Postembryonic Development of Amplifying Neuroblast Lineages in the *Drosophila* Brain: Proliferation, Differentiation and Projection Patterns.

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Prof. Dr. E. Parlow

to my Mother

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Summary

We identified a novel mode of neurogenesis in the larval brain of *Drosophila* that involves the amplification of neuroblast proliferation through intermediate progenitors (IPs). These intermediate neural progenitors are generated by asymmetric division of a subset of the *Drosophila* brain neuroblasts, which we refer to as dorsomedial neuroblasts (DM neuroblasts). These neuroblasts divide asymmetrically to self-renew, but unlike the other brain neuroblasts do not segregate the cell fate determinant Prospero to the daughter cells. As a result, in contrast to conventional ganglion mother cells (GMC), intermediate progenitors undergo multiple divisions and express molecular markers of self-renewing neuroblasts. The novel IPs described here have remarkable similarities to the IPs that have been identified recently in mammalian brain development.

We analyzed the type and fate of cells generated in the DM lineages. With a combination of neuronal and glial cell markers we show that the DM lineages generate not only neurons but also glial cells. The DM neuroblasts thus represent the first identified multipotent precursor cells in the fly brain during postembryonic development. We also show that the adult-specific neurons of each DM lineage form several spatially separated axonal fascicles some of which project along larval brain commissural structures which are primordia of future adult midline neuropil. By taking advantage of a DM-specific Gal4 reporter line we identify and follow DM-derived neuronal cells into early pupal stages and demonstrate that neurons of the DM lineages make a major contribution to the developing central complex, in that the numerous columnar elements are likely to be DM lineage-derived. These findings suggest that the amplification of proliferation which characterizes DM lineages may be an important requirement for generating the large number of neurons required in highly complex neuropil structures such as the central complex in the *Drosophila* brain.

Index

1. Introduction

Time flies like an arrow. Fruit flies like a banana.

Groucho Marx

So many flies, so upside down! Did sickness or some silly clown
Cause to be so impeded a *course of study, newly seeded?*
The moral of this little tale is *that it's quite beyond the pale*
To experiment with noxious fumes where *flies are kept in closed up rooms.*

Prof. Nicholas Strausfeld "Earlier days"

1.1 Introduction into *Drosophila* neurogenesis

In insects, the brain consists of a supraoesophageal ganglion that can be subdivided into the protocerebral, deutocerebral, and tritocerebral neuromeres, and a suboesophageal ganglion that is subdivided into the mandibular, maxillary, and labial neuromeres. The developing ventral nerve cord extends posteriorly from the suboesophageal ganglion into the trunk [1]. In this work, we will use the term 'brain' equivalent to the supraoesophageal ganglion.

The brain of insects and some other arthropod taxa is formed by a unique type of stem cell-like progenitor cell called a neuroblast [2]. Neural progenitors of this type are not found in vertebrates or other invertebrate phyla [3]. The *Drosophila* brain is shaped during three developmental phases that include the embryonic, larval and pupal phase [4] (Fig. 1.1).
In the early embryo, a population of neuroblasts (primary neuroblasts) delaminates from a special neurogenic region, the neuroectoderm, to undergo sequential cycles of self-renewing divisions.

Each neuroblast produces a highly invariant lineage of cells that, at least temporarily, stay together and extend processes that fasciculate into a common bundle (primary axon tract). These postmitotic neural cells (primary glia and neurons) differentiate into the fully functional larval brain. After a phase of mitotic dormancy where most brain neuroblasts persist in a cell-cycle arrested state, the same neuroblasts that had proliferated to form primary neurons during the embryonic period become active again during larval period and produce a stereotyped set of secondary lineages that finally give rise to the adult brain [2, 5].

During larval life the adult-specific progeny of each neuroblast accumulates in a growing cluster of immature neurons that extend fasciculated neurites (secondary lineage axon tracts) close to the neuropil but wait until metamorphosis to complete their extension to adult specific synaptic targets [6-8]. Whereas the primary, larval-functional progeny of each NB show a high degree of phenotypic diversity [9, 10], the adult-specific cells in a given lineage are remarkably similar and typically project to only one or two initial targets in the larva [7, 8, 11]. During the pupal phase

(metamorphosis) the adult brain forms by neuronal remodeling of larval functional neurons and final morphogenesis of adult-specific neurons [7, 12, 13].

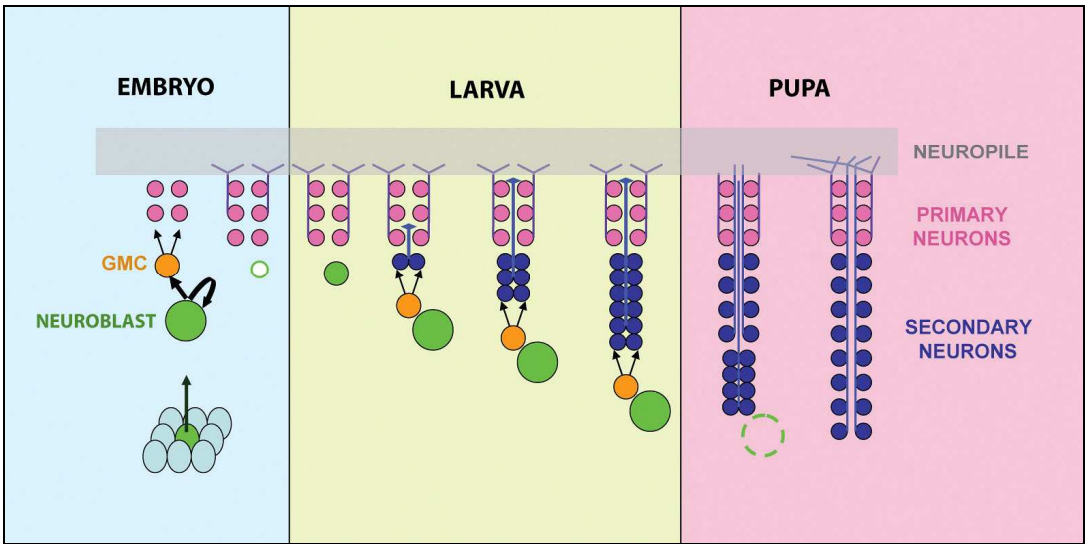

Fig1.1 Neurogenesis in *Drosophila*. Two phases of neurogenesis, separated by a quiescent state of the neuroblast, produce primary and secondary neurons of the same lineage. Whereas the primary progeny of the neuroblast quickly differentiates into functional neurons of the larva, cell of the secondary lineage wait until metamorphosis to fully extend their projections (see text for more detail).

Each neuroblast gives birth to a series of clonal progeny during neurogenesis. Thereby, the *Drosophila* brain is composed of groups of clonally related cells. A number of recent publications have addressed the analysis of the developmental origin of adult brain units taking advantage of the MARCM (Mosaic Analysis with a Repressible Cell Marker) system [7, 13-16]. Upon heat-shock induced mitotic recombination in the neuroblasts all clonally related cells are labelled with a membrane-bound marker, and therefore, projection patterns of neurons can be studied in the context of overall brain architecture. Furthermore, clonal mutant analysis enables us to study homozygous mutant clones in a heterozygous background. In addition, fine neuronal morphology or the timing of developmental processes can be studied at single-cell clone resolution with MARCM [16].

The *Drosophila* nervous system is made up by two major cell types, neurons and glial cells. Neurons play the leading role in processing and transmitting information, while

glia play the supporting role, nourishing and insulating neurons. Both neurons and glia are generated from multipotent neural progenitors or pure neural, or glial stem cells in Drosophila and vertebrates [17-25]. While much effort has been made to identify neural progenitors and the mechanisms controlling their fates, the mechanisms that control whether neural progenitor cells will adopt glial vs. neuronal cell fates are only beginning to be understood.

In the mature *Drosophila* brain cell bodies of neurons and glial cells form an outer layer, or cortex, around an inner neuropil that consists of highly branched axons and dendrites, as well as synapses formed in between these processes. Dendritic and axonal branches are assembled into neuropil compartments [3]. Glial sheaths envelop the cortex surface (surface glia), groups of neuronal cell bodies (cortex glia) and the neuropil (neuropil glia) [26-28]. A recently published neuroblast lineage atlas of developing adult brain in the late larva subdivides each brain hemisphere into approximately 100 clonal lineages, each represented by a fasciculated neurite bundle that forms an invariant pattern in the neuropil [11]. Therefore, the question arises how does each family of clonally related neurons contribute to the formation of the adult neural circuits?

1.2 Asymmetric cell division in the *Drosophila* central nervous system

The central nervous system (CNS) of *Drosophila* develops from the stem-cell like precursors, neuroblasts [29]. The definitive feature of a stem cell is an ability to divide asymmetrically to self-renew, generating at the same time an identical copy of itself and a more differentiated progeny [30]. The term “asymmetric cell division” is used to refer to any division in which sister cells have different fates, which means they have differences in size, shape, morphology, gene expression pattern, biochemical features, or the number of subsequent cell divisions undergone by the two daughter cells [30]. In *Drosophila* neurogenesis, asymmetric cell division is the major mechanism for generating cell-fate diversity. There are two mechanisms by which diversity can be achieved with respect to the neuroblast: intrinsic and extrinsic. When an intrinsic mechanism is used, regulators of self-renewal are localized asymmetrically during mitosis so that they are inherited by only one of the two daughter cells [31, 32]. Intrinsic mechanisms are more common for stem cells during development (for example, *Drosophila* neuroblasts). Alternatively, in case of extrinsic regulation, the stem cell is in close contact with the stem cell niche and depends on this contact for maintaining the potential to self-renew [33]. Niche mechanisms are more common in adult stem cells, for example, ovarian stem cells.

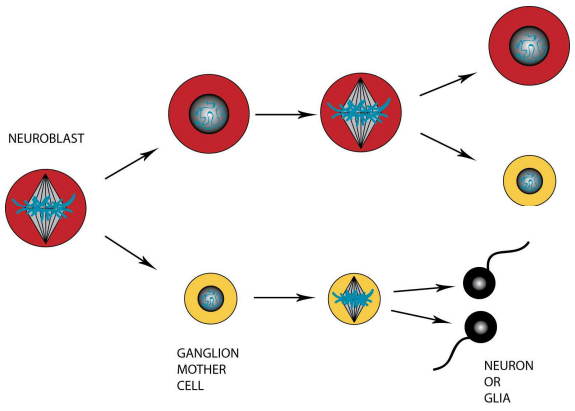

Fig 1.2 Asymmetric division of neuroblast in Drosophila (see text for details)

Drosophila neuroblasts are well-studied example for the intrinsically induced asymmetric cell division. After the delamination from the embryonic neuroectoderm, neuroblasts start to divide asymmetrically, generating two cells in each division. The first cell is a neuroblast, which continues to divide in a stem cell-like fashion. The

second cell is called ganglion mother cell, which undergo one final division to produce pair of neuronal cells (neurons or glia). (Fig. 1.2)

The different fate of two neuroblast daughter cells is induced by the unequal segregation of several proteins into one of the two daughter cells. Before mitosis, the cell fate determinants of the so-called apical complex segregate to the apical side of the cell cortex (these proteins are: Par-3/Bazooka, Par-6, atypical PKC (aPKC), Inscuteable, Partner of Inscuteable, Gαi, Mud). [31, 34-38]. Another group of determinants called basal complex and segregated to the basal side of the cell (these are: Numb, Prospero and Prospero mRNA, Partner of Numb, Miranda, Staufen) (Fig. 1.3) [39-44].

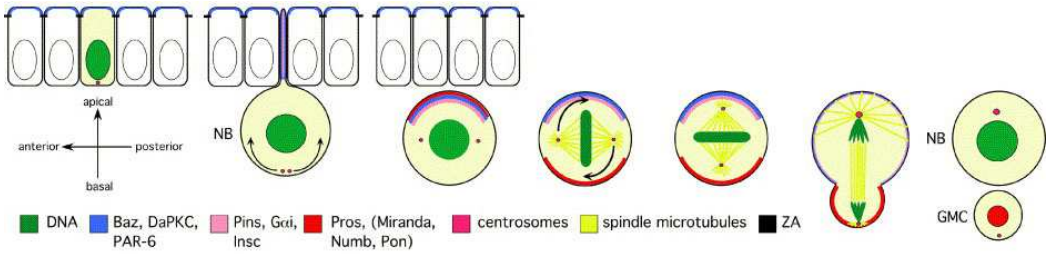

Fig. 1.3 Asymmetric division of neuroblasts in Drosophila

The panel shows a simulated time course of delamination and division of a single NB in the ventral neurogenic region of the *Drosophila* embryo. The subcellular localization of several polarity regulators, cell fate determinants and their adaptor proteins is indicated in different colors. For simplicity, in the epithelium and in the delaminating NB only the subcellular localization of the PAR/aPKC complex is indicated. The red color represents the localization of Pros. In meta- through anaphase, Miranda, Numb and Pon are localized in a very similar fashion, but there are differences in the localization of these proteins in pro- and late telophase. For abbreviations, see text (Modified after Wodarz A and Huttner WB, 2003).

Bazooka/Par-3, Par-6 and aPKC make up an evolutionarily conserved core protein complex that is involved in cell polarity in a variety of contexts (reviewed in [36]). Inscuteable is an adapter protein that recruits Pins (a receptor-independent regulator of Gαi) via its GoLoco domain [38, 45, 46] to the Bazooka/Par-3, Par-6, aPKC complex. However, recent live imaging experiments [47, 48] have suggested that Insc, Pins and Gαi act differently in embryonic and larval neuroblasts.

The apical complex does not influence cell fate directly, but it guides three fundamental aspects of neuroblast asymmetric cell division: regulating the orientation

of the mitotic spindle along the apical–basal axis, determining the strong daughter cell size asymmetry between neuroblast and GMC, and targeting cell-fate determinants of the basal complex to the basal side of the cell during asymmetric cell division[49]. Numb acts as a tissue-specific repressor of the Notch pathway. It binds to α-Adaptin and might control the intracellular trafficking of Notch intermediates [44]. In the Numb mutant larval brains, the neuroblasts overproliferate and form a tumor-like phenotype [37, 50].

Prospero (Pros) is a transcription factor which segregates asymmetrically in neuroblasts. Pros is cytoplasmic in neuroblasts during interphase, and it only enters the nucleus once asymmetrically segregated into the GMC [31]. When Pros is mutated in embryonic neuroblasts, the GMC does not exit a cell cycle and continues to divide. Pros contains a homeodomain and binds upstream of over 700 genes, acting as a transcriptional activator for genes which are involved in differentiation and as an inhibitor for genes involved in neuroblast self-renewal [42]. The asymmetric segregation of Pros and Numb is mediated by two adaptor proteins called Miranda and Pon (Partner of Numb) [31]. Miranda is a coiled-coil protein that binds to Pros. Miranda also binds to the RNA binding protein Staufen which in turn transports *pros* RNA but is not required for cell-fate specification in neuroblasts. Miranda acts as an obligatory molecular adaptor that connects Pros and Staufen to the machinery for asymmetric protein localization.The adaptor protein for Numb is a coiled-coil protein called Pon. Pon binds to Numb and assists the asymmetric localization of Numb but is not required during late stages of mitosis [51].

In the mammalian brain, neural stem cells divide asymmetrically and often amplify the number of progeny they generate via symmetrically dividing intermediate progenitors [52-54]. In the CNS of *Drosophila* neuroblasts undergo sequential cycles of self-renewing divisions, dividing asymmetrically to produce ganglion mother cells which in turn divide once more to generate two neural progeny [2]. Therefore, the logical question arises whether specific neural stem cell-like neuroblasts in the brain of *Drosophila* might also amplify neuronal proliferation by generating symmetrically dividing intermediate progenitors and by what mechanisms it can be achieved?

1.3 Glial development in the *Drosophila* central nervous system

In the *Drosophila* central nervous system about 10% of cells are of glial nature. In recent years it has become clear that glia contribute to virtually all aspects of nervous system development and function. Glia help to shape the fly's nervous system by presenting growth cones with permissive migrational substrates, determine neuronal survival via trophic interactions and pruning axons during metamorphosis [55-59]. Besides developmental functions, glia have non-developmental functions too: it contributes to the blood-brain barrier, metabolic and homeostatic functions and potentially modulatory roles during synaptic transmission [60, 61].

