. A., S. Holbrook, et al. (1998). "Dorsoventral patterning in the Drosophila central nervous system: the vnd homeobox gene specifies ventral column identity." Genes Dev **12**(22): 3603-3612.

Meinertzhagen IA, Hanson TE. (1993). "The development of the optic lobe". The development of *Drosophila*. (Bate M, Martinez-Arias A.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1363–1492.

Meloche, S. and J. Pouyssegur (2007). "The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition." Oncogene **26**(22): 3227-3239.

Mieko Mizutani, C. and E. Bier (2008). "EvoD/Vo: the origins of BMP signalling in the neuroectoderm." Nat Rev Genet **9**(9): 663-677.

Miguel-Aliaga, I. and S. Thor (2004). "Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity." Development **131**(24): 6093-6105.

Miljkovic-Licina, M., S. Chera, et al. (2007). "Head regeneration in wild-type hydra requires de novo neurogenesis." Development **134**(6): 1191-1201.

Miljkovic-Licina, M., D. Gauchat, et al. (2004). "Neuronal evolution: analysis of regulatory genes in a first-evolved nervous system, the hydra nervous system." Biosystems **76**(1-3): 75-87.

Miller, A. A., R. Bernardoni, et al. (1998). "Positive autoregulation of the glial promoting factor glide/gcm." The EMBO journal **17**(21): 6316-6326.

Miya, T., K. Morita, et al. (1996). "An ascidian homologue of vertebrate BMPs-5-8 is expressed in the midline of the anterior neuroectoderm and in the midline of the ventral epidermis of the embryo." Mech Dev **57**(2): 181-190.

Miyata, T., D. Kawaguchi, et al. (2010). "Mechanisms that regulate the number of neurons during mouse neocortical development." Curr Opin Neurobiol **20**(1): 22-28.

Mizutani, C. M., N. Meyer, et al. (2006). "Threshold-dependent BMP-mediated repression: a model for a conserved mechanism that patterns the neuroectoderm." PLoS Biol **4**(10): e313.

Mizutani, C. M., Q. Nie, et al. (2005). "Formation of the BMP activity gradient in the Drosophila embryo." Dev Cell **8**(6): 915-924.

Moens, C. B. and V. E. Prince (2002). "Constructing the hindbrain: insights from the zebrafish." Dev Dyn **224**(1): 1-17.

Morimoto, A. M., K. C. Jordan, et al. (1996). "Pointed, an ETS domain transcription factor, negatively regulates the EGF receptor pathway in Drosophila oogenesis." Development **122**(12): 3745-3754.

Moroz, L. L. (2009). "On the independent origins of complex brains and neurons." Brain Behav Evol **74**(3): 177-190.

Morrison, S. J. and J. Kimble (2006). "Asymmetric and symmetric stem-cell divisions in development and cancer." Nature **441**(7097): 1068-1074.

Muller, P., N. Yanze, et al. (1999). "The homeobox gene Otx of the jellyfish Podocoryne carnea: role of a head gene in striated muscle and evolution." Dev Biol **216**(2): 582-594.

Munera, J., G. Cecena, et al. (2011). "Ets2 regulates colonic stem cells and sensitivity to tumorigenesis." Stem cells **29**(3): 430-439.

Mwinyi, A., X. Bailly, et al. "The phylogenetic position of Acoela as revealed by the complete mitochondrial genome of Symsagittifera roscoffensis." BMC Evol Biol **10**: 309.

Nassif, C., A. Noveen, et al. (1998). "Embryonic development of the Drosophila brain. I. Pattern of pioneer tracts." The Journal of comparative neurology **402**(1): 10-31.

Nassif, C., A. Noveen, et al. (2003). "Early development of the Drosophila brain: III. The pattern of neuropile founder tracts during the larval period." The Journal of comparative neurology **455**(4): 417-434.

Nederbragt, A. J., P. te Welscher, et al. (2002). "Novel and conserved roles for orthodenticle/ otx and orthopedia/ otp orthologs in the gastropod mollusc Patella vulgata." Dev Genes Evol **212**(7): 330-337.

Neumuller, R. A., C. Richter, et al. (2011). "Genome-wide analysis of self-renewal in Drosophila neural stem cells by transgenic RNAi." Cell stem cell **8**(5): 580-593.

Neuser, K., T. Triphan, et al. (2008). "Analysis of a spatial orientation memory in Drosophila." Nature **453**(7199): 1244-1247.

Newsome, T. P., B. Asling, et al. (2000). "Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics." Development **127**(4): 851-860.

Nieuwenhuys, R. (2002). "Deuterostome brains: synopsis and commentary." Brain Res Bull **57**(3-4): 257-270.

Nomaksteinsky, M., E. Rottinger, et al. (2009). "Centralization of the deuterostome nervous system predates chordates." Curr Biol **19**(15): 1264-1269.

Noveen, A., A. Daniel, et al. (2000). "Early development of the Drosophila mushroom body: the roles of eyeless and dachshund." Development **127**(16): 3475-3488.