To determine further roles of glia in the adult brain we need a systematic characterisation of glia diversity and development. Cell lineage analysis techniques have been used to analyse most of the embryonic neuroblast lineages. These studies have elucidated the cellular composition and the specific nature of each neuroblast lineage and the morphologies of cells they consist of [17, 20, 21, 62]. Not much is known about glial postembryonic development. According to position, features and presumable function in the adult brain glial cells are subdivided into five classes: perineurial surface glia, subperineurial surface glia, cortex glia, ensheathing neuropil-associated glia and astrocyte-like neuropil-associated glia. Recent studies demonstrated that distinct glial types derive from different precursors and that most adult perineurial, ensheathing and astrocyte-like glia are produced after embryogenesis. Perineurial glial cells are made locally on the brain surface. In contrast, the wide-spread ensheathing and astrocyte-like glia derive from specific brain regions [27].

In contrast to adult-specific glia, embryonic glia is very well studied. Embryonic glia is subdvided according to its origin into two classes: lateral and midline glia. Lateral glia derives from the neuroectoderm. Midline glia derives from mesectoderm [1, 17]. Differentiation of almost all embryonic glia except for midline glia is promoted by expression of *glial cell missing/gcm2* gene complex [63-66]. Fly *gcm* gene encodes a transcription factor, that is transiently expressed in all lateral glia. *gcm2* is a gene homologous to *gcm*, but displays weaker and delayed expression [67, 68]. Lack of

gcm/gcm2 causes the complete loss of all lateral glial cells, in gain-of-function conditions presumptive neurons transformed into glia (Fig. 1.4) [63-66].

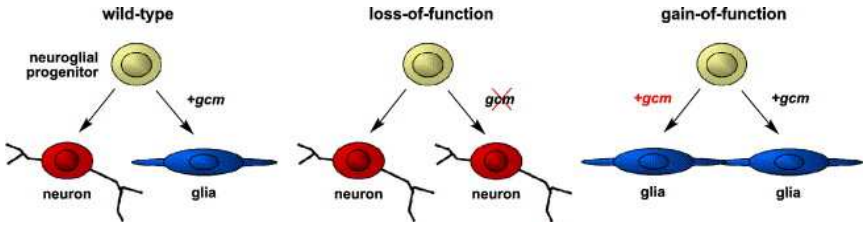

Fig 1.4 *gcm* acts as a binary switch for glia versus neurons in *Drosophila.* Phenotypes are shown for a neural progenitor that gives rise to a neuron and a glia in a wild-type animal, *gcm* loss-of-function mutant animal, and *gcm* gain-of-function mutant animal in which a transgenic construct drives ectopic *gcm* expression (red text) in presumptive neurons. Expression of *gcm* induces glial cell fate (modified after Jones et al., 1995).

Not much is known about the postembryonic requirement of *gcm*. Recent study demonstrated that there are novel lineages of postembryonic-born glia in the ventral ganglia which require *gcm* [69]. It was also shown that the progenitors of surface perineurial glia require *gcm* during embryonic stage but do not need it postembryonically to generate glial cells [27].

In the *Drosophila* embryonic CNS glial cells are known to be generated either from glioblasts (GB), which produce exclusively glia (e.g. embryonic anterior GB [70]) or from multipotent precursors, neuroglioblasts (NGB) (Fig 1.5). There are two types of multipotent neuroblasts known to date. For the first type (e.g. NGB6-4T and NGB5-6) it has been demonstrated that the early bifurcation of the glial versus neuronal sublineages takes place during the first division after embryonic delamination from the neuroectoderm. *gcm* is expressed asymmetrically in the glial sublineage, and the decision is made on the level of the neuroglioblast [71]. The second type of neuroglioblasts proliferation (e.g. embryonic NB1-1A) involves Notch acting upstream of a *gcm.* NB1-1A first produces neurogenic GMC that gives rise to a pair of neurons. During next three divisions NB1-1A produces three ganglion mother cells each of which divide asymmetrically producing sibling neuron and glial cell. In this case, *gcm* acts as an effector of Notch signalling during sibling cell fate specification [62].

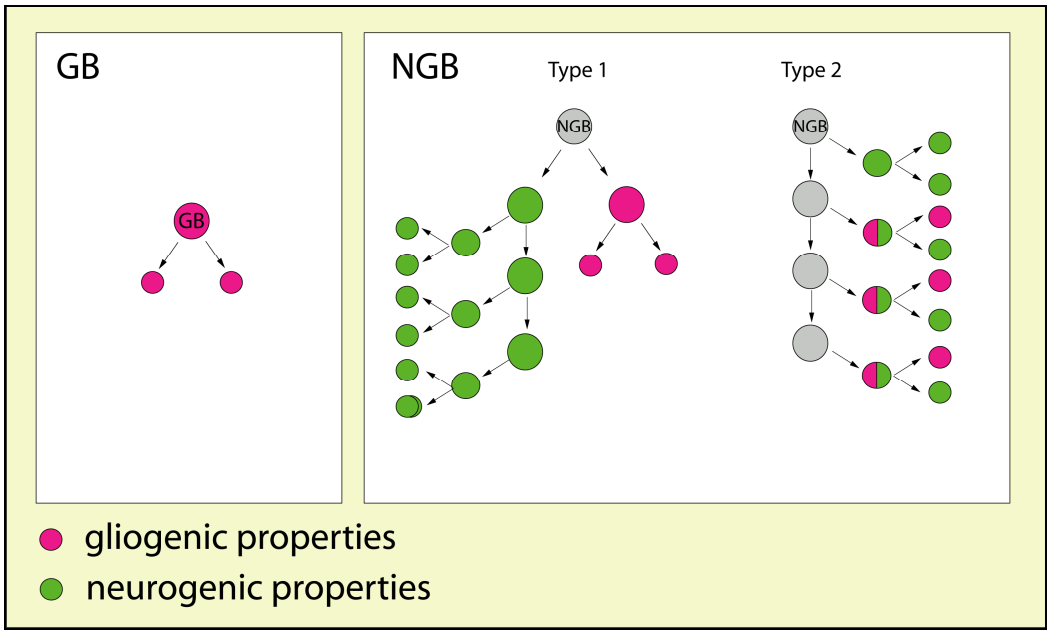

Fig 1.5 Three modes of gliogenesis. Glia cells originate from different types of progenitors, glioblasts or neuroglioblast. GBs possess only gliogenic properties and give rise exclusively to glial cells. NGBs generate glial and neuronal components in a mixed lineage. Two different types of NGBs exist (see text for details) (modified after Udolph et al., 2001).

Recent studies demonstrated that *gcm* transcription is controlled by a combination of tissue-specific and lineage-specific modular elements,but not by glial subtype-specific elements, nor by elements that control expression in progenitors that undergo a specific mode of division [72].

gcm is thought to initiate gliogenesis through the transcriptional activation of glial-specific target genes. These potential target genes include the glial-specific transcription factors encoded by the *reversed polarity (repo), pointed, tramtrack (ttk)* and *loco* genes (Fig. 1.6). The *repo* gene encodes a homeodomain transcription factor that is expressed in all lateral glial cells [73]. Transient expression of *gcm* is followed by maintained expression of *repo*, which appears to control only terminal glial differentiation. *pointed* promotes different aspects of glial cell differentiation, and is required for the expression of several glial markers [74]. *ttk* acts to repress neuronal differentiation and inhibits the expression of the pan-neural bHLH genes *asense* and *deadpan*, which promote the neuronal potential of neural progenitors[75]. Results of other studies suggest that repo may also cooperate with ttk to suppress neuronal fates [76]. loco gene encodes a family member of the Regulators of G-Protein Signaling

(RGS) proteins expressed in lateral glia. A *cis*-regulatory DNA element of *loco* can direct glial-specific expression of a reporter gene in vivo [77].

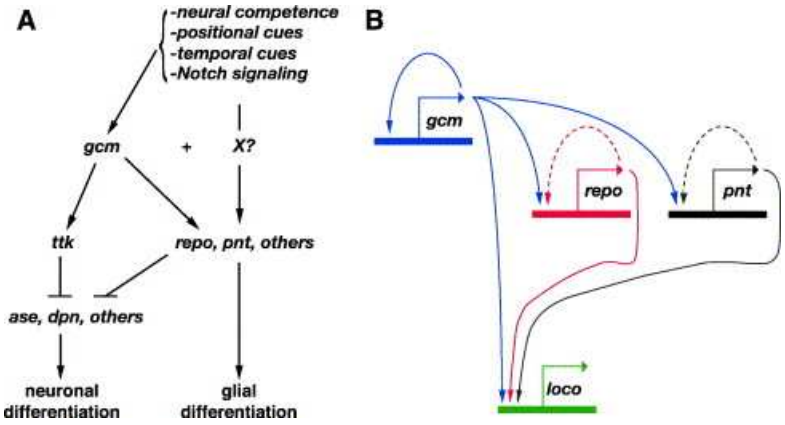

Fig. 1.6 Transcriptional regulatory networks controlling gliogenesis in *Drosophila*. (A) Summary of *gcm* pathway. *gcm* transcription is regulated by multiple inputs in different neural lineages. *gcm* initiates glial cell development by the simultaneous activation of glial differentiation and repression of neuronal differentiation. Additional neural factors (*X*) may be required to activate glial fate. Glial differentiation is promoted by the factors *repo*, *pointed* (*pnt*), and others. Neuronal differentiation is blocked by *tramtrack* (*ttk*) through the repression of neural factors such as *asense* (*ase*) and *deadpan* (*dpn*). *repo* may be required as a co-factor for neuronal repression (see text for additional detail). (B) Circuit diagram for the transcriptional regulation of the glial-specific gene *loco*. *gcm* cooperates with downstream factors *repo* and *pnt* to initiate and maintain *loco* expression. *gcm* autoregulates to boost its own expression. Dashed lines represent hypothetical autofeedback loops regulating *repo* and *pnt*. Transient expression of *gcm* activates the circuit; *loco* expression is maintained by *repo* and *pnt*. (modified after Jones et al., 2005).

1.4 Central complex in the *Drosophila* brain.

A recently published neuroblast lineage atlas of developing adult brain in the late larva subdivides each brain hemisphere into approximately 100 clonal lineages, each represented by a fasciculated neurite bundle that forms an invariant pattern in the neuropil [11]. Therefore, the question arises how does each family of clonally related neurons contribute to the formation of the adult neural circuits?

The central complex is a prominent midline neuropil complex of adult insect brains. Its gross structure is quite similar even amongst species from diverse habitats [78, 79]. The central complex is a putative center related to different functions ranging from locomotor control to visual information processing [80-84].

Central complex consists of four substructures: the ellipsoid body, the fan-shaped body, the protocerebral bridge and the paired noduli (Fig. 1.7). The fan-shaped body is the largest of the other parts and has a shape of a saucer. It is subdivided into a dorsal and ventral part, has 6 horizontal layers and 8 vertical segments which made of columnar and tangential arborisations of different lineages. The ellipsoid body is situated anteriorly to the fan-shaped body and consists of anterior and the posterior rings. Ventral to the fan-shaped body lie two noduli, which are roughly spherical and segmented into two subunits. The protocerebral bridge looks like the handlebar of a bicycle and lies at the dorso-posterior margin of the brain. It is composed of 16 glomeruli, 8 on each side of the midline. Closely associated with the central complex are two accessory areas: the lateral accessory lobes and the bulb, also called the lateral complex [85, 86].

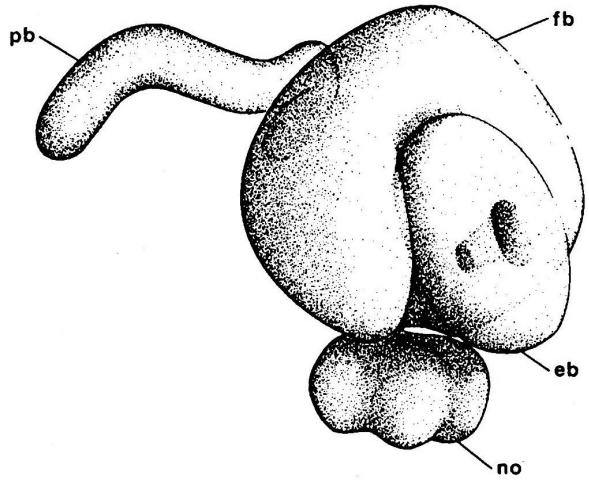

Fig. 1.7 The four substructures of central complex: protocerebral bridge (pb), fa-shaped body (fb), ellipsoid body (eb), and noduli (no).

Neurons of the central complex were studied in different insect species including *Drosophila* [86, 87]. Based on the analysis of the Golgi-stained brain preparations one characteristic features of the neurons of the central complex were described. First, they have no more than three branching regions. Another characteristic feature is that the most neurons of the central complex belong to one of two categories: large-field or small-field neurons. A large-field neuron typically arborizes in only a single substructure and links it to one or two central brain regions outside the central complex. In contrast, small-field neurons connect small domains of substructures. Some cells connect two domains in the same substructure. The majority of the small-field cells are intrinsic to the central complex [86].

It was suggested that central complex is built of clonal units. Nevertheless, it was demonstrated only partially. Several clonal units were mentioned very shortly for all the substructures, but their lineages were never identified [88].

1.5 This Thesis

The overall goal of the research study done here was to analyze developmental features of one of the lineage groups of *Drosophila* larval brain: we identify them here as dorsomedial (DM) lineages.

In the first part of this thesis, results are presented that provide cellular and molecular evidence for a new mode of neurogenesis that involves the amplification of neuroblast proliferation through intermediate progenitors in the larval brain of *Drosophila*. Together, these DM neuroblast lineages comprise over 5000 adult-specific neural cells and thus represent a substantial part of the larval, and possibly adult, brain. However, currently there is no information available about the structure or function of any of the neural cells in these DM lineages. We used MARCM-based clonal analysis together with immunocytochemical labeling techniques to investigate the type and fate of neural cells generated in the DM lineages.

In the second part of this thesis, results are presented that further investigate the development of the progeny of DM lineages. Our findings provide cellular and molecular evidence for the fact that DM neuroblasts are multipotent progenitors; they thus represent the first identified progenitor cells in the fly brain that have neuroglioblast functions during postembryonic development. We analyzed the projection pattern of DM-lineages at high resolution. Our results demonstrate that the adult specific neurons of the DM lineages arborize widely in the brain and also make a major contribution to the developing central complex. These findings suggest that the amplification of proliferation which characterizes DM lineages may be an important requirement for generating the large number of neurons required in highly complex neuropil structures such as the central complex in the *Drosophila* brain.

2 Amplification of neural stem cell proliferation by interemediate progenitor cells in *Drosophila* brain development

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2.1 Summary

In the mammalian brain, neural stem cells divide asymmetrically and often amplify the number of progeny they generate via symmetrically dividing intermediate progenitors. Here we investigate whether specific neural stem cell-like neuroblasts in the brain of *Drosophila* might also amplify neuronal proliferation by generating symmetrically dividing intermediate progenitors. Cell lineage-tracing and genetic marker analysis show that remarkably large neuroblast lineages exist in the dorsomedial larval brain of *Drosophila*. These lineages are generated by brain neuroblasts that divide asymmetrically to self renew but, unlike other brain neuroblasts, do not segregate the differentiating cell fate determinant Prospero to their smaller daughter cells. These daughter cells continue to express neuroblast-specific molecular markers and divide repeatedly to produce neural progeny, demonstrating that they are proliferating intermediate progenitors. The proliferative divisions of these intermediate progenitors have novel cellular and molecular features; they are morphologically symmetrical, but molecularly asymmetrical in that key differentiating cell fate determinants are segregated into only one of the two daughter cells.

Our findings provide cellular and molecular evidence for a new mode of neurogenesis in the larval brain of *Drosophila* that involves the amplification of neuroblast proliferation through intermediate progenitors. This type of neurogenesis bears remarkable similarities to neurogenesis in the mammalian brain, where neural stem cells as primary progenitors amplify the number of progeny they generate through generation of secondary progenitors. This suggests that key aspects of neural stem cell biology might be conserved in brain development of insects and mammals.

2.2 Introduction

Neural stem cells are primary precursors that have the ability to renew themselves at each division such that one of the two daughter cells retains stem cell identity, while the other enters a program of differentiation and contributes to a continuous supply of neural cell types. Understanding how neural stem cells maintain their pluripotent state and how their progeny differentiate into distinct neural fates is of central importance for understanding nervous system development (for recent reviews, see [52-54]). Neural stem cells must exert a tight control over proliferative divisions so as to generate the appropriate number of neural progeny necessary to populate the nervous system but not to produce so many self-renewing daughters that neoplastic overgrowth occurs [89]. Therefore, a better comprehension of the mechanisms that control the behavior of neuronal stem cells and their progeny may also be important for understanding brain tumors [90, 91].

The *Drosophila* central nervous system is an excellent simple model system for analyzing the molecular mechanisms that control neural stem cell divisions (for recent reviews, see [32, 92]). *Drosophila* neural stem cells, called neuroblasts (NBs), delaminate as single cells from the neuroectoderm and undergo repeated asymmetric cell divisions, each of which self-renew the NB while producing a smaller neural progenitor cell called a ganglion mother cell (GMC). Compared to the NB, the GMC adopts a radically opposite fate and undergoes a single neurogenic division to produce two cells that exit the cell cycle and differentiate (reviewed in [93-95]). During embryogenesis, each NB produces a lineage of 10–20 primary neural cells that contribute to the functional circuitry of the larva. Following a period of quiescence, most NBs resume their asymmetric mode of proliferative divisions during post-embryonic development and generate the lineage-related clusters of secondary adult-specific neurons that make up the bulk of the adult central brain and thoracic ganglia [2, 5, 8].

Mechanisms involved in NB division and neural proliferation during embryogenesis have been studied in great detail (reviewed in [31, 32, 96, 97]). NB divisions are known to be molecularly as well as morphologically asymmetric, and a number of key intrinsic and extrinsic factors that control the asymmetrical and self-renewing

divisions of these NBs have been identified. Among these, a central role is played by molecular polarity cues that establish the apico-basal polarity of the NB and enable the asymmetric segregation of localized cell-fate determinants from the NB to the GMCs at each asymmetric cell division. Although considerable insight has been attained into the mechanisms by which NB polarity is established and maintained, little is known about the function of the proteins that are asymmetrically localized to the GMC. The best characterized of these fate determinants is the homeodomain protein Prospero, which is synthesized in the NB and localized at the cell cortex in a polarized manner. Upon segregation to the GMC, Prospero acts in the nucleus to repress NB-specific gene expression (including genes required for self-renewal) and activate genes for GMC fate specification and terminal differentiation of post-mitotic neurons [42, 43, 98, 99]. Asymmetric segregation of Prospero protein is mediated by the adaptor coiled-coil protein Miranda. Once segregated from the NB to the GMC, Miranda is degraded, thereby releasing Prospero from the cell cortex and allowing it to enter the nucleus [39-41]. Indeed, the nuclear localization of Prospero is one of the first molecular differences between the self-renewing NB and a differentiating cell [100, 101].

During the postembryonic period of neurogenesis, the NBs of the central brain and thoracic ganglia are thought to undergo a similar proliferation program and express many of the asymmetric cell fate determinants that characterize embryonic neurogenesis [102, 103]. Nuclear localization of Prospero is manifest in GMCs and postmitotic neurons of the larval brain, and loss of *prospero* in somatic clones results in massive overproliferation of cells that express molecular markers of NBs [104-106]. Additionally, numerous other molecular control elements are likely to be required for the continuous mitotic activity of NBs during postembryonic life (reviewed in [107]).

Controlled neuronal proliferation is especially important for the generation of the adult brain. The mature brain of *Drosophila* is an exceedingly complex structure with numerous highly organized neuropil assemblies, such as the mushroom bodies, central complex and antennal lobes, as well as other specialized neuropils and major fiber tracts required for complex behavioral functions [108]. Remarkably, approximately 95% of the neurons that make up the adult brain are post-embryonic in origin, and in

the central brain all of these neurons are produced by a set of only about 100 bilaterally symmetrical NBs [109, 110]. Given the fact that 100 NB pairs generate the tens of thousands of differentiated, spatially heterogeneous neurons in the adult central brain, sophisticated mechanisms for lineage- and region-specific amplification control of NB proliferation are likely to be required during post-embryonic brain development. However, with the exception of rough estimates, which suggest that each brain NB might undergo between 40 and 60 rounds of post-embryonic mitosis to produce lineages of 100–150 neurons, very little is known about this process and the underlying molecular mechanisms.

Here we report that a striking amplification of neuronal proliferation is achieved by specific brain NBs during postembryonic development through the generation of intermediate progenitor cells (IPs). Using cell lineage-tracing and marker analysis, we show that remarkably large NB lineages develop in the dorsomedial (DM) area of the larval brain. Like any other lineages in the brain, they derive from unique NB precursors that remain associated with their post-mitotic neuronal progeny. In addition, they contain a large pool of cells that do not express neuronal differentiation markers, are engaged in the cell cycle, and show mitotic activity. While some of these mitotically active cells are GMCs, the others express NB-specific molecular markers and divide repeatedly to produce neural progeny, implying that they are IPs. The proliferative divisions of these IPs are morphologically symmetrical, but molecularly asymmetrical in that cell fate determinants such as Prospero and Miranda are segregated into only one of the daughter cells. The IPs are generated by a specific set of NBs that do not segregate Prospero to their smaller daughter cell, thereby allowing this cell to retain proliferative capacity instead of undergoing its final neurogenic division. The amplification of NB proliferation through IPs reported here for *Drosophila* bears remarkable similarities to mammalian neurogenesis, where neural stem cells as primary progenitors often amplify the number of progeny they generate via symmetrically dividing secondary progenitors (reviewed in [53]). This suggests that key aspects of neural stem cell biology might be conserved in brain development of flies and mammals.

2.3 Results

2.3.1 Large neuroblast lineages are located in the dorsomedial brain hemispheres

Since most of the secondary, adult-specific neurons of the brain are generated during larval development [4], we used mosaic-based MARCM techniques to label NB lineages (hereafter referred to as 'NB lineages' or 'NB clones') in the developing larval nervous system [16]. Random mitotic recombination was induced in NBs within a few hours after larval hatching (ALH) in order to achieve positive labeling of their clonal post-mitotic progeny (Figure 2.1a). Labeled NB clones typically consisted of a single NB, unequivocally recognizable as a large cell of roughly 10 μm in diameter, and an associated cluster of smaller cells representing its larval progeny (Figure 2.1a,b) [111, 112].

Prominent among these were unusually large clones recoverable at the DM margins of the brain hemispheres (Figure 2.1b). Six NBs located in the most medial position of each hemisphere were found to generate this type of clone, hereafter referred to as 'DM lineages' or 'DM clones'. As detailed below, the parental DM NBs were easily identifiable owing to the signature pattern of Miranda-positive cells that followed the lateral to medial orientation of their progeny in these labeled clones. Morphologically, DM NBs were indistinguishable from other NBs in the central brain or in the ventral ganglia. Thus, cell volume measurements of DM and non-DM NBs in third larval instar brains gave comparable values of 344 ± 94 μm^3 (n = 12) and 424 ± 110 μm^3 (n = 13), respectively. Preliminary analysis of the axonal tracts suggests that the large NB clones in the dorsal brain correspond to the pl and pm subgroups of the Dorsoposterior medial (DPM) lineages previously described (data not shown) [11].

To compare the proliferative capacity of the DM NBs with that of other NBs in the larval central nervous system, we quantified the number of cells in DM NB lineages, in mushroom body NB lineages, and in other NB lineages scored randomly in different brain and ventral ganglion regions of the late third instar larvae shortly before pupation (96 h ALH). The number of cells in the DM lineages had an average value of 450 (range 370–580). Remarkably, this was more than twice the average number of cells observed for the larval lineages of the mushroom body NBs (184 ±

17, n = 17) or for other larval NB lineages scored in other areas of the central nervous system (Figure 2.1c).

To determine the rate of clone size increase during larval central nervous system development, we counted the number of cells in MARCM-labeled DM NB clones, mushroom body NB clones and other dorsal brain NB clones at various larval stages (Figure 2.1d). Following a quiescent phase in the early developing larva, most NBs had entered mitosis by the late second larval instar stage [4]. Our observations show that at this stage (48 h ALH), NBs in the dorsal brain had generated only a small number of postembryonic cells and that no pronounced lineage-specific differences in progeny number was apparent (Figure 2.1d, 48 h ALH). However, at 72 h and 96 h ALH, the DM lineages had increased markedly in size when compared to other dorsal brain NB lineages, indicating an approximate four-fold increase in their rate of proliferation (Figure 2.1d).

To investigate this further, we cultured MARCM-labeled brain explants in 5-bromodeoxyuridine (BrdU) and then used anti-BrdU immunocytochemistry to determine the number of cells engaged in S-phase in DM clones compared to other NB clones of the central brain. Following a 90 minute pulse of BrdU incorporation in L3 brain explants, we found a markedly higher number of BrdU-positive cells in DM clones (38 ± 8 BrdU positive cells, n = 8 clones) than in the other NB clones scored at random in dorsal brain regions of the same specimens (4 ± 1.5, n = 27). (This higher rate of BrdU incorporation in DM clones was also observed at earlier stages and in various conditions of incubation; data not shown.)

These data indicate that a significant amplification of proliferation occurs in the DM lineages when compared to other NB lineages of the central brain (hereafter collectively referred to as 'non-DM' lineages).

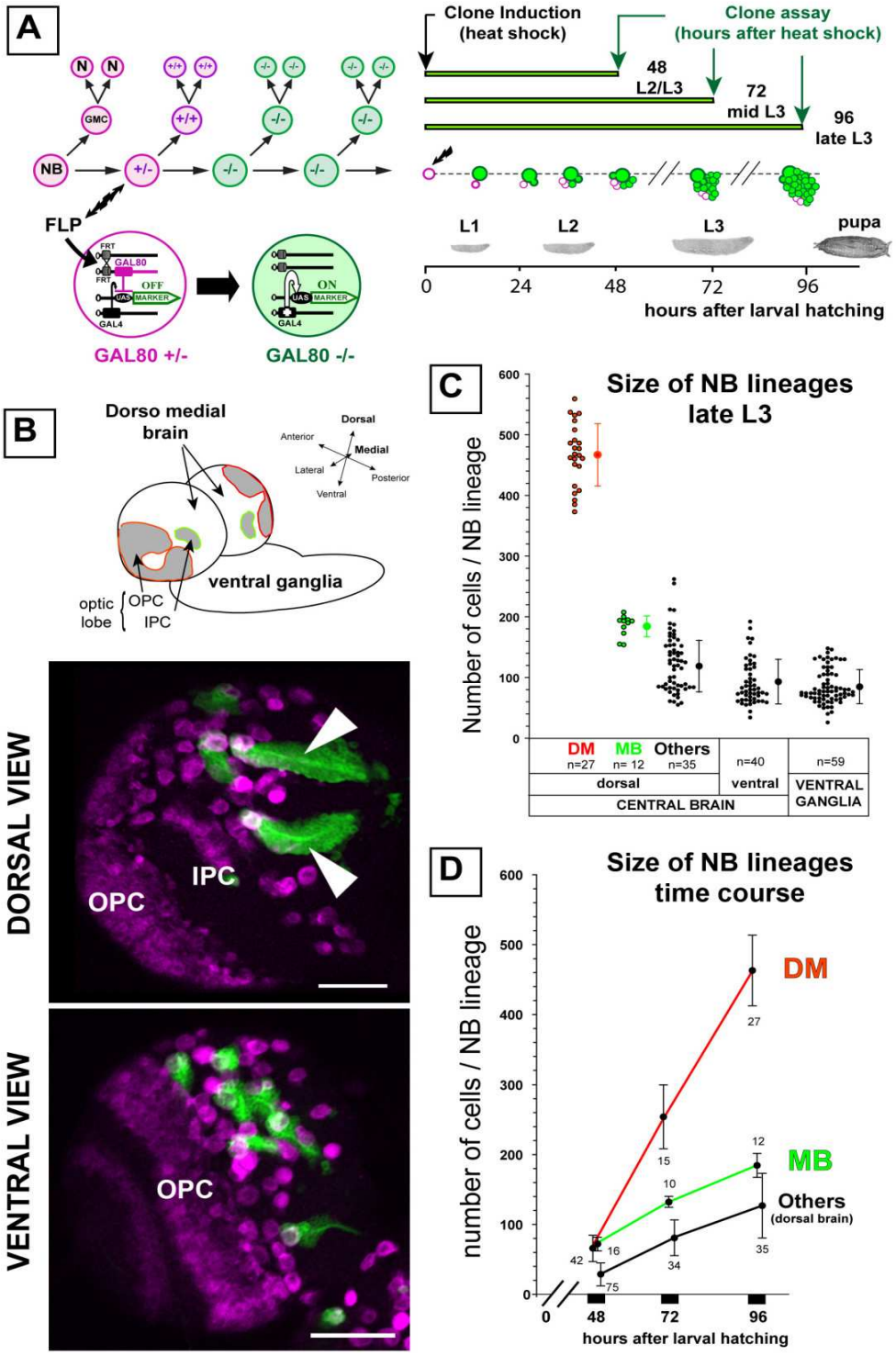

Fig. 2.1 The DM brain NBs generate a large number of progeny during larval development.(a) Lineage labeling of a NB by MARCM. Left: schematic representation of a NB lineage in transgenic flies carrying a repressor transgene GAL80 distal to an FRT site in heterozygous (±) conditions. Ubiquitous expression of GAL80 under tubulin promoter control (pink) prevents GAL4-driven expression of the mCD8::GFP marker gene (green). Heat shock-induced FLP recombinase (FLP) at a given time point mediates the FRT site-specific mitotic recombination. Segregation of recombinant chromosomes at

mitosis may result in the loss of the GAL80 repressor transgene in the NB daughter, which allows stable expression of the marker in this cell and its progeny. After several rounds of division such a positively labeled clone contains the NB, one or more GMCs and numerous post-mitotic neurons (N). Right: following random heat-shock induced NB recombination in newly hatched larvae, the size and composition of isolated NB lineages were examined at different time points during larval development. **(b)** NB clones were examined in all parts of the brain and ventral ganglia with the exception of optic lobes. The latter are easily recognizable in a single brain hemisphere by their lateral position and the high density of cells that express the progenitor marker Miranda (magenta, lower panels). On confocal images of brain hemispheres at low magnification (lower panels), GFP-labeled NB clones are easily identifiable by the presence of a large Miranda-positive NB and an associated cluster of clonal progeny. Unusually large clones could be identified in the dorsomedial part of the brain hemispheres (arrowheads). Anterior is to the top and lateral is to the left for each view. OPC and IPC, outer and inner proliferating centers, respectively. Scale bars: 50 μm. **(c)** The size of NB lineages was determined by counting cells in isolated clones plotted on the diagram according to their position in the nervous system (x axis). Each dot represents a clone with the mean ± standard deviation indicated by dots and error bars next to each group. DM, dorsomedial NB lineage; MB, mushroom body NB lineage; n, number of clones examined in each area. **(d)** Growth rate of different lineages examined at different time points after clone induction. Dots and bars represent the average size and standard deviation determined from the indicated number of clones.

2.3.2 DM lineages contain a large population of mitotically active progenitor cells

The large number of cells found in the DM NB clones could, in principle, be due to an unusually high rate of mitotic activity of the DM NBs. However, immunodetection of mitotic DNA in MARCM clones (via the phospho-histone H3 (PH3) epitope) revealed a comparable mitotic frequency in these NBs (22.5%, n = 40) compared to NBs found in dorsal (16.7 %, n = 48) or ventral (21.6 %, n = 97) brain lineages. This prompted us to search for other types of progenitor cells in these lineages. To this aim, we first characterized molecular markers enabling *in situ* detection of mitotically active versus post-mitotic cells in labeled NB lineages of the larval brain.

Typically, in all NB clones examined, the majority of the labeled cells expressed the neuronal identity marker Elav. Prominent exceptions were the large NBs and a set of smaller cells closely associated with the NBs, all of which were Elav-negative (Figure 2.2). Quantification of the number of these Elav-negative cells revealed a striking difference in DM lineages compared to non-DM lineages (Figure 2e). DM lineages contained an average of 56.7 ± 11.8 Elav-negative cells (n = 10 clones) closely associated with the Elav-negative NBs. This was over 10 times more than in non-DM NB clones (4.7 ± 1.7 cells, n = 114), suggesting that the DM lineages contain a markedly higher number of mitotically active progenitor cells.

Could these smaller Elav-negative cells associated with the NBs be GMCs? To investigate this, we first studied the expression of the coiled-coil protein Miranda. The *miranda* gene has been reported to be expressed in larval NBs but not in their GMCs [113]; Miranda expression might, therefore, be a useful marker for differentiating NB-like cells from GMCs. In non-DM lineages, Miranda was strongly expressed in the NBs but only very weakly expressed in the set of smaller, Elav-negative cells associated with the NBs, suggesting that these Elav-negative cells were GMCs (Figure 2.2a,a'). (Their weak expression of Miranda could be due to perdurance of the protein during cell divisions; see also [102, 103]). In DM lineages, Miranda was strongly expressed in the NB; however, in contrast to non-DM lineages, distinct Miranda expression was also observed in many of the smaller, Elav-negative cells associated with the NBs (Figure 2.2b,b'). This suggests that the smaller Elav-negative/Miranda-positive cells in the DM lineages might not be GMC-like, but might have properties that are more NB-like. To investigate this further, we next attempted to find other markers for progenitor cells and, thus, examined the expression of Cyclin E (CycE) and PH3 as markers of mitotically active cells.