O'Neill, E. M., I. Rebay, et al. (1994). "The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway." Cell **78**(1): 137-147.

Oland, L. A. and L. P. Tolbert (1996). "Multiple factors shape development of olfactory glomeruli: insights from an insect model system." Journal of neurobiology **30**(1): 92-109.

Oland, L. A. and L. P. Tolbert (2003). "Key interactions between neurons and glial cells during neural development in insects." Annual review of entomology **48**: 89-110.

Oliver, G., A. Mailhos, et al. (1995). "Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development." Development **121**(12): 4045-4055.

Pabst, O., H. Herbrand, et al. (1998). "Nkx2-9 is a novel homeobox transcription factor which demarcates ventral domains in the developing mouse CNS." Mech Dev **73**(1): 85-93.

Pan, Y., Y. Zhou, et al. (2009). "Differential roles of the fan-shaped body and the ellipsoid body in Drosophila visual pattern memory." Learning & memory **16**(5): 289-295.

Panov, A., A. (1959). "Struture of the insect brain at succesive stages of postemrbyonic development. II. The central body". Entomol. Rev. **38**:276-83.

Park, Y., V. Filippov, et al. (2002). "Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency." Development **129**(2): 493-503.

Parkefelt, L. and P. Ekstrom (2009). "Prominent system of RFamide immunoreactive neurons in the rhopalia of box jellyfish (Cnidaria: Cubozoa)." J Comp Neurol **516**(3): 157-165.

Parkefelt, L., C. Skogh, et al. (2005). "Bilateral symmetric organization of neural elements in the visual system of a coelenterate, Tripedalia cystophora (Cubozoa)." J Comp Neurol **492**(3): 251-262.

Patel, N. H., P. M. Snow, et al. (1987). "Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila." Cell **48**(6): 975-988.

Pattyn, A., A. Vallstedt, et al. (2003). "Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors." Genes Dev **17**(6): 729-737.

Pauls, D., M. Selcho, et al. (2010). "Drosophila larvae establish appetitive olfactory memories via mushroom body neurons of embryonic origin." The Journal of neuroscience : the official journal of the Society for Neuroscience **30**(32): 10655-10666.

Pearson, B. J. and C. Q. Doe (2003). "Regulation of neuroblast competence in Drosophila." Nature **425**(6958): 624-628.

Pearson, B. J. and C. Q. Doe (2004). "Specification of temporal identity in the developing nervous system." Annual review of cell and developmental biology **20**: 619-647.

Pera, E. M. and M. Kessel (1998). "Demarcation of ventral territories by the homeobox gene NKX2.1 during early chick development." Dev Genes Evol **208**(3): 168-171.

Pereanu, W. and V. Hartenstein (2006). "Neural lineages of the Drosophila brain: a three-dimensional digital atlas of the pattern of lineage location and projection at the late larval stage." J Neurosci **26**(20): 5534-5553.

Pereanu, W., A. Kumar, et al. (2010). "Development-based compartmentalization of the Drosophila central brain." The Journal of comparative neurology **518**(15): 2996-3023.

Pereanu, W., D. Shy, et al. (2005). "Morphogenesis and proliferation of the larval brain glia in Drosophila." Developmental biology **283**(1): 191-203.

Perez, S. E. and H. Steller (1996). "Migration of glial cells into retinal axon target field in Drosophila melanogaster." Journal of neurobiology **30**(3): 359-373.

Peterson, K. J., J. B. Lyons, et al. (2004). "Estimating metazoan divergence times with a molecular clock." Proc Natl Acad Sci U S A **101**(17): 6536-6541.

Pfeiffer, B. D., A. Jenett, et al. (2008). "Tools for neuroanatomy and neurogenetics in Drosophila." Proc Natl Acad Sci U S A **105**(28): 9715-9720.

Philippe, H., H. Brinkmann, et al. "Acoelomorph flatworms are deuterostomes related to Xenoturbella." Nature **470**(7333): 255-258.

Philippe, H., H. Brinkmann, et al. (2007). "Acoel flatworms are not platyhelminthes: evidence from phylogenomics." PLoS One **2**(1): e717.

Pielage, J. and C. Klambt (2001). "Glial cells aid axonal target selection." Trends in neurosciences **24**(8): 432-433.

Pignoni, F. and S. L. Zipursky (1997). "Induction of Drosophila eye development by decapentaplegic." Development **124**(2): 271-278.

Piraino, S., G. Zega, et al. "Complex neural architecture in the diploblastic larva of Clava multicornis (Hydrozoa, Cnidaria)." J Comp Neurol **519**(10): 1931-1951.

Poeck, B., S. Fischer, et al. (2001). "Glial cells mediate target layer selection of retinal axons in the developing visual system of Drosophila." Neuron **29**(1): 99-113.

Poeck, B., T. Triphan, et al. (2008). "Locomotor control by the central complex in Drosophila-An analysis of the tay bridge mutant." Developmental neurobiology **68**(8): 1046-1058.