In green fluorescent protein (GFP)-labeled non-DM NB clones, used as control, a small number of GMCs were observed as small NB-associated cells expressing either CycE or PH3 (Figure 2.2c,c'). At 96 h ALH we found an average of two CycE-positive cells (range one to five) and a maximum of one cell engaged in mitosis as visualized by anti-PH3 (Figure 2.2e) [111]. This pattern was consistent with live imaging data obtained in experiments on cultured nervous systems to monitor asymmetric NB divisions [48]. Thus, as in the embryo, these larval NBs divide by a budding process that generates a set of smaller GMCs, each GMC is born adjacent to the previous one, and the division of the 'oldest' GMC is delayed compared to that of the NB.

Contrasting with this simple pattern, DM lineages contained an average of 38 CycE-positive cells located around the NB, and many scattered mitoses, up to 14 per clone, were observed by PH3 immunoreactivity (Figure 2.2d,d',e). This strikingly high level of ongoing mitotic activity and engagement in the cell cycle in DM lineages compared to other central brain lineages (including mushroom body lineages) was seen at all stages of larval development examined (Figure 2.2f). These findings

indicate that significantly elevated mitotic activity occurs among the numerous small NB-associated cells in larval DM lineages. Moreover, they are in accordance with the idea that these cells do not adopt a GMC fate, but rather remain mitotically active and continue to proliferate. In this case, these cells would have the characteristics of IPs that amplify the proliferation of their parent NBs (primary progenitors) in the DM lineages.

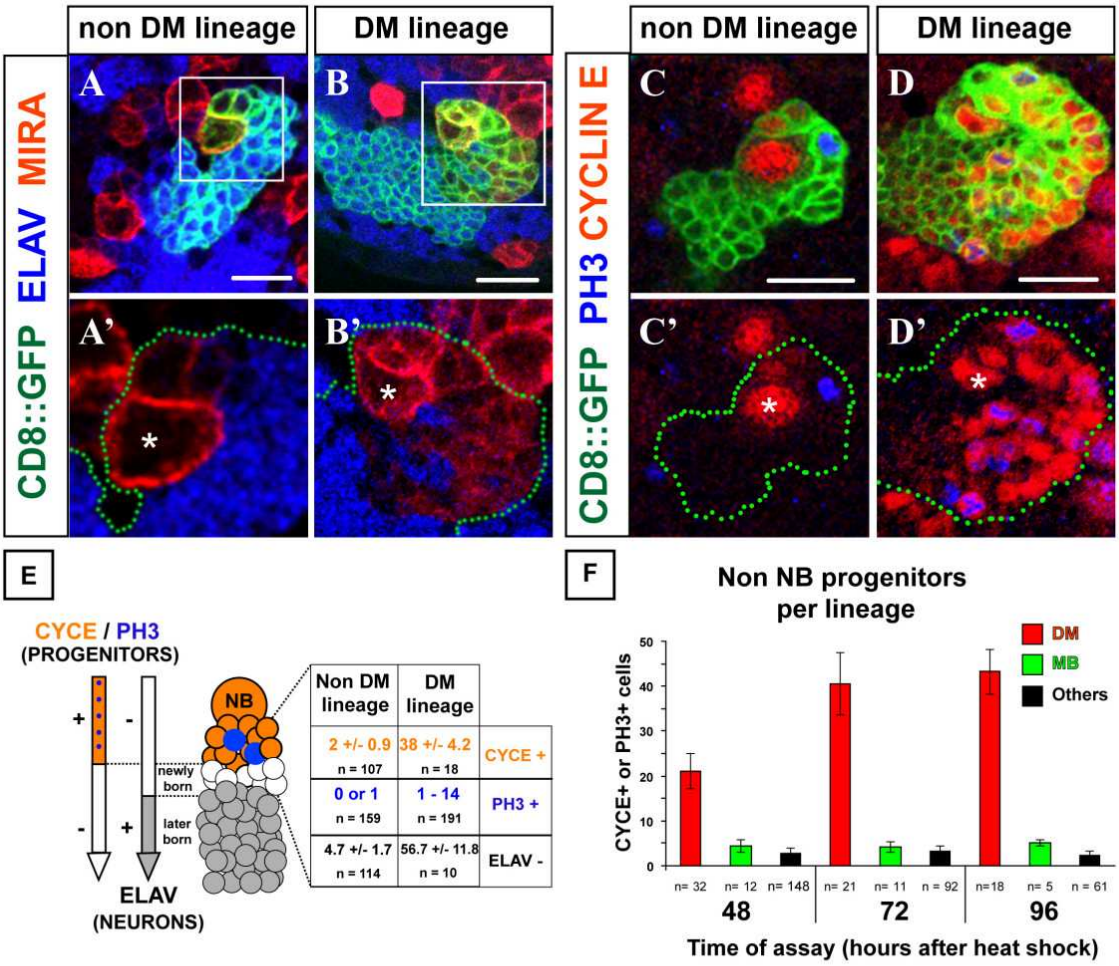

Fig. 2.2 The DM NBs generate an exceptional number of neuronal progenitors.(a-d') Confocal images of representative non-DM and DM lineages labelled with mCD8::GFP (membrane marker, green) in larval brains stained for the markers indicated. Each panel shows the most superficial area of a single NB clone viewed around the NB (asterisk) in the dorsal brain. The GFP channel is omitted for clarity in the lower panels and green dots outline the clones. Note that (a', b') show close up views of the areas boxed in (a, b). Progenitor cells in an NB lineage include the NB identifiable by its size (asterisk) and the most recently born cells in its associated progeny. These cells are found in close spatial proximity to the NB and are characterized by a weak level of cortical Miranda (red in a-b') and

the absence of the neuronal marker ELAV (blue in a-b'). **(c-d')** NB-associated cells are unambiguously defined as progenitors by the expression of the cell cycle markers Cyclin E and/or PH3. **(e)** Quantification of various markers in NB clones at 96 h ALH underscores the high number of small progenitor cells among the progeny of the DM NBs. **(f)** DM NBs are always associated with the highest number of non-NB progenitors during larval development. Scale bars: 10 μm.

2.3.3 Molecular markers reveal two types of non-neuroblast progenitor cells in DM lineages

If some of the mitotically active cells in DM NB clones are amplifying IPs, they might be expected to have cellular and molecular features in common with proliferating NBs. To investigate this, we first examined the expression patterns of Prospero, Miranda, and CycE in NBs of non-DM lineages, used as control, as well as in the small NB-associated progenitors of the DM lineages. For this, MARCM clones induced at larval hatching were scored at 96 h ALH. Importantly, we further restricted our analysis to cells engaged in mitosis (PH3-positive) in order to identify progenitor cells unambiguously and to obtain valid comparisons, since all markers showed cell-cycle dependent expression (see below). (Clones analyzed at 48 h or 72 h ALH gave comparable results; data not shown.)

In non-DM clones, Prospero was specifically detected at the cellular cortex of the NBs, accumulating on one side during mitosis (Figure 2.3a; n = 57; 100%). All other cells in the clones expressed Prospero in the nucleus or uniformly throughout the cell, thus including both GMCs and post-mitotic cells. Localization of Prospero was more specifically revealed in the GMCs by co-staining with anti-PH3 (Figure 2.3b; n = 37; 100%) or CycE (not shown). In striking contrast, in DM lineages 31% of PH3-positive small NB-associated cells expressed Prospero at the cortex in a polarized manner. This expression pattern was, thus, similar to that observed in dividing NBs (Figure 2.3g,g", arrow). The remaining dividing, NB-associated cells showed uniform expression of Prospero throughout the cell at mitosis; their pattern was, thus, GMC-like (Figure 2.3g,g' arrowheads).

As expected, the adaptor protein Miranda formed prominent cortical crescents in dividing NBs of non-DM clones (Figure 2.3c, asterisks). In the associated GMCs, Miranda was detected at weaker levels with uniform cortical distribution both at

interphase and during mitosis (Figure 2.3c, inset, and Figure 2.3d, arrowheads). Strikingly, in DM lineages, 36% of the NB-associated cells showed strong and polarized expression of Miranda during mitosis, as described for dividing NBs (Figure 2.3h,h", arrows). The remaining dividing cells showed weak and uniform cortical localization of Miranda; their Miranda expression pattern was, thus, GMC-like (Figure 2.3h,h' arrowheads).

To confirm the presence of both NB-like and GMC-like progenitors in the DM NB lineages, we searched for markers of cellular identity that did not rely on the conventional criteria of cell size and/or cortical polarity. Significantly, we found that in non-DM lineages (taken as reference lineages), CycE was detected in virtually all the self-renewing NBs during mitosis (Figure 2.3e, asterisks; n = 74), but never during the terminal division of the GMCs (Figure 2.3f, arrowheads; n = 48). This distinctive criterion for cell identity was only applicable during mitosis because all progenitor cells expressed CycE at interphase, irrespective of their size (Figure 2.3e,f; $PH3^-$ nuclei; see also Figure 2.2c,d). In DM lineages, some of the small PH3-positive cells were negative for CycE but other small PH3-positive cells were positive for CycE (Figure 2.3i,i', arrow and arrowhead). Thus, in agreement with the data obtained using markers of cell polarity, both NB-like and GMC-like progenitors could be identified simultaneously in the progeny of a single DM NB (Figure 2.3g–i). Furthermore these two types of progenitors were observed specifically in these lineages and at all larval stages examined. Thus, the small CycE-positive/PH3-positive progenitors represented 55% (n = 64), 45% (n = 93) and 40% (n = 105) of the mitotic cells found in DM NB clones at 48 h ALH, 72 h ALH and 96 h ALH, respectively. The small CycE-positive/PH3-positive progenitors were never found associated with NBs of the ventral brain or the ventral ganglia at the corresponding stages (114 PH3-positive cells in 297 clones examined).

Taken together, these data indicate that the larval DM lineages contain two types of molecularly distinct progenitor cells other than NBs. Although not readily identifiable by their size, approximately two-thirds of these cells have molecular expression patterns of Prospero, Miranda and CycE that are characteristic of GMCs. In contrast, the remaining third have expression patterns of Prospero, Miranda and CycE that are remarkably similar to the patterns found in proliferative NBs. These novel NB-like

progenitors are hereafter referred to as IPs. Our data further show that IPs are generated by DM NBs throughout larval neurogenesis in a quantitatively stable and balanced ratio with GMC-like progenitors and post-mitotic neurons.

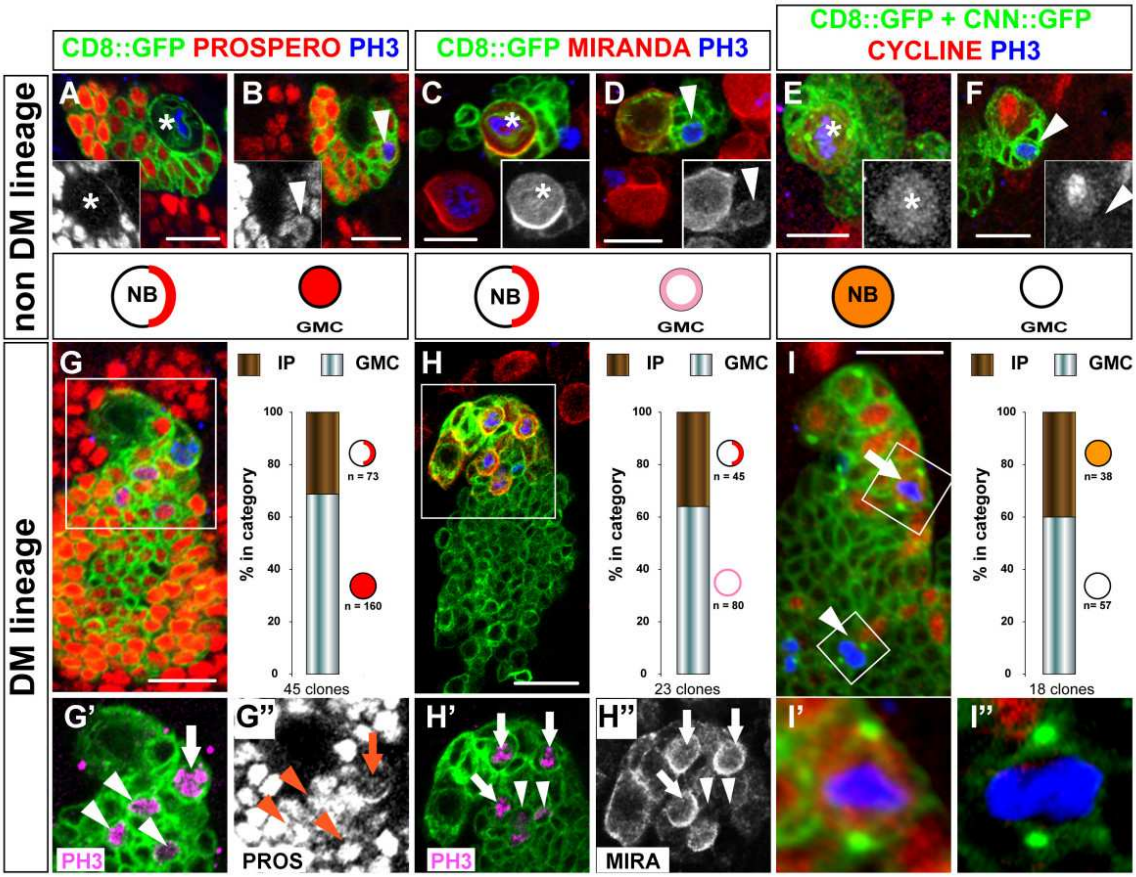

Fig. 2.3 Molecular characterization of NB-like and GMC-like progenitors in the progeny of DM NBs. Confocal images of MARCM-labeled NB clones in the dorsal part of larval brains stained for the markers indicated on the top of the columns. Representative views of **(a-f)** non-DM lineages are used as a reference for **(g-i")** the DM lineages. Clones were labeled with CD8::GFP (membrane marker, green in all panels) and CNN::GFP (centrosomes visualized as bright green spots in e, f, i-i"). Proliferative cells are detected by anti-Cyclin (red in e, f, i-i') and anti-PH3 during mitosis (blue in all panels). In a non-DM NB clone, mitosis is restricted to two cell types: the NB and a single GMC in close proximity (a-f, asterisks and arrowheads, respectively). NBs show a unique pattern of polarized expression of Prospero and Miranda at the cell cortex during mitosis (a, c) and stable expression of Cyclin E throughout the cell cycle (e, mitosis; f, interphase). In contrast, the GMC is uniquely defined when engaged in mitosis (PH3 positive) by nuclear localization of Prospero (b, inset), weak uniform cortical localization of Miranda (d, inset) and lack of Cyclin E (f, inset). (g-i) In DM clones many progenitors other than the NB are identified as PH3-positive nuclei. These cells show patterns of marker expression usually found in mitotic NBs (IP; arrows) or mitotic GMCs (arrowheads). Lower panels show close up views of the areas boxed in (g-i). The two types of mitotic progenitors can be detected simultaneously in a single DM lineage (images) and are found at a comparable ratio when quantified in multiple clones using the three independent markers (histograms). IP, small NB-associated intermediate progenitor with NB-like marker expression. Scale bars: 10 μm (a-f) or 15 μm (g-i).

2.3.4 Intermediate progenitor cells divide repeatedly and produce multicellular neuronal clones

The NB-like molecular expression pattern of IPs suggests that this novel type of progenitor might share some of the mitotic properties of NBs. Indeed, if the augmentation of proliferation observed in the DM lineage is mediated by amplifying IPs, these cells would be expected to divide repeatedly. To investigate this possibility, we first performed live imaging of MARCM clones on cultured brain explants dissected from third instar larvae. Clones were labeled simultaneously with CD8::GFP and tau::GFP to visualize both cell membranes and mitotic spindles (see Materials and methods). In agreement with anti-PH3 staining on fixed tissue, we observed numerous cell divisions among the small cells that were closely associated with the NB in DM NB clones (Figure 2.4a). With the exception of the asymmetric divisions of the NB itself, all of the observed cell divisions in the clones were symmetrical (n = 75, 10 clones). Importantly, we repeatedly observed small, NB-associated cells that divided more than once. Two subsequent symmetrical divisions of such a progenitor cell are visible in the still images taken from a time-lapse laser confocal movie (Figure 2.4b).