Pontious, A., T. Kowalczyk, et al. (2008). "Role of intermediate progenitor cells in cerebral cortex development." Developmental neuroscience **30**(1-3): 24-32.

Popov, A. V., A. I. Peresleni, et al. (2005). "The role of the flabellar and ellipsoid bodies of the central complex of the brain of Drosophila melanogaster in the control of courtship behavior and communicative sound production in males." Neuroscience and behavioral physiology **35**(7): 741-750.

Popperl, H., M. Bienz, et al. (1995). "Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx." Cell **81**(7): 1031-1042.

Prieto-Godino, L. L., S. Diegelmann, et al. (2012). "Embryonic origin of olfactory circuitry in Drosophila: contact and activity-mediated interactions pattern connectivity in the antennal lobe." PLoS biology **10**(10): e1001400.

Prokop, A. and I. A. Meinertzhagen (2006). "Development and structure of synaptic contacts in Drosophila." Seminars in cell & developmental biology **17**(1): 20-30.

Prokop, A. and G. M. Technau (1991). "The origin of postembryonic neuroblasts in the ventral nerve cord of Drosophila melanogaster." Development **111**(1): 79-88.

Qiu, M., K. Shimamura, et al. (1998). "Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development." Mech Dev **72**(1-2): 77-88.

Quiquand, M., N. Yanze, et al. (2009). "More constraint on ParaHox than Hox gene families in early metazoan evolution." Dev Biol **328**(2): 173-187.

Ramaekers, A., E. Magnenat, et al. (2005). "Glomerular maps without cellular redundancy at successive levels of the Drosophila larval olfactory circuit." Current biology : CB **15**(11): 982-992.

Rangarajan, R., H. Courvoisier, et al. (2001). "Dpp and Hedgehog mediate neuron-glia interactions in Drosophila eye development by promoting the proliferation and motility of subretinal glia." Mechanisms of development **108**(1-2): 93-103.

Rangarajan, R., Q. Gong, et al. (1999). "Migration and function of glia in the developing Drosophila eye." Development **126**(15): 3285-3292.

Ray, K. and V. Rodrigues (1995). "Cellular events during development of the olfactory sense organs in Drosophila melanogaster." Developmental biology **167**(2): 426-438.

Ready, D. F., T. E. Hanson, et al. (1976). "Development of the Drosophila retina, a neurocrystalline lattice." Developmental biology **53**(2): 217-240.

Rebay, I., F. Chen, et al. (2000). "A genetic screen for novel components of the Ras/Mitogen-activated protein kinase signaling pathway that interact with the yan gene of Drosophila identifies split ends, a new RNA recognition motif-containing protein." Genetics **154**(2): 695-712.

Reichert, H. (2009). "Evolutionary conservation of mechanisms for neural regionalization, proliferation and interconnection in brain development." Biol Lett **5**(1): 112-116.

Reichert, H. (2011). "Drosophila neural stem cells: cell cycle control of self-renewal, differentiation, and termination in brain development." Results and problems in cell differentiation **53**: 529-546.

Reichert, H. and A. Simeone (2001). "Developmental genetic evidence for a monophyletic origin of the bilaterian brain." Philos Trans R Soc Lond B Biol Sci **356**(1414): 1533-1544.

Rentzsch, F., C. Guder, et al. (2007). "An ancient chordin-like gene in organizer formation of Hydra." Proc Natl Acad Sci U S A **104**(9): 3249-3254.

Rhinn, M. and M. Brand (2001). "The midbrain--hindbrain boundary organizer." Curr Opin Neurobiol **11**(1): 34-42.

Ridgel, A. L., B. E. Alexander, et al. (2007). "Descending control of turning behavior in the cockroach, Blaberus discoidalis." Jo comp physio. **193**(4): 385-402.

Riebli, N., G. Viktorin, et al. (2013). "Early-born neurons in type II neuroblast lineages establish a larval primordium and integrate into adult circuitry during central complex development in Drosophila." Neural development **8**: 6.

Rodriguez Moncalvo, V. G. and A. R. Campos (2009). "Role of serotonergic neurons in the Drosophila larval response to light." BMC neuroscience **10**: 66.

Rogers, E. M., C. A. Brennan, et al. (2005). "Pointed regulates an eye-specific transcriptional enhancer in the Drosophila hedgehog gene, which is required for the movement of the morphogenetic furrow." Development **132**(21): 4833-4843.

Rogulja-Ortmann, A., K. Luer, et al. (2007). "Programmed cell death in the embryonic central nervous system of Drosophila melanogaster." Development **134**(1): 105-116.

Roux, P. P. and J. Blenis (2004). "ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions." Microbiology and molecular biology reviews : MMBR **68**(2): 320-344.

Rowitch, D. H. and A. P. McMahon (1995). "Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of Wnt-1 and En-1." Mech Dev **52**(1): 3-8.

Rubin, G. M., H. C. Chang, et al. (1997). "Signal transduction downstream from Ras in Drosophila." Cold Spring Harbor symposia on quantitative biology **62**: 347-352.