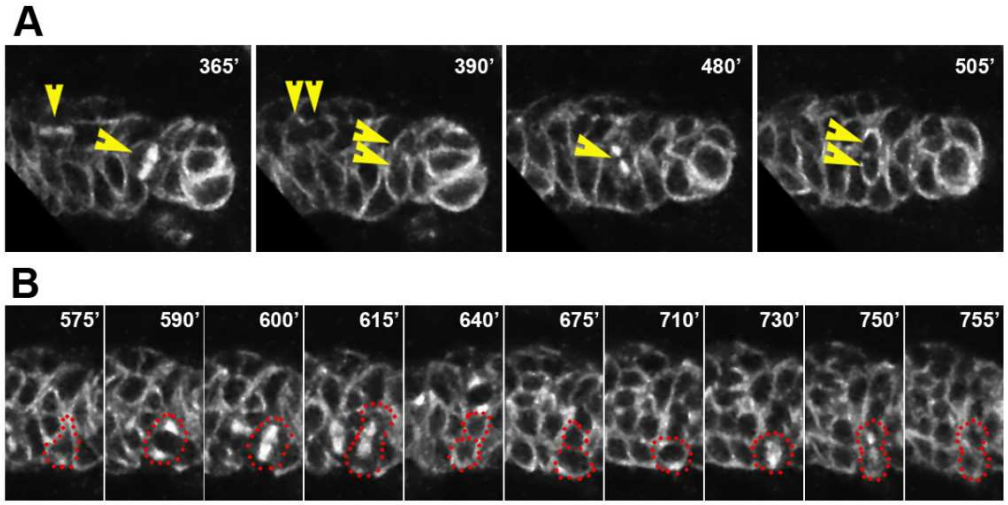

Fig 2.4 Live imaging of multiple and repeated division of DM NB daughter cells in MARCM-labeled clones. Frames from time-lapse recordings of a DM clone labeled with CD8::GFP and tau::GFP in larval brain cultured over 13 hours. The large NB, not visible in these frames, divided twice during this time period. The time is indicated in minutes relative to the start of the recording. **(a)**

Multiple divisions of small NB-associated cells may be ongoing simultaneously in the clone and each gives rise to two daughter cells of equal size (single and double arrowheads at following intervals). **(b)** A single NB daughter cell may undergo several rounds of division. Shown are two consecutive divisions of a cell outlined with dots. Following a first symmetric division (575'–675'), the lower daughter cell underwent a second division (710'–755') while its sibling did not divide further during the recording.

Next, we performed a more detailed analysis of the different types of MARCM clones that were recoverable in the DM lineages. To date, only two types of multicellular clones have been observed in the central brain following a somatic recombination event in a parental NB and the loss of the GAL80 repressor in one of the post-mitotic siblings. Thus, the NB clones described above derive from the proliferation of GAL80-minus NB founders, while two cell clones are obtained from GAL80-minus GMCs (Figure 2.5a). Other possible recombination events may occur in a GMC, but they result in the labeling of a single post-mitotic daughter cell [16, 48]. In DM lineages containing repeatedly dividing IPs, a third type of non-NB clone consisting of more than two labeled cells would be predicted to occur following the loss of the GAL80 repressor (Figure 2.5a).

Mitotic recombination was randomly induced in progenitor cells at 24 h and 48 h ALH and progenies were examined in isolated GFP-labeled clones 48 hours later (Figure 2.5b). As expected, single cell-, two cell-, and NB clones were recovered throughout the central nervous system. Prominent among the latter were the exceptionally large DM NB clones identifiable in the dorsal brain by their medial position and the spatial orientation of the labeled progeny that extend from the typical large cluster of late born Miranda-positive cells (Figure 2.5d,d'). Consistent with their linear growth rate (Figure 2.1d), we measured comparable clone sizes for DM NB clones generated during each of the two overlapping 48 hour windows (157 cells ± 33, n = 14 clones, and 220 cells ± 43, n = 16 clones, respectively). Likewise, non-DM NBs selected at random in the dorsal brain also generated comparable, albeit smaller, NB clones in the same time periods (63 cells ± 20, n = 40 clones, and 66 cells ± 23, n = 48 clones, respectively). Importantly, however, numerous clones lacking a NB and consisting of more than two cells were recovered in these experiments. These multicellular non-NB clones were found only in close spatial association with DM NBs and their progeny (Figure 2.5e,e'). Cell counts revealed a wide range of clone

sizes in these lineages. Most clones, however, comprised 6–25 cells and this class was observed at comparable frequency in the two time windows examined (73% and 67%, respectively; Figure 2.5c). In over 90% of the cases examined, the cells in these multicellular clones expressed Elav, indicating that they were composed exclusively of post-mitotic neurons (Figure 2.5e,e').

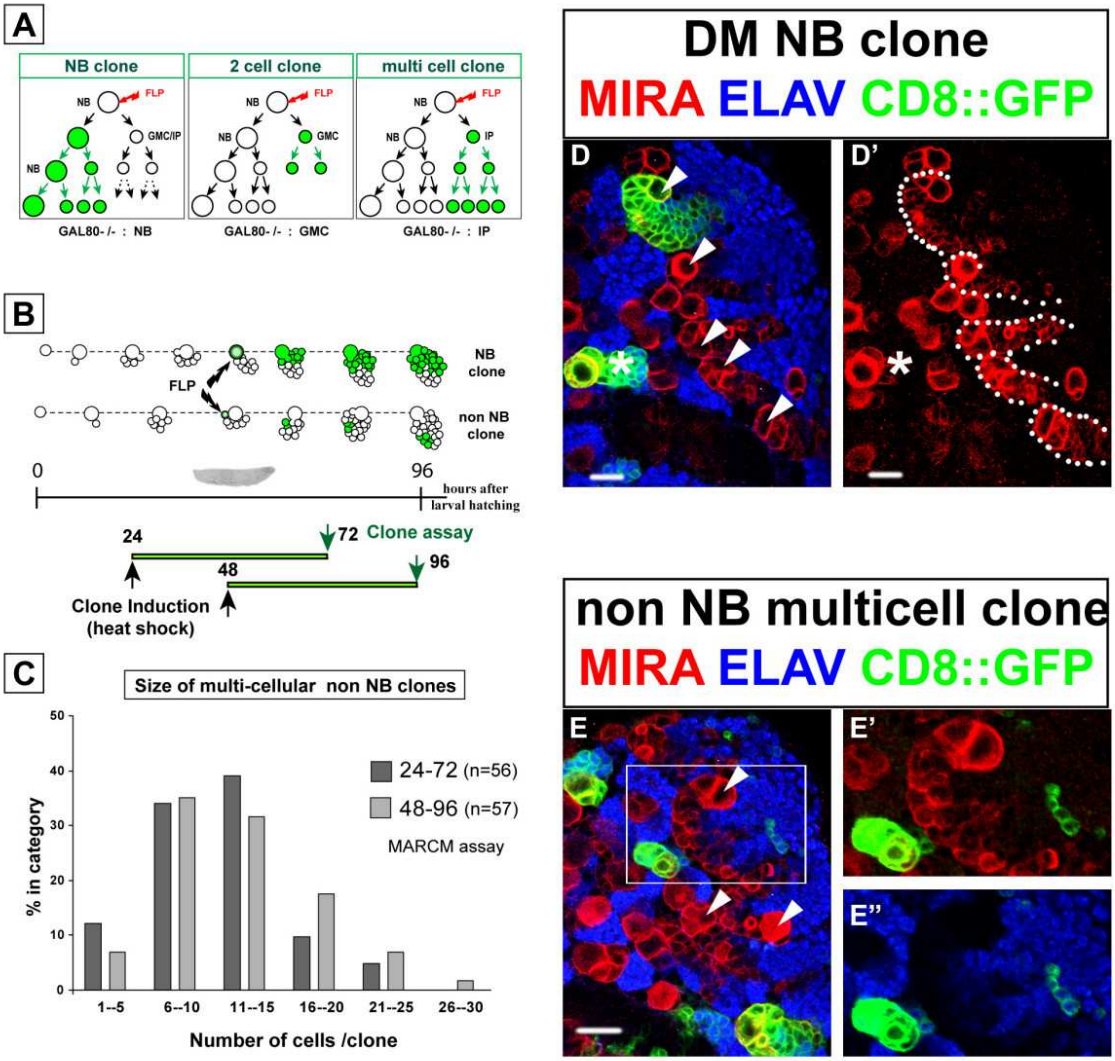

Fig. 2.5
Clonal expansion of IPs analyzed by MARCM.(a) Schematic representation of the different types of MARCM clones that can be recovered following FLP-mediated recombination in a NB (red arrow) and segregation of homozygous GAL80 chromosomes into one of its two daughter cells (green). A multicellular clone lacking the NB (right panel) reveals the ability of the IP daughter cell to undergo several rounds of division. Not shown are FLP-mediated recombination events in the GMC or in the IP that give rise to multicellular clones only in the latter case. Recombination in the GMC gives a single

labeled cell. **(b)** Top: schematic organization of multicellular GFP-labeled clones (green) after time-controlled recombination (heat-shocked FLP, black arrows) in two developing NB lineages. Bottom: unlike NB clones (upper lineage), IP clones were identified as GFP-labeled cell clusters lacking the large Miranda-positive NB and pushed away from this founder cell by proliferation (non-NB clone, lower lineage). The size and composition of clonal progenies were examined 48 hours after two independent heat-induced recombination events. **(c)** Size distribution of multicellular non-NB clones generated by recombination at 24 h (light grey bars) or 48 h (dark grey bars) ALH and assayed 48 hours later. The similar histogram profile reveals the comparable mitotic potential of progenitors present in the DM lineage at 24 or 48 h ALH. **(d, d')** Representative confocal image of NB clones induced at 48 h ALH and examined at 96 h in a dissected brain stained for the markers indicated (dorsal view, lateral to the left, anterior to the top). DM NBs are identifiable in the most medial row of large cells (arrowheads) by their association to a large cluster of Miranda-positive progenitors (various DM lineages are outlined by dots in (d); the GFP channel was omitted for clarity). The GFP-labeled progeny of a single DM NB follows the orientation of the Miranda-positive cell cluster. A typical non-DM NB clone is found on the lateral site of the brain (asterisk). This single large NB is associated with a few Miranda-positive GMCs. **(e)** Representative IP clone of four cells among the presumptive progeny of the nearest DM NB (arrowheads); same scale and conditions as in (d). A magnification of the area boxed in (e) is shown in (e', e''), with one channel omitted for clarity. The cells in the clone have undetectable level of Miranda (red) and all express the neuronal marker ELAV (blue). Scale bars: 15 μm.

The observed variability in clone size could be due to intrinsic variations in the mitotic capacity of different IPs and/or may result from mitotic recombination occurring in an IP that had already completed a variable number of divisions after its birth. Interestingly, the distribution of clonal cell number appeared remarkably similar when FLP/FRT recombination was induced at 24 h or at 48 h ALH (Figure 2.5c). This suggests that the mitotic potential of IPs is independent of their birth date from their parental DM NBs during larval development.

These findings imply that IPs in DM lineages can divide several times and produce differentiated progeny in less than 48 hours. Thus, they allow considerable amplification of the number of neurons produced in comparison to the standard mode of division adopted by other lineages in the central brain.

2.3.5 DM neuroblasts do not segregate Prospero protein to their daughter cells

The experiments described above show that DM NBs generate multiply dividing daughter cells that produce neural progeny. Surprisingly, these amplifying IP cells appear to be restricted to the DM lineages. What might explain this restriction? DM and non-DM NBs are not morphologically distinguishable and both divide asymmetrically to generate smaller progeny cells (Figure 2.3 and below).

A large amount of evidence indicates that the polarized assembly of multiprotein complexes at the cellular cortex during mitosis is both a characteristic hallmark of NBs and a key determinant in promoting their self-renewing ability. As exemplified in non-DM lineages (Figure 2.6a,c), Prospero and Miranda are synthesized in the NB and they co-localize on one side of the cortex at metaphase (Figure 2.6a, asterisk). This asymmetric distribution results in unequal segregation of these proteins to the budding new GMC as visualized at telophase or soon after cytokinesis (Figure 2.6c, asterisk). (Older GMCs located in close proximity to the newly generated GMC show a much lower level of Miranda and manifest the same type of nuclear localization of Prospero as do all other post-mitotic nuclei of the clone; Figure 2.6c, $n > 50$ clones). Importantly, the loss of these fate determinants in mosaic clones leads to unrestricted proliferation of the GMC *in situ* and the acquisition of neoplastic characters of mutant cells in transplantation assays[104-106, 114].

Remarkably, and in contrast to all other *Drosophila* NBs described to date, Prospero was undetectable in the DM NBs during mitosis (Figure 2.6b,d). In all DM NB clones examined ($n = 25$), Miranda, but not Prospero, formed a cortical crescent in the dividing NB at metaphase (Figure 2.6b, asterisk) and segregated to the smaller daughter cell (Figure 2.6d). As a result, the IPs that derived directly from the DM NB lacked nuclear Prospero. GFP-labeled DM lineages typically contained 28 ± 9 Prospero-negative cells close to the NB (Figure 2.6b, white dots, $n = 14$ clones). These are likely to be accumulating IPs in interphase because they showed weak uniform expression of Miranda at the cortex and did not express PH3 (Figure 2.6b and data not shown). At IP mitosis, however, Prospero was unambiguously detected in these progenitors and showed co-localization with Miranda in a polarized manner (Figure 2.6b, arrows).

These data identify the DM NBs as a unique subset of neural stem cell-like progenitors that do not express and segregate Prospero during mitosis, thereby generating daughter cells that are molecularly distinct from GMCs.

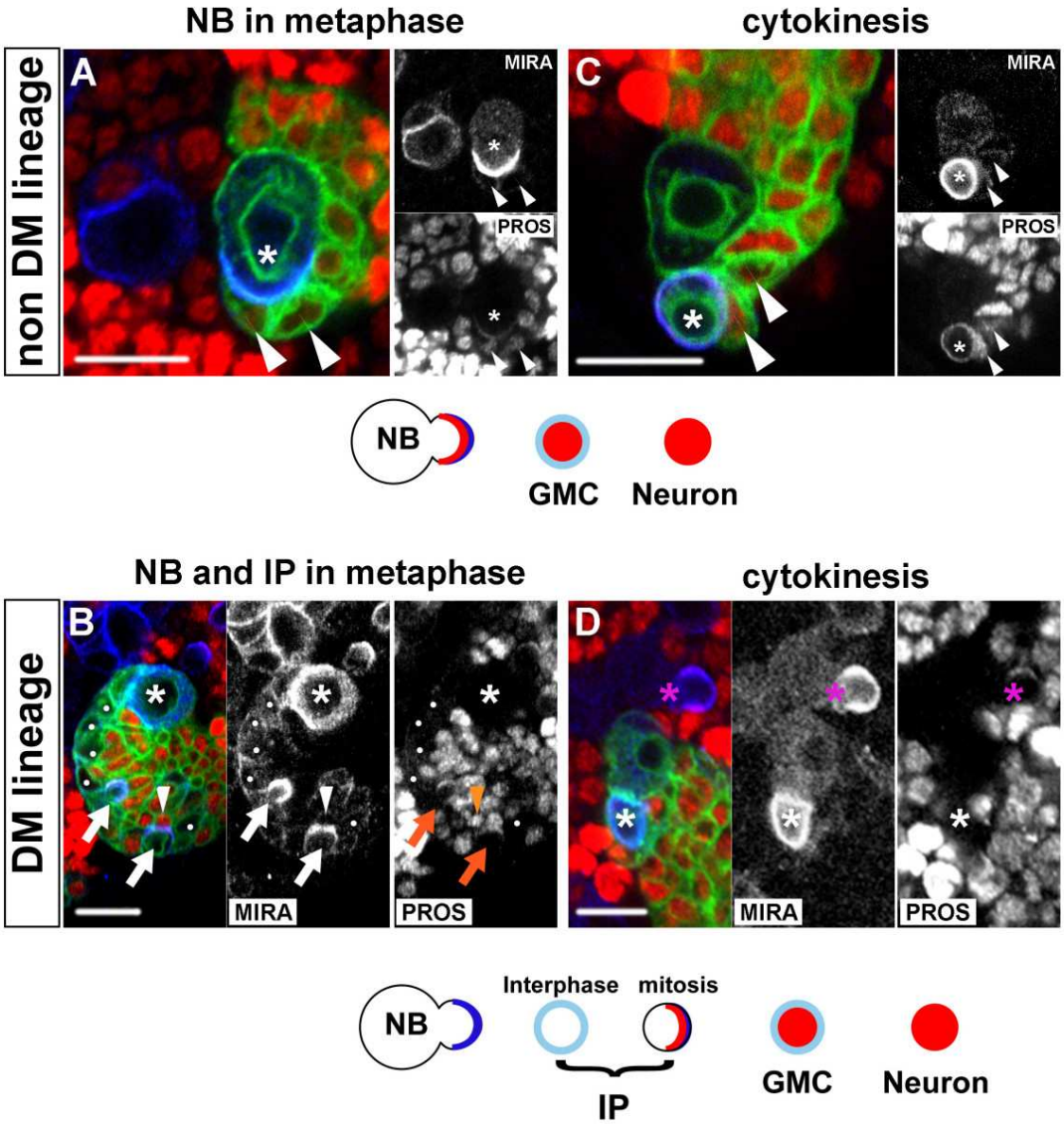

Fig. 2.6
Asymmetrically dividing DM NBs do not express Prospero. Confocal images of NB divisions in a canonical NB lineage (top panels) compared to a DM lineage (bottom panels). Shown are representative CD8::GFP-labeled clones (green), seen around the NB in late larval brains stained for Miranda (MIRA, blue) and Prospero (PROS, red). Single channels are also shown in gray scale for better contrast. **(a, b)** Miranda forms cortical crescents at metaphase in both non-DM and DM NBs (asterisk). **(c, d)** Following asymmetric division, Miranda segregates into the small daughter cell and remains associated at high levels at the cortex soon after cytokinesis (the small newborn daughter cell is marked by an asterisk). Prospero co-localizes with Miranda in the dividing non-DM NBs (a, c, asterisks) and is nuclear in the oldest GMCs, which retain a low level of Miranda at the cortex (a, c, arrowheads), and in all other post-mitotic cells in the clone. In the DM NBs, Prospero is undetectable during mitosis (b, d, asterisks). (Note in (d) a canonical NB outside the clone (magenta asterisk) that shows co-localization of Miranda and Prospero and serves as internal control.) Recently born NB daughter cells show weak uniform cortical Miranda and lack Prospero (white dots in b). Polarized cortical Miranda during mitosis identifies these cells as IPs (b, arrows) and co-localization with Prospero is once again observed in these cells (b, insets). Cells with GMC-like (arrowheads) or neuronal expression of the markers are also observed as in canonical non-NB lineages. Scale bars: 10 μm.

2.3.6 Intermediate progenitor cell divisions are morphologically symmetrical but molecularly asymmetrical

Studies on asymmetric neural stem cell division in *Drosophila* have established a simple scheme that links cell size of sibling daughter cells, restriction of mitotic potential and partitioning of fate determinants. Thus, in the canonical scheme exemplified in MARCM-labeled non-DM clones, the only self-renewing cell is the large NB that segregates Miranda/Prospero to its small GMC daughter cell during mitosis (Figure 2.6a,c). In contrast, the terminal division of the GMC involves the formation of equal-sized daughter cells at telophase and equal partitioning of Miranda/Prospero to both cells (n = 27; Figure 2.7a,c and data not shown).

The asymmetric division of DM NBs is also associated with the unequal segregation of Miranda to the smaller daughter cell (Figure 2.6b,d). Moreover, the resulting IP divides symmetrically to generate sibling cells of similar size as examined at telophase (n = 14; Figure 2.7b,d). Thus, in terms of the morphology of their cell divisions, IP cells are more like GMCs than like NBs. However, in sharp contrast to GMCs, mitotic IPs show cortical crescents of Miranda and Prospero (Figure 2.6b) and unequal partitioning of these two proteins at telophase (Figure 2.7d; n = 7). Thus, in terms of the segregation of cell fate determinants, dividing IP cells are remarkably more NB-like and differ substantially from GMCs.

Taken together, these findings demonstrate that the proliferative divisions of amplifying IPs in DM lineages have novel cellular and molecular features. These divisions are morphologically symmetrical and lead to two daughter cells of similar size, but molecularly asymmetrical in that the differentiating cell fate determinants Prospero and Miranda are segregated into only one cell. The ensuing absence of these differentiating cell fate determinants in the remaining daughter cell is likely to be a significant factor in the mitotic activity of amplifying IP cells.

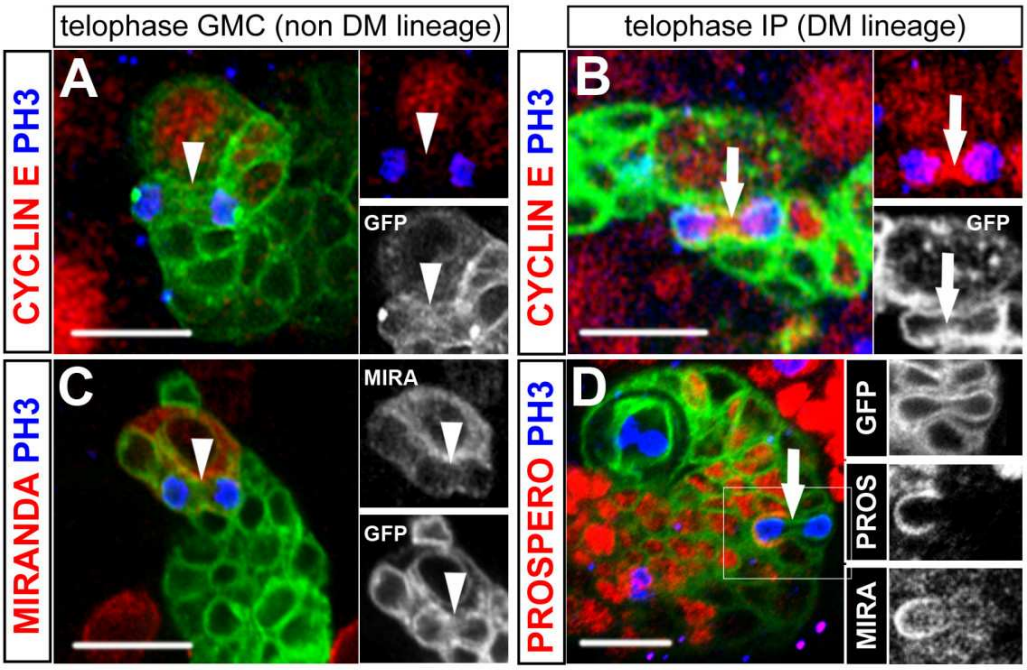

Fig. 2.7
Unequal segregation of Prospero/Miranda during symmetric division of IPs. Confocal images of representative CD8::GFP labeled clones (green) in **(a, c)** canonical non-DM or **(b, d)** DM lineages. Shown are mitotic figures of small NB-associated cells at anaphase/telophase, visualized by anti- PH3 staining of DNA (blue). Separate channels are also shown in insets for better contrast. The outline of the plasma membrane stained by CD8::GFP shows that both the GMC (a, c, arrowheads) and the IP (b, d, arrows) divide symmetrically and give rise to daughter cells of similar sizes. The dividing IP is identified by NB-like expression of Cyclin E during mitosis (b) while GMC division lacks Cyclin E expression at this phase of the cell cycle (a). In the mitotic GMC, Miranda distributes equally to both daughter cells (c, inset) while Prospero is nuclear (see Figure 3b). In IP division, Prospero and Miranda co-segregate to only one of the two daughter cells (d, insets). Scale bars: 10 μm.

2.4 Discussion

In this report, we present cellular and molecular evidence for a new mode of neurogenesis in the larval brain of *Drosophila*. In the canonical model for postembryonic neurogenesis exemplified by the non-DM lineages of the brain and the lineages of the ventral ganglia, NBs divide asymmetrically in a stem cell mode to self-renew and generate a GMC that divides once to produce two post-mitotic cells that differentiate (Figure 2.8a). Associated with this process is the asymmetric segregation of the cell fate determinants Prospero and Miranda from the parent NB into the GMC, whereupon Prospero acquires a nuclear localization that is retained in the GMC's post-mitotic progeny.

The data presented here are consistent with a novel model for neurogenesis exemplified by the DM NBs, which divide asymmetrically in a stem cell mode to self-renew and generate IP daughter cells (Figure 2.8b). In this process, they do not segregate the cell fate determinant Prospero into the IP cells, which subsequently repeatedly divide symmetrically (in morphological aspects) yet asymmetrically segregate the cell fate determinants Prospero and Miranda during mitosis. The daughter cell that receives the Prospero and Miranda determinants is fated to become a differentiating GMC-like cell, whereas the other daughter cell retains its ability to divide several more times.

This novel model postulates that DM NBs produce exclusively IPs and not GMCs. The alternative notion, that the NB sometimes produces an IP and sometimes a GMC, is unlikely given that Prospero is never detected in the NB and, thus, cannot be segregated to one of its daughter cells as would be required for GMC generation. The model also posits that GMCs are produced by IPs through (functionally) asymmetrical divisions that result in one daughter cell becoming a GMC while the other daughter cell self-renews as an IP. Alternative scenarios, such as one in which IPs first divide symmetrically to expand in numbers and then adopt a GMC fate to generate differentiating neurons, are unlikely given the spatiotemporal pattern of Prospero/Miranda expression and the stable ratio of IPs versus GMCs observed in DM NB clones throughout larval development.

The experimental findings that support this novel model have implications for our understanding of neural stem cells and proliferation control. These are discussed in the following.

The NBs of the developing central brain and ventral ganglia divide asymmetrically in a stem cell mode in which the larger NB self renews and the smaller daughter cell differentiates into a different cell type, usually a GMC (reviewed by [31, 32, 92, 94, 95, 115]). This asymmetric division of the parent NB has been thought to be tightly coupled with the asymmetric segregation of cell fate determinants, and central among these molecular determinants is the transcription factor Prospero, which is required in GMCs to inhibit self-renewal and to promote differentiation [42, 43, 98-101]. Our findings indicate that the asymmetric segregation of Prospero does not occur in all dividing brain NBs. Indeed, in the DM NBs the lack of asymmetric segregation of Prospero to the IPs may be a key element in imparting (transient) NB-like features to these proliferating cells.

The GMCs of the developing nervous system divide symmetrically and generate two postmitotic progeny of equal size. Our findings indicate that IP cells also divide symmetrically in morphological terms, although Prospero and Miranda are partitioned to only one of their daughter cells. Thus, the morphologically symmetric cell division of a NB-derived daughter cell does not necessarily engender equal portioning of differentiation factors into both resulting cells. It has been assumed that only cells of a certain critical size show NB-like proliferative properties. The small size of the GMC would be a key factor promoting cell cycle exit and differentiation of its progeny (see [92]). This simple link between cell size and self renewing/terminal division is also called into question by our findings, since IPs are comparable in size to GMCs and yet they possess a very distinct mitotic potential.

The only repeatedly dividing progenitor cell type identified to date in the central nervous system of *Drosophila* is the NB. Our studies identify the IP cell as a second progenitor type with the capacity to undergo multiple rounds of divisions. This characteristic is coupled with several cellular and molecular features that are shared with NBs. Among these are the specific expression patterns of Prospero, Miranda and CycE during mitosis as well as the ability to asymmetrically segregate Prospero and Miranda during cell division. The number of divisions that IPs typically carry out is

currently not known with precision. Our observations based on quantification of cell number in multicellular clones suggest an average of three-to five divisions as a conservative estimate. If, as assumed by our model, each IP cell division results in the generation of one GMC-like daughter cell, this estimate would predict a three- to five-fold amplification of the number of neuronal progeny in DM lineages compared with other lineages of the central brain and ventral ganglia. This prediction is in reasonable accordance with the amplified cell numbers observed in NB clones of DM versus non-DM lineages. The ultimate fate of the IPs is currently not known. The fact that almost all intermediate precursor-derived multicellular clones are composed exclusively of postmitotic neurons suggests that, after multiple divisions, these cells are either eliminated by programmed cell death or that they terminally divide and differentiate.

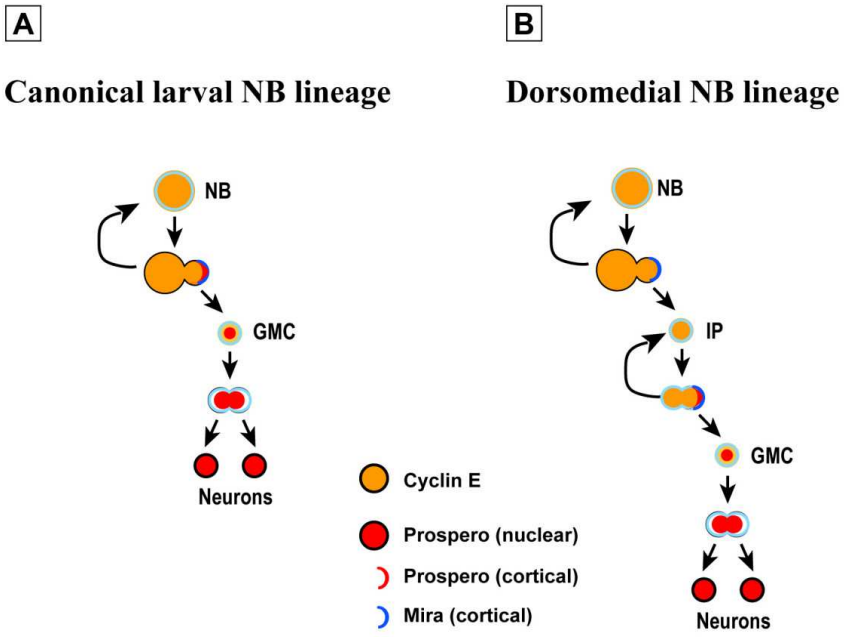

Fig. 2.8
Model for a transient amplifying progenitor cells in DM NB lineages.(a) In the canonical model of asymmetric NB division, a single neurogenic division of the small GMC progenitor cell produces two neurons (N) at each round of NB division. Unequal partitioning of Prospero promotes neurogenic division by inhibiting self-renewing factors in the GMC. **(b)** The DM NB divides asymmetrically without Prospero, which enables the small daughter cell to retain self-renewing potential and to behave as an IP. In this cell, expression of Prospero and unknown polarization cues re-established the asymmetric segregation of fate determinants and the generation of the neurogenic progenitor GMC. This novel mode of neurogenesis increases the number of post-mitotic neurons that individual NBs in the dorsomedial brain can generate at each round of divisions.

Although the DM NBs do not express and segregate Prospero to their daughter intermediate precursors, these daughter cells do express Prospero in a cortical and polarized manner during mitosis. The off/on state of Prospero must be kept under tight control for a controlled amplification of proliferation achieved in DM lineages since complete mutational loss of Prospero in brain clones leads to uncontrolled proliferative activity and brain tumor formation [104-106, 114]. Indeed, our observations on the DM lineages imply that deregulated IPs that fail to express Prospero might be an important source of tumor cells in the brain. Interestingly, region-specific action of tumor suppressor genes in the larval brain has been previously reported using somatic cell clones [105].

3 Postembryonic development of transit amplifying neuroblast lineages in the *Drosophila* brain.

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3.1 Summary

Specific dorsomedial (DM) neuroblast lineages of the *Drosophila* brain amplify their proliferation through generation of transit amplifying intermediate progenitor cells [116]. Together, these DM neuroblast lineages comprise over 5000 adult-specific neural cells and thus represent a substantial part of the brain. However, currently there is no information available about the structure or function of any of the neural cells in these DM lineages. In this report we use MARCM-based clonal analysis together with immunocytochemical labeling techniques to investigate the type and fate of neural cells generated in the DM lineages.

Genetic cell lineage-tracing and immunocytochemical marker analysis reveal that DM neuroblasts are multipotent progenitors which produce a set of postembryonic brain glia as well as a large number of adult-specific protocerebral neurons. During larval development the adult-specific neurons of each DM lineage form several spatially separated axonal fascicles, some of which project along larval brain commissural structures which are primordia of midline neuropil. By taking advantage of a specific Gal4 reporter line, the DM-derived neuronal cells can be identified and followed into early pupal stages. During pupal development the neurons of the DM lineages arborize in many parts of the brain and contribute to neuropil substructures of the developing central complex, such as the fan-shaped body, noduli and protocerebral bridge.

Our findings provide cellular and molecular evidence for the fact that DM neuroblasts are multipotent progenitors; they, thus, represent the first identified progenitor cells in the fly brain that have neuroglioblast functions during postembryonic development. Moreover, our results demonstrate that the adult specific neurons of the DM lineages arborize widely in the brain and also make a major contribution to the developing central complex. These findings suggest that the amplification of proliferation which characterizes DM lineages may be an important requirement for generating the large number of neurons required in highly complex neuropil structures such as the central complex in the *Drosophila* brain.