Rubinfeld, H. and R. Seger (2005). "The ERK cascade: a prototype of MAPK signaling." Molecular biotechnology **31**(2): 151-174.

Rusten, T. E., R. Cantera, et al. (2002). "The role of TGF beta signaling in the formation of the dorsal nervous system is conserved between Drosophila and chordates." Development **129**(15): 3575-3584.

Ryan, J. F., M. E. Mazza, et al. (2007). "Pre-bilaterian origins of the Hox cluster and the Hox code: evidence from the sea anemone, Nematostella vectensis." PLoS One **2**(1): e153.

Saalfeld, S., A. Cardona, et al. (2009). "CATMAID: collaborative annotation toolkit for massive amounts of image data." Bioinformatics **25**(15): 1984-1986.

Saalfeld, S., A. Cardona, et al. (2010). "As-rigid-as-possible mosaicking and serial section registration of large ssTEM datasets." Bioinformatics **26**(12): i57-63.

Saalfeld, S., R. Fetter, et al. (2012). "Elastic volume reconstruction from series of ultra-thin microscopy sections." Nature methods **9**(7): 717-720.

Saina, M., G. Genikhovich, et al. (2009). "BMPs and chordin regulate patterning of the directive axis in a sea anemone." Proc Natl Acad Sci U S A **106**(44): 18592-18597.

Saini, N. and H. Reichert (2012). "Neural stem cells in Drosophila: molecular genetic mechanisms underlying normal neural proliferation and abnormal brain tumor formation." Stem cells international **2012**: 486169.

Sanchez-Soriano, N., G. Tear, et al. (2007). "Drosophila as a genetic and cellular model for studies on axonal growth." Neural development **2**: 9.

Sanes, J. R. and S. L. Zipursky "Design principles of insect and vertebrate visual systems." Neuron **66**(1): 15-36.

Sasai, Y., B. Lu, et al. (1995). "Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus." Nature **377**(6551): 757.

Satterlie, R. A. "Do jellyfish have central nervous systems?" J Exp Biol **214**(Pt 8): 1215-1223.

Scantlebury, N., X. L. Zhao, et al. (2010). "The Drosophila gene RanBPM functions in the mushroom body to regulate larval behavior." PloS one **5**(5): e10652.

Schilling, T. F. and R. D. Knight (2001). "Origins of anteroposterior patterning and Hox gene regulation during chordate evolution." Philos Trans R Soc Lond B Biol Sci **356**(1414): 1599-1613.

Schindelin, J: "Fiji is just ImageJ – Batteries Included". Luxembourg: ImageJ User and Developer Conference; 6-7 November; 208

Schindelin, J., I. Arganda-Carreras, et al. (2012). "Fiji: an open-source platform for biological-image analysis." Nature methods **9**(7): 676-682.

Schlosser, G. and K. Ahrens (2004). "Molecular anatomy of placode development in Xenopus laevis." Dev Biol **271**(2): 439-466.

Schmid, A., A. Chiba, et al. (1999). "Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets." Development **126**(21): 4653-4689.

Schmidt, H., C. Rickert, et al. (1997). "The embryonic central nervous system lineages of Drosophila melanogaster. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm." Developmental biology **189**(2): 186-204.

Schober, M., I. Rebay, et al. (2005). "Function of the ETS transcription factor Yan in border cell migration." Development **132**(15): 3493-3504.

Seibert, J., D. Volland, et al. (2009). "Ems and Nkx6 are central regulators in dorsoventral patterning of the Drosophila brain." Development **136**(23): 3937-3947.

Semmler, H., M. Chiodin, et al. "Steps towards a centralized nervous system in basal bilaterians: insights from neurogenesis of the acoel Symsagittifera roscoffensis." Dev Growth Differ **52**(8): 701-713.

Sen, S., S. Biagini, et al. (2014). "Orthodenticle is required for the development of olfactory projection neurons and local interneurons in Drosophila." Biology open **3**(8): 711-717.

Sen, S., B. Hartmann, et al. "Expression and function of the empty spiracles gene in olfactory sense organ development of Drosophila melanogaster." Development **137**(21): 3687-3695.

Shimamura, K., D. J. Hartigan, et al. (1995). "Longitudinal organization of the anterior neural plate and neural tube." Development **121**(12): 3923-3933.

Silies, M., Y. Yuva, et al. (2007). "Glial cell migration in the eye disc." The Journal of neuroscience : the official journal of the Society for Neuroscience **27**(48): 13130-13139.

Silva, B., N. I. Goles, et al. (2014). "Serotonin receptors expressed in Drosophila mushroom bodies differentially modulate larval locomotion." PloS one **9**(2): e89641.

Simeone, A., M. Gulisano, et al. (1992). "Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex." Embo J **11**(7): 2541-2550.

Skeath, J. B. and S. Thor (2003). "Genetic control of Drosophila nerve cord development." Current opinion in neurobiology **13**(1): 8-15.

Smith, K. M., L. Gee, et al. (1999). "CnOtx, a member of the Otx gene family, has a role in cell movement in hydra." Dev Biol **212**(2): 392-404.

Song, W., M. Onishi, et al. (2007). "Peripheral multidendritic sensory neurons are necessary for rhythmic locomotion behavior in Drosophila larvae." Proceedings of the National Academy of Sciences of the United States of America **104**(12): 5199-5204.

Sonnenfeld, M. J. and J. R. Jacobs (1995). "Macrophages and glia participate in the removal of apoptotic neurons from the Drosophila embryonic nervous system." The Journal of comparative neurology **359**(4): 644-652.

Sousa-Nunes, R., L. L. Yee, et al. (2011). "Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in Drosophila." Nature **471**(7339): 508-512.

Soustelle, L. and A. Giangrande (2007). "Novel gcm-dependent lineages in the postembryonic nervous system of Drosophila melanogaster." Dev Dyn **236**(8): 2101-2108.

Spindler, S. R. and V. Hartenstein (2010). "The Drosophila neural lineages: a model system to study brain development and circuitry." Development genes and evolution **220**(1-2): 1-10.

Spindler, S. R., I. Ortiz, et al. (2009). "Drosophila cortex and neuropile glia influence secondary axon tract growth, pathfinding, and fasciculation in the developing larval brain." Developmental biology **334**(2): 355-368.

Sprecher, S. G., H. Reichert, et al. (2007). "Gene expression patterns in primary neuronal clusters of the Drosophila embryonic brain." Gene expression patterns : GEP **7**(5): 584-595.

Steinmetz, P. R., R. P. Kostyuchenko, et al. "The segmental pattern of otx, gbx, and Hox genes in the annelid Platynereis dumerilii." Evol Dev **13**(1): 72-79.

Steinmetz, P. R., R. Urbach, et al. "Six3 demarcates the anterior-most developing brain region in bilaterian animals." Evodevo **1**(1): 14.

Stocker, R. F. (2008). "Design of the larval chemosensory system." Advances in experimental medicine and biology **628**: 69-81.

Strausfeld N. (1976). Atlas of an Insect Brain. Springer

Strausfeld, N. J. (2009). "Brain organization and the origin of insects: an assessment." Proceedings. Biological sciences / The Royal Society **276**(1664): 1929-1937.

Strausfeld, N. J., L. Hansen, et al. (1998). "Evolution, discovery, and interpretations of arthropod mushroom bodies." Learn Mem **5**(1-2): 11-37.

Strauss, R. (2002). "The central complex and the genetic dissection of locomotor behaviour." Current opinion in neurobiology **12**(6): 633-638.

Strauss, R. and M. Heisenberg (1993). "A higher control center of locomotor behavior in the Drosophila brain." The Journal of neuroscience : the official journal of the Society for Neuroscience **13**(5): 1852-1861.

Struhl, G. and K. Basler (1993). "Organizing activity of wingless protein in Drosophila." Cell **72**(4): 527-540.

Studer, M., A. Gavalas, et al. (1998). "Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning." Development **125**(6): 1025-1036.

Studer, M., A. Lumsden, et al. (1996). "Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1." Nature **384**(6610): 630-634.

Sussel, L., O. Marin, et al. (1999). "Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum." Development **126**(15): 3359-3370.

Suzuki, Y., M. D. Yandell, et al. (1999). "A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in Caenorhabditis elegans." Development **126**(2): 241-250.

Sweeney, L. B., Y. H. Chou, et al. (2011). "Secreted semaphorins from degenerating larval ORN axons direct adult projection neuron dendrite targeting." Neuron **72**(5): 734-747.

Sweeney, L. B., A. Couto, et al. (2007). "Temporal target restriction of olfactory receptor neurons by Semaphorin-1a/PlexinA-mediated axon-axon interactions." Neuron **53**(2): 185-200.

Sweeney, L. B. and L. Luo "'Fore brain: a hint of the ancestral cortex." Cell **142**(5): 679-681.

Technau, G. M., C. Berger, et al. (2006). "Generation of cell diversity and segmental pattern in the embryonic central nervous system of Drosophila." Developmental dynamics : an official publication of the American Association of Anatomists **235**(4): 861-869.

Technau, U., S. Rudd, et al. (2005). "Maintenance of ancestral complexity and non-metazoan genes in two basal cnidarians." Trends Genet **21**(12): 633-639.

Technau, U. and R. E. Steele "Evolutionary crossroads in developmental biology: Cnidaria." Development **138**(8): 1447-1458.

Thum, A. S., B. Leisibach, et al. (2011). "Diversity, variability, and suboesophageal connectivity of antennal lobe neurons in D. melanogaster larvae." The Journal of comparative neurology **519**(17): 3415-3432.

Tissot, M., N. Gendre, et al. (1997). "Larval chemosensory projections and invasion of adult afferents in the antennal lobe of Drosophila." Journal of neurobiology **32**(3): 281-297.