3.2 Introduction

The *Drosophila* brain is a highly complex structure composed of tens of thousands of neurons that are interconnected in numerous exquisitely organized neuropil structures such as the mushroom bodies, antennal lobes and central complex. The neurons of the central brain, defined as the supraesophageal ganglion without the optic lobes, derive from approximately 100 bilaterally symmetrical pairs of neural stem cell-like neuroblasts, each of which is thought to generate a characteristic lineage of neural progeny (see [117], [3]). A number of studies indicate that each developing neuroblast acquires an intrinsic capacity for neuronal proliferation in a cell autonomous manner and generates a specific lineage of neural progeny which is nearly invariant and unique. This implies that each neuroblast acquires a specific identity which determines the number and types of neural progeny it generates. This specification of neuroblasts has been shown to occur through a combination of positional information, temporal cues, and combinatorial cues provided by the suite of developmental control genes expressed by each precursor (for reviews see [94];[92];[115]).

Neuroblasts begin to proliferate during embryonic development and during this initial phase of proliferation they generate the primary neurons of the larval brain. After a period of mitotic quiescence during the early larval period, most brain neuroblasts reactivate proliferation and produce secondary neurons which make up the bulk of the adult brain and are, hence, referred to as adult-specific neurons ([5]; [2]). Indeed 95% of the neurons in the adult brain are secondary neurons generated during postembryonic development. These adult-specific neurons initially form a lineage-related cluster of immature neurons that extend fasciculated primary neurites into the neuropil but wait until metamorphosis to complete their extension to synaptic targets and final morphogenesis [6-8, 11].

Most neuroblasts in the central brain generate lineages comprising on average 100-120 adult-specific cells [116]. (The neuroblasts that generate the intrinsic cells of the mushroom bodies each produce an average of approximately 200 adult-specific cells; these neuroblasts do not enter a quiescent state in early larva.) In contrast, remarkably large neuroblast lineages are generated in the dorsomedial (DM) area of the larval

brain. The number of adult-specific cells in these DM neuroblast lineages has an average value of 450 and is thus more than twice the average number of cells in the mushroom body lineages [116]. The large number of neurons in these lineages is achieved by an amplification of neuroblast proliferation through generation of intermediate progenitor cells. Most neuroblasts in the central brain divide asymmetrically in a stem cell mode whereby they self-renew and generate smaller daughter cells called ganglion mother cells (GMCs) which divide once to produce two postmitotic progeny ([92]; [115]; [93]; [118]; [119]). In contrast, dividing DM neuroblasts (also referred to as a PAN neuroblasts or Type II neuroblasts) self-renew and generate intermediate progenitor cells which act as transit amplifying cells and can generate numerous GMC-like cells by retaining their ability to divide several more times ([116, 120, 121]). In this respect, neurogenesis in DM lineages is similar to that seen in the mammalian CNS in which the primary progenitors amplify the progeny they produce through the generation of proliferating intermediate progenitors [89, 122]. (In addition to the six pairs of DM neuroblasts located in the dorsomedial area of the brain, there are two additional pairs of PAN (Type II) neuroblasts located more laterally in the brain [121]; because they are easier to identify, we focused our analysis on the 6 dorsomedial DM neuroblasts.)

The six bilaterally symmetrical pairs of DM (Type II) neuroblast lineages together generate over 5000 adult-specific cells due to the amplification of neuroblast proliferation [116]. Given current estimates of total cell number in the *Drosophila* brain [88], this cell number would roughly correspond to one fourth of the total number of cells in the central brain. The DM lineages thus represent a substantial part of the brain. However, currently there is no information available about the phenotypic fate of any of the neural cells in the DM lineages. It is not known if the cells in these lineages are exclusively neuronal or if glial cells are also generated. Nor is it known if the neurons in these lineages are involved in the formation of specific complex neuropil structures or if they project widely throughout the brain. This total lack of information on the type of cells generated and their roles in brain circuitry, thus, represents a major obstacle in understanding the development of the fly brain.

3.3 Results

3.3.1 Dm neuroblast lineages contain adult-specific neurons and glial cells

During larval development, six DM neuroblasts generate large lineages each of which consists of an average of 450 cells that are located at the dorsomedial midline of each hemisphere [116, 121]. These DM lineages can be identified based on their overall size and position by using mosaic-based MARCM techniques to label neuroblasts and their postembryonic progeny in the larval brain. For this, random mitotic recombination was induced in neuroblasts by heat-shock induction of FLP within a few hours after larval hatching (ALH) in order to obtain positive labeling of the neuroblasts and their clonal post-mitotic progeny (hereafter referred to as neuroblast clones). GFP-labelled neuroblast clones corresponding to each of the six DM lineages were recovered at the late third instar stage and co-stained with neural cell-specific molecular labels. All of these clones consisted of more than 350 cells and were thus significantly larger than any of the other neuroblast lineages in the larval brain [116]. Clonal cell numbers were not significantly different if MARCM labelling was induced in the larva a few hours after hatching (450 cells; range 370-580) or in the embryo at stage 13 (468 cells; range 362-545), underscoring the fact that most of neurons in the fly brain are generated postembryonically.

All postembryonic DM lineages contained a set of intermediate neuronal progenitors located near the neuroblast; these cells have been described previously [116, 120, 121] and are not considered further here. To determine if the post-mitotic cells in the DM lineages were all adult-specific neuronal cells or if they also comprised glial cells, GFP labelled neuroblast clones were co-labelled with the neuron-specific marker anti-Elav and the glial cell-specific marker anti-Repo. In all cases, we found that the great majority of the cells in DM lineages were Elav-positive and thus corresponded to neuronal cells, however, we also found that DM lineages consistently contained Repo-positive, Elav-negative glial cells (Fig. 3.1A-C). These DM-derived glial cells were located distal to the neuroblast in the clone of labelled cells suggesting that they might be among the more early born cells in the lineage. (Birth order and clonal position are correlated; cells located proximal to the neuroblast and near the cell cortex in labelled clones tend to be late born, those located more distally and

deeper in labelled clones are usually early born; see [3]). However, since glial cells can migrate following their generation, more precise birth-dating experiments are required in order to substantiate this notion. The average number of GFP-labelled glial cells in DM neuroblast clones at 96h ALH was 13 ± 4.4. DM clones recovered at 72 h ALH also contained an average of 13 ± 6.1 cells suggesting that most of the glia had been generated by this time.

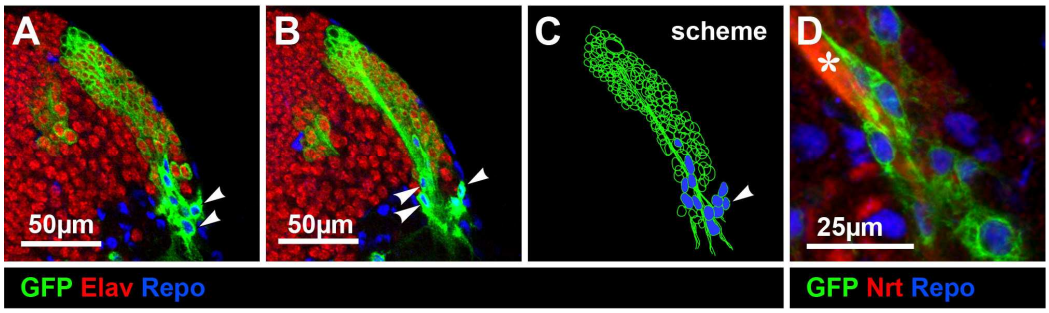

Figure 3.1 DM neuroblast lineages contain both neurons and glial cells
(A, B) Consecutive confocal images (from dorsal to ventral) of a DM MARCM clone (GFP, green). Neuronal cells are Elav-positive and glial cells are Repo-positive (blue, arrowheads). (C) Scheme of the DM lineage: note that localization of the glial cells is distal to the neuroblast in the clone. (D) A close-up view of the DM glial cells. Secondary neuronal cells, their projections and common axonal bundle are stained with anti-Neurotactin (red) and glial cells are Repo-positive (blue). Glial cells extend processes along the axonal bundle (asterisk).

The DM lineage glial cells had the general structural features of neuropil glia. In the late third larval instar, their cell bodies were generally clustered in the vicinity of the emergent secondary axon tracts of the DM lineages, and their processes were associated with these tracts (Fig. 3.1D). Furthermore, DM lineage glial processes were also often associated with larval interhemispheric commissures. Repo-positive surface and cortex glia located at the dorsal midline immediately adjacent to the DM clones were never GFP-labelled. Since the DM lineages contain both neurons and glia, the DM neuroblasts are, in terms of their proliferative potential, neuroglioblast-like. They are likely to be the only neural progenitors with neuroglioblast features present in the postembryonic brain; all other postembryonically generated glia are thought to be generated by symmetrically dividing glial precursors [27]. In accordance with this, when we examined 153 non-DM (canonical) postembryonic neuroblast lineages we never found glial cells in the labelled clones.

Taken together, these findings uncover an unexpected feature of these neuroblast lineages; they are not only by far the largest in terms of overall cell number, they also comprise glial cells and thus represent the only known neuroglioblast lineages in the postembryonic central brain.

3.3.2 DM neurons form commissural and longitudinal secondary axon tracts in the larval brain

To study the axonal projections of the adult-specific neurons of DM lineages in the larval brain, we recovered MARCM-labelled neuroblast clones corresponding to each of the six DM lineages and co-immunostained these with an anti-Neurotactin antibody which labelled secondary neurons and their axon tracts (clones were induced at early first instar stage and recovered at the late third instar). In all DM lineages, axons from the adult-specific neuronal cells fasciculated to form initial secondary axon tracts within the cortical layer. As soon as these initial secondary axon tracts reached the brain neuropil, they split into several subsidiary tracts. This contrasted with the behavior of secondary tracts in most other brain lineages which generally formed a single, discrete fascicle of axons within the brain cortex and neuropil during larval stages ([3]; [11]). In all six DM lineages, some of these subsidiary secondary tracts projected into the interhemispheric commissures while others formed ipsilateral descending or ascending projections. Similar to the secondary axon tracts formed by other neuroblast lineages in the late larval brain, these DM-derived secondary axon tracts traveled for variable distances within the brain hemispheres but had not yet evolved into the long axon tracts that characterize the adult brain.

The DM lineages formed a set of secondary axon tracts with a characteristic and relatively invariant trajectory in the larval brain neuropil. (Based on the rostral-to-caudal arrangement of the cell body clusters of the six DM lineages in the larval brain hemispheres we numbered each of these as follows: DM1 (most rostral), DM2, DM3, DM4, DM5, DM6. The characteristic neuroanatomical features of the secondary axon tracts formed by all six DM lineages in the late third instar larval brain are described below. For a given DM lineage, these anatomical features were the same irrespective of whether MARCM clones were induced in the stage 13 embryo or in the early larva.

The DM1 lineage formed a secondary axon tract that projected towards the interhemispheric region, where it separated into two fascicles: the first fascicle entered a larval interhemispheric commissure and immediately split into multiple commissural fiber bundles; the second fascicle formed a descending projection, from which an additional small commissural bundle also branched off (Fig. 3.2A-D). The commissural fiber bundles crossed the midline and a subset of these entered the neuropil of the contralateral hemisphere; within the commissures these fiber bundles appeared to defasciculate into a network of smaller axon bundles which projected along multiple commissural pathways (Fig.3.2 E, F). The larval commissure in which these fibers projected corresponds to the DPC1 commissure as defined by Pereanu and Hartenstein [11]. The descending fascicle, which in contrast remained tightly bundled, projected along the medial edge of the ipsilateral hemisphere.

The DM2 lineage formed a secondary axon tract that projected toward the commissural system and also split into multiple commissural fiber bundles and one descending fascicle (Fig. 3.3A-D). The set of DM2 commissural bundles entered the same larval commissure as the DM1 commissural fascicle, but using a different site of entry from that used by the DM1 fibers. (We refer to these commissure entry sites as site 1 and site 2, respectively.) DM2-derived commissural bundles defasciculated into smaller lattice-like projections that crossed the midline, and some of these entered the contralateral hemisphere (Fig. 3.3E, F). Glial cells from the DM2 lineage were often observed near the site of commissure entry. The single descending fascicle projected along a pathway that was roughly parallel to the one formed by the DM1 descending fascicle but was located more laterally in the hemisphere.

The DM3 lineage initial secondary axon tract, upon entering the neuropil split into three fiber bundles (Fig. 3.4A-D). Two of these bundles entered the commissural system at two sites; one site was the same as used by DM2 commissural fascicles (site 2) while the other was DM3-specific (site 3). Within the larval commissure, both axon bundles defasciculated into a meshwork of smaller bundles, that crossed the midline and entered the contralateral hemisphere (Fig. 3.4E, F). The third axon bundle formed a descending fascicle which projected along a pathway that differed from the two used by DM1 and DM2 descending fascicles.

The DM4 lineage had the most complex projection pattern of all the larval DM lineages. Its initial secondary axonal tract first split into three main axon bundles, and each of these formed several subsidiary axonal bundles upon entering the neuropil (Fig.3.5A-D). The result of this subdivision process was that the axons from this lineage formed three different and spatially separated commissural fascicles as well as into one ascending fascicle and one descending fascicle. It is noteworthy that among the three commissural fascicles, only one projected in the same larval interhemispheric commissural structure as did the other DM lineages, but it did so via an entry site (site 4) different from those used by axons of the DM1-3 lineages (Fig. 3.5E, F). The other two fascicles which projected across the midline did so in other, more dorsally located larval brain commissural structures.

The DM5 lineage secondary axon tract, upon entering the neuropil, split into several commissural fascicles and one major ascending fiber bundle (Fig. 3.6A-D). The single ascending fiber bundle subdivided into two short branches. (A minor short descending fascicle was also observed; see arrowheads Fig. 3.6B, C). All commissural fascicles of the DM5 lineage projected in the same larval commissural structure shared by the other DM lineages using the commissure entry site 4. Upon entering this commissure, the axon bundles defasciculated into smaller projections forming cross bridging structures (Fig. 3.6E, F).

The secondary axon tract of the DM6 also split into several commissural fibers and one major ascending fiber bundle (and one minor descending bundle) upon entering the neuropil. The anatomical features of all of these fiber bundles were very similar to those of the DM5 lineage (Fig. 3.7A-F). Thus, commissural fascicles entered the commissure at site 4 and subsequently defasciculated, and the ascending fiber bundle split into two branches. Indeed, in comparison to the DM1-4 lineages, which are individually distinct in their axon projection pattern types, the DM5 and DM6 lineages appear to form axonal projections patterns that are largely indistinguishable.

Taken together, these findings indicate that all of the DM lineages generate highly complex secondary axon tract projects in the larval brain. Our data reveal five types of axonal projection patterns all of which have pronounced (ipsilateral) longitudinal as well as commissural components. Four different types of axonal projection

patterns can be ascribed to the DM1, DM2, DM3, DM4 lineages and a common fifth DM5/6 type can be attributed to both DM5 and DM6 lineages.