Tix, S., E. Eule, et al. (1997). "Glia in the chiasms and medulla of the Drosophila melanogaster optic lobes." Cell and tissue research **289**(3): 397-409.

Tix, S., J. S. Minden, et al. (1989). "Pre-existing neuronal pathways in the developing optic lobes of Drosophila." Development **105**(4): 739-746.

Tomer, R., A. S. Denes, et al. "Profiling by image registration reveals common origin of annelid mushroom bodies and vertebrate pallium." Cell **142**(5): 800-809.

Tomlinson, A. (1985). "The cellular dynamics of pattern formation in the eye of Drosophila." Journal of embryology and experimental morphology **89**: 313-331.

Tomlinson, A. (1988). "Cellular interactions in the developing Drosophila eye." Development **104**(2): 183-193.

Tomsa, J. M. and J. A. Langeland (1999). "Otx expression during lamprey embryogenesis provides insights into the evolution of the vertebrate head and jaw." Dev Biol **207**(1): 26-37.

Traganos, F., Z. Darzynkiewicz, et al. (1977). "Simultaneous staining of ribonucleic and deoxyribonucleic acids in unfixed cells using acridine orange in a flow cytofluorometric system." The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society **25**(1): 46-56.

Treisman, J. E. (2013). "Retinal differentiation in Drosophila." Wiley interdisciplinary reviews. Developmental biology **2**(4): 545-557.

Triphan, T., B. Poeck, et al. (2010). "Visual targeting of motor actions in climbing Drosophila." Current biology : CB **20**(7): 663-668.

Truman, J. W. and M. Bate (1988). "Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster." Developmental biology **125**(1): 145-157.

Truman, J. W., W. Moats, et al. (2010). "Role of Notch signaling in establishing the hemilineages of secondary neurons in Drosophila melanogaster." Development **137**(1): 53-61.

Truman, J. W., H. Schuppe, et al. (2004). "Developmental architecture of adult-specific lineages in the ventral CNS of Drosophila." Development **131**(20): 5167-5184.

Tsuji, T., E. Hasegawa, et al. (2008). "Neuroblast entry into quiescence is regulated intrinsically by the combined action of spatial Hox proteins and temporal identity factors." Development **135**(23): 3859-3869.

Umesono, Y., K. Watanabe, et al. (1999). "Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes." Dev Genes Evol **209**(1): 31-39.

Unhavaithaya, Y. and T. L. Orr-Weaver (2012). "Polyploidization of glia in neural development links tissue growth to blood-brain barrier integrity." Genes & development **26**(1): 31-36.

Urbach, R. (2007). "A procephalic territory in Drosophila exhibiting similarities and dissimilarities compared to the vertebrate midbrain/hindbrain boundary region." Neural Dev **2**: 23.

Urbach, R. and G. M. Technau (2003). "Early steps in building the insect brain: neuroblast formation and segmental patterning in the developing brain of different insect species." Arthropod structure & development **32**(1): 103-123.

Urbach, R. and G. M. Technau (2004). "Neuroblast formation and patterning during early brain development in Drosophila." BioEssays : news and reviews in molecular, cellular and developmental biology **26**(7): 739-751.

Urbach, R. and G. M. Technau (2008). "Dorsoventral patterning of the brain: a comparative approach." Adv Exp Med Biol **628**: 42-56.

Valerius, M. T., H. Li, et al. (1995). "Gsh-1: a novel murine homeobox gene expressed in the central nervous system." Dev Dyn **203**(3): 337-351.

Vandendries, E. R., D. Johnson, et al. (1996). "orthodenticle is required for photoreceptor cell development in the Drosophila eye." Dev Biol **173**(1): 243-255.

Varnam, C. J., R. Strauss, et al. (1996). "Larval behavior of Drosophila central complex mutants: interactions between no bridge, foraging, and Chaser." Journal of neurogenetics **11**(1-2): 99-115.

Vieille-Grosjean, I., P. Hunt, et al. (1997). "Branchial HOX gene expression and human craniofacial development." Dev Biol **183**(1): 49-60.

Viktorin, G., N. Riebli, et al. (2011). "Multipotent neural stem cells generate glial cells of the central complex through transit amplifying intermediate progenitors in Drosophila brain development." Developmental biology **356**(2): 553-565.

Vincent, S., J. L. Vonesch, et al. (1996). "Glide directs glial fate commitment and cell fate switch between neurones and glia." Development **122**(1): 131-139.

Voas, M. G. and I. Rebay (2004). "Signal integration during development: insights from the Drosophila eye." Developmental dynamics : an official publication of the American Association of Anatomists **229**(1): 162-175.

Wada, H., J. Garcia-Fernandez, et al. (1999). "Colinear and segmental expression of amphioxus Hox genes." Dev Biol **213**(1): 131-141.

Wada, H., H. Saiga, et al. (1998). "Tripartite organization of the ancestral chordate brain and the antiquity of placodes: insights from ascidian Pax-2/5/8, Hox and Otx genes." Development **125**(6): 1113-1122.

Wada, H. and N. Satoh (2001). "Patterning the protochordate neural tube." Curr Opin Neurobiol **11**(1): 16-21.

Wagh, D. A., T. M. Rasse, et al. (2006). "Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila." Neuron **49**(6): 833-844.

Wallace, J. A., F. Li, et al. (2013). "Ets2 in tumor fibroblasts promotes angiogenesis in breast cancer." PloS one **8**(8): e71533.

Wang, W., X. Chen, et al. (1996). "Msx3: a novel murine homologue of the Drosophila msh homeobox gene restricted to the dorsal embryonic central nervous system." Mech Dev **58**(1-2): 203-215.

Wang, Y. C., J. S. Yang, et al. (2014). "Drosophila intermediate neural progenitors produce lineage-dependent related series of diverse neurons." Development **141**(2): 253-258.

Wassarman, K. M., M. Lewandoski, et al. (1997). "Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function." Development **124**(15): 2923-2934.

Wasylyk, C., A. P. Bradford, et al. (1997). "Conserved mechanisms of Ras regulation of evolutionary related transcription factors, Ets1 and Pointed P2." Oncogene **14**(8): 899-913.

Watanabe, H., T. Fujisawa, et al. (2009). "Cnidarians and the evolutionary origin of the nervous system." Dev Growth Differ **51**(3): 167-183.

Watts, R. J., O. Schuldiner, et al. (2004). "Glia engulf degenerating axons during developmental axon pruning." Current biology : CB **14**(8): 678-684.

Wegerhoff, R., Breidbach, O. (1992). "Structure and development of the larval central complex in a holometabolous insect, the beetle Tenebrio molitor. Cell Tissue Res. **268**:341-59.

Wegerhoff, R., O. Breidbach, et al. (1996). "Development of locustatachykinin immunopositive neurons in the central complex of the beetle Tenebrio molitor." Journal of comparative neurology **375**(1): 157-166.

Weigmann, K., R. Klapper et al. (2003). „FlyMove – a new way to look at development of Drosophila. Trends in Genetics **19**: 310.

Weiss, J. B., T. Von Ohlen, et al. (1998). "Dorsoventral patterning in the Drosophila central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity." Genes Dev **12**(22): 3591-3602.

Weng, M., K. L. Golden, et al. (2010). "dFezf/Earmuff maintains the restricted developmental potential of intermediate neural progenitors in Drosophila." Developmental cell **18**(1): 126-135.

Weng, M. and C. Y. Lee (2011). "Keeping neural progenitor cells on a short leash during Drosophila neurogenesis." Current opinion in neurobiology **21**(1): 36-42.

White, K. and H. Steller (1995). "The control of apoptosis in Drosophila." Trends in cell biology **5**(2): 74-78.

Williams, L. (1975). "Anatomical studies of the insect central nervous system: a ground plan of the midbrain and an introduction to the central complex in the locust, *Schistocerca gregaria* (Orthoptera)". J. Zool. (Lond.) **176**, 67-86.

Wilkinson, D. G., S. Bhatt, et al. (1989). "Segmental expression of Hox-2 homoeobox-containing genes in the developing mouse hindbrain." Nature **341**(6241): 405-409.

Winberg, M. L., S. E. Perez, et al. (1992). "Generation and early differentiation of glial cells in the first optic ganglion of Drosophila melanogaster." Development **115**(4): 903-911.

Wistrand, M., L. Kall, et al. (2006). "A general model of G protein-coupled receptor sequences and its application to detect remote homologs." Protein Sci **15**(3): 509-521.

Wolff, T. and D. F. Ready (1991). "The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave." Development **113**(3): 841-850.

Wong, A. M., J. W. Wang, et al. (2002). "Spatial representation of the glomerular map in the Drosophila protocerebrum." Cell **109**(2): 229-241.

Wong, D. C., J. K. Lovick, et al. (2013). "Postembryonic lineages of the Drosophila brain: II. Identification of lineage projection patterns based on MARCM clones." Developmental biology **384**(2): 258-289.

Wurst, W. and L. Bally-Cuif (2001). "Neural plate patterning: upstream and downstream of the isthmic organizer." Nat Rev Neurosci **2**(2): 99-108.

Xiao, Q., H. Komori, et al. (2012). "klumpfuss distinguishes stem cells from progenitor cells during asymmetric neuroblast division." Development **139**(15): 2670-2680.

Xie, T., E. Kawase, et al. (2005). "Intimate relationships with their neighbors: tales of stem cells in Drosophila reproductive systems." Devl dyn Am Asso Ana **232**(3): 775-790.

Xiong, W. C., H. Okano, et al. (1994). "repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system." Genes & development **8**(8): 981-994.

Yang, J. S., T. Awasaki, et al. (2013). "Diverse neuronal lineages make stereotyped contributions to the Drosophila locomotor control center, the central complex." The Journal of comparative neurology **521**(12): 2645-2662, Spc2641.

Yanze, N., J. Spring, et al. (2001). "Conservation of Hox/ParaHox-related genes in the early development of a cnidarian." Dev Biol **236**(1): 89-98.

Yasugi, T., A. Fischer, et al. (2014). "A regulatory transcriptional loop controls proliferation and differentiation in Drosophila neural stem cells." PloS one **9**(5): e97034.

Yoon, S. and R. Seger (2006). "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions." Growth factors **24**(1): 21-44.

Young, J. M. and J. D. Armstrong (2010). "Building the central complex in Drosophila: the generation and development of distinct neural subsets." The Journal of comparative neurology **518**(9): 1525-1541.

Young, J. M. and J. D. Armstrong (2010). "Structure of the adult central complex in Drosophila: organization of distinct neuronal subsets." The Journal of comparative neurology **518**(9): 1500-1524.

Younossi-Hartenstein, A., P. Green, et al. (1997). "Control of early neurogenesis of the Drosophila brain by the head gap genes tll, otd, ems, and btd." Dev Biol **182**(2): 270-283.

Younossi-Hartenstein, A., C. Nassif, et al. (1996). "Early neurogenesis of the Drosophila brain." The Journal of comparative neurology **370**(3): 313-329.

Younossi-Hartenstein, A., B. Nguyen, et al. (2006). "Embryonic origin of the Drosophila brain neuropile." The Journal of comparative neurology **497**(6): 981-998.

Younossi-Hartenstein, A., P. M. Salvaterra, et al. (2003). "Early development of the Drosophila brain: IV. Larval neuropile compartments defined by glial septa." The Journal of comparative neurology **455**(4): 435-450.

Yu, F., C. T. Kuo, et al. (2006). "Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology." Neuron **51**(1): 13-20.

Yu, H. H., T. Awasaki, et al. (2013). "Clonal development and organization of the adult Drosophila central brain." Current biology : CB **23**(8): 633-643.

Yu, H. H., C. F. Kao, et al. (2010). "A complete developmental sequence of a Drosophila neuronal lineage as revealed by twin-spot MARCM." PLoS biology **8**(8).

Yu, J. K., Y. Satou, et al. (2007). "Axial patterning in cephalochordates and the evolution of the organizer." Nature **445**(7128): 613-617.

Zacharias, D., Williams J., Meier T. and Reichert H. (1993). "Neurogenesis in the insect brain: cellular identification and molecular characterization of brain neuroblasts in the grasshopper embryo". Development **118**, 941-955.

Zheng, X., C. T. Zugates, et al. (2006). "Baboon/dSmad2 TGF-beta signaling is required during late larval stage for development of adult-specific neurons." The EMBO journal **25**(3): 615-627.

Zhou, B., Williams, D. W. et al., (2009). " Temporal patterns of broad isoform expression during the development of neuronal lineages in Drosophila. " Neural Development **4** (39): 1749-8104

Zhu, H., T. Hummel, et al. (2006). "Dendritic patterning by Dscam and synaptic partner matching in the Drosophila antennal lobe." Nat Neurosci **9**(3): 349-355.

Zhu, S., S. Barshow, et al. (2011). "Ets transcription factor Pointed promotes the generation of intermediate neural progenitors in Drosophila larval brains." Proceedings of the National Academy of Sciences of the United States of America **108**(51): 20615-20620.

Zhu, S., J. Wildonger, et al. (2012). "The bHLH repressor Deadpan regulates the self-renewal and specification of Drosophila larval neural stem cells independently of Notch." PloS one **7**(10): e46724.

Appendix

CURRICULUM VITAE
Nadia Riebli

Home address Hauptstrasse 28
4102 Binningen

Place of Birth Liestal, Switzerland
Date of birth 19th April 1986
Nationality Swiss
Marital status unmarried

Education

2011- 2014 Ph.D studies in *Drosophila* brain development at the Biozentrum, University of Basel; in the group of Prof. Heinrich Reichert
Thesis title: "Contribution of transit amplifying type-II NB lineages to central complex primordium formation and optic lobe glial cells in *Drosophila melanogaster*"

2009- 2011 *M.Sc. in Animal Biology*, University of Basel
2006- 2009 *B.Sc. in Biology*, University of Basel
2001 - 2005 *Matura*, Gymnasium Liestal, Baselland, Switzerland

List of Publications

RIEBLI, N., VIKTORIN, G. & REICHERT, H. 2013. Early-born neurons in type II neuroblast lineages establish a larval primordium and integrate into adult circuitry during central complex development in Drosophila. Neural development, 8, 6.

VIKTORIN, G., RIEBLI, N., POPKOVA, A., GIANGRANDE, A. & REICHERT, H. 2011. Multipotent neural stem cells generate glial cells of the central complex through transit amplifying intermediate progenitors in Drosophila brain development. Developmental biology, 356, 553-65.

VIKTORIN, G., RIEBLI, N. & REICHERT, H. 2013. A multipotent transit-amplifying neuroblast lineage in the central brain gives rise to optic lobe glial cells in Drosophila. Developmental biology, 379, 182-94.