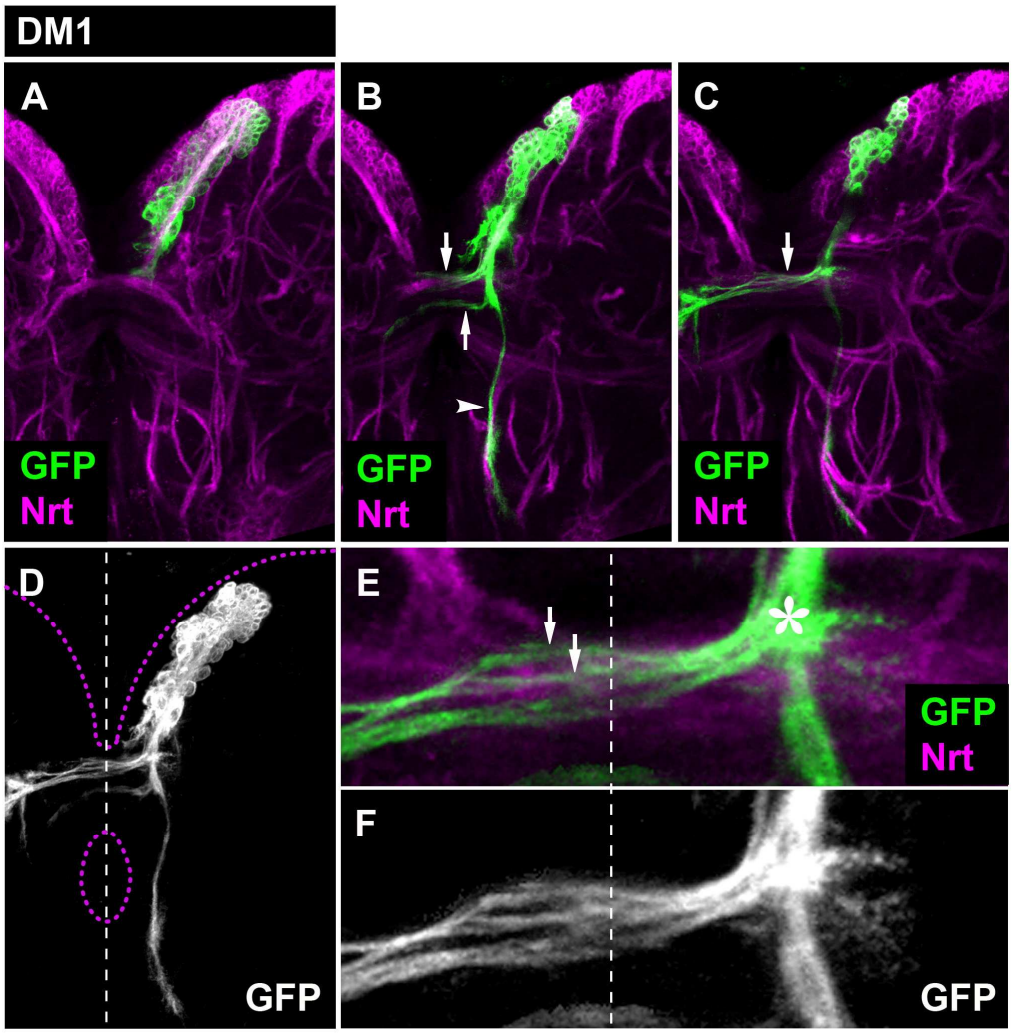

Figure 3.2 Projection pattern of the larval DM1 lineage
(A, B, C) Consecutive confocal images (from dorsal to ventral) of DM1 MARCM-labeled clone (GFP, green). Secondary neurons and their projections are stained with anti-Neurotactin (magenta). Note commissural (arrows) and longitudinal (arrowheads) projections. (D) Z-projection of the entire DM1 lineage. Brain and oesophagus outline shown as a magenta dashed line, midline is a white dashed line. (E, F) A close-up view of the commissural projections. Note how the axonal bundle defasciculates (arrows) upon entering the commissure. Also note that DM1 axons enter the commissure at a most medial site (asterisk).

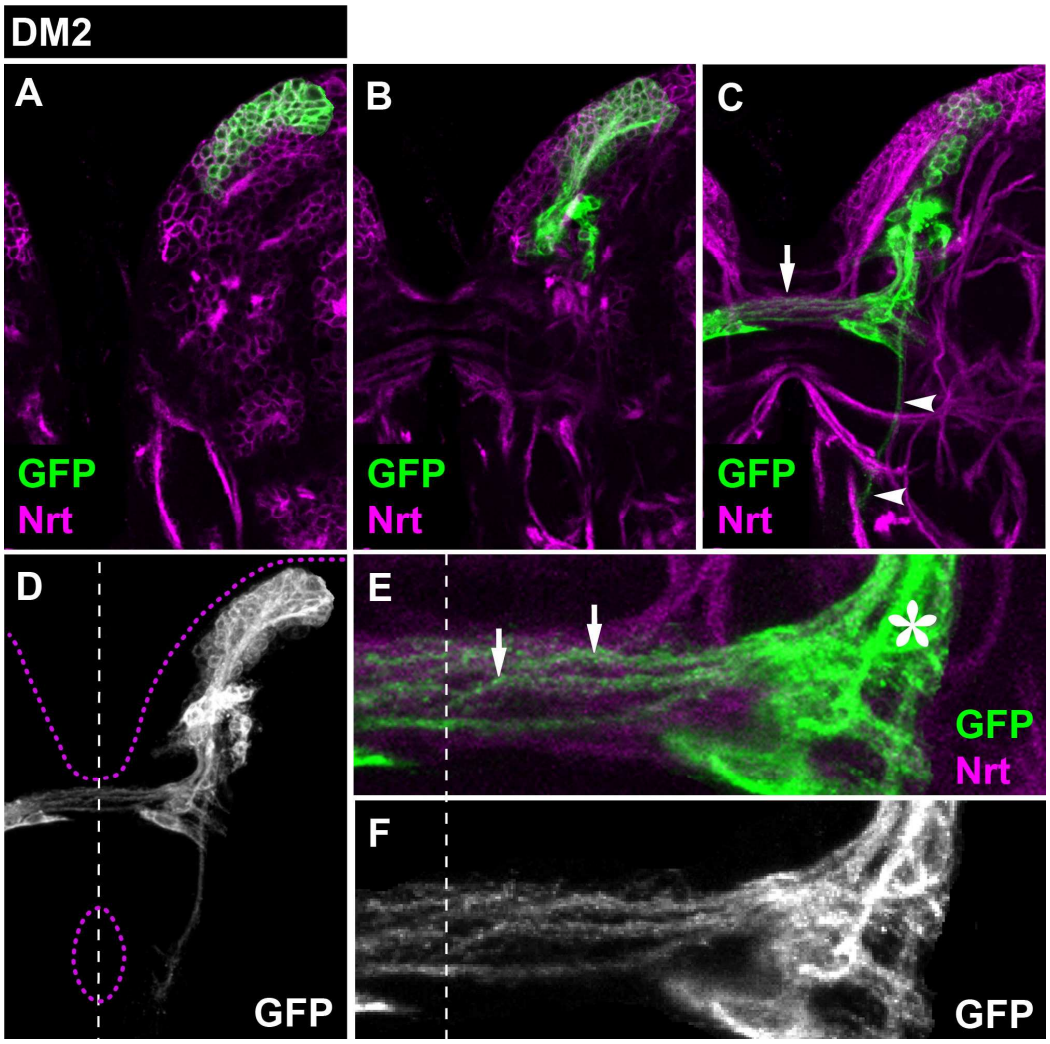

Figure 3.3 Projection pattern of the larval DM2 lineage
(A, B, C) Consecutive confocal images (from dorsal to ventral) of DM2 MARCM-labeled clone (GFP, green). Secondary neurons and their projections are stained with anti-Neurotactin (magenta). Note commissural (arrows) and longitudinal (arrowheads) projections. (D) Z-projection of the entire DM2 lineage. Brain and oesophagus outline shown as a magenta dashed line, midline is a white dashed line. (E, F) A close-up view of the commissural projections. Note how the axonal bundle defasciculates (arrows) upon entering the commissure. Also note the site where DM2 axons enter the commissure (asterisk).

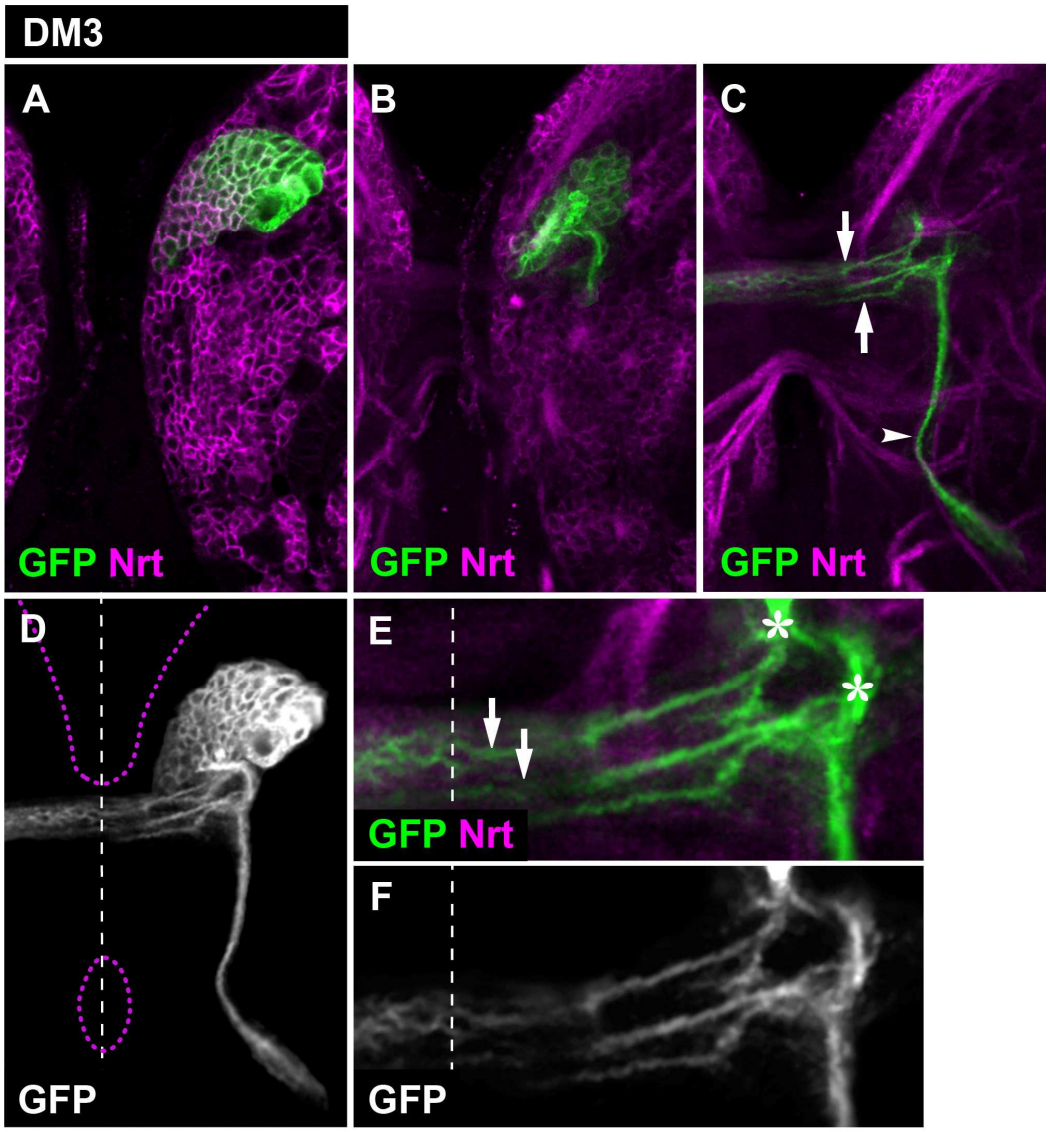

Figure 3.4 Projection pattern of the larval DM3 lineage
(A, B, C) Consecutive confocal images (from dorsal to ventral) of DM3 MARCM-labeled clone (GFP, green). Secondary neurons and their projections are stained with anti-Neurotactin (magenta). Note commissural (arrows) and longitudinal (arrowheads) projections. (D) Z-projection of the entire DM3 lineage. Brain and oesophagus outline shown as a magenta dashed line, midline is a white dashed line. (E, F) A close-up view of the commissural projections. Note how the axonal bundle defasciculates (arrows) upon entering the commissure. Also note the site where DM3 axons enter the commissure (asterisk).

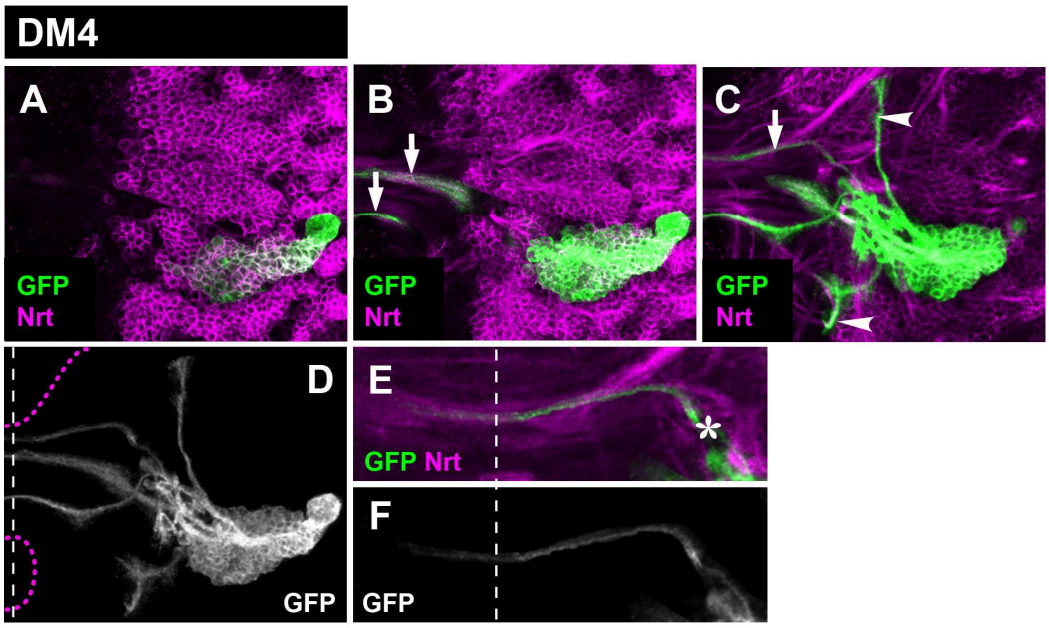

Figure 3.5 Projection pattern of the larval DM4 lineage
(A, B, C) Consecutive confocal images (from dorsal to ventral) of DM4 MARCM-labeled clone (GFP, green). Secondary neurons and their projections are stained with anti-Neurotactin (magenta). Note commissural (arrows) and longitudinal (arrowheads) projections. (D) Z-projection of the entire DM4 lineage. Brain and oesophagus outline shown as a magenta dashed line, midline is a white dashed line. (E, F) A close-up view of the commissural projections. Note the site where DM4 axons enter the commissure (asterisk).

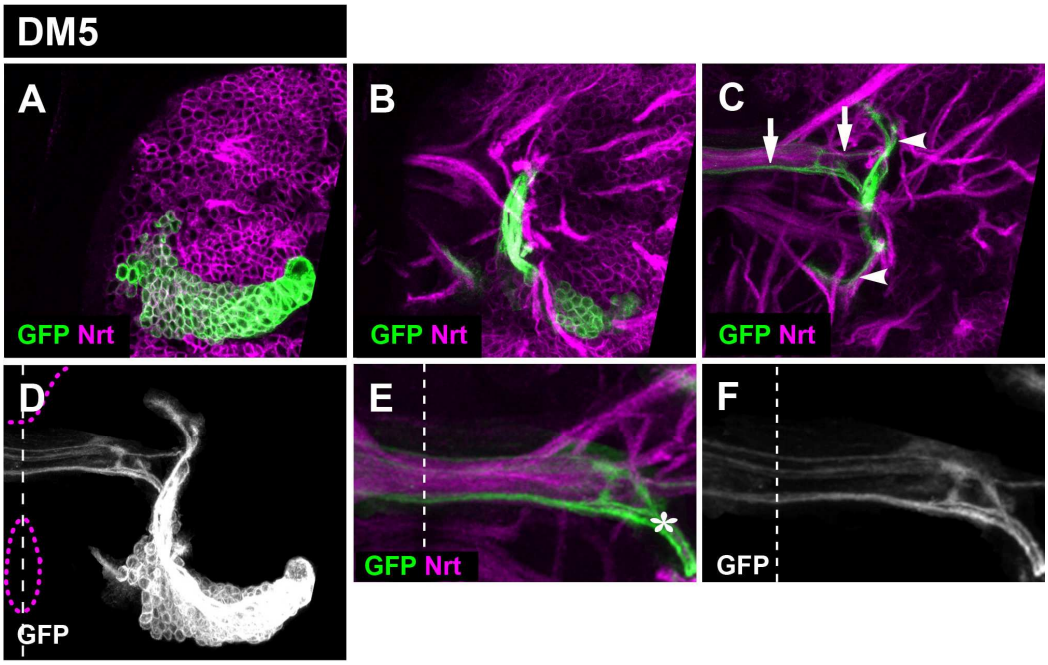

Figure 3.6 Projection pattern of the larval DM5 lineage
(A, B, C) Consecutive confocal images (from dorsal to ventral) of DM5 MARCM-labeled clone (GFP, green). Secondary neurons and their projections are stained with anti-Neurotactin (magenta). Note commissural (arrows) and longitudinal (arrowheads) projections. (D) Z-projection of the entire DM5 lineage. Brain and oesophagus outline shown as a magenta dashed line, midline is a white dashed line. (E, F) A close-up view of the commissural projections. Note the site where DM5 axons enter the commissure (asterisk).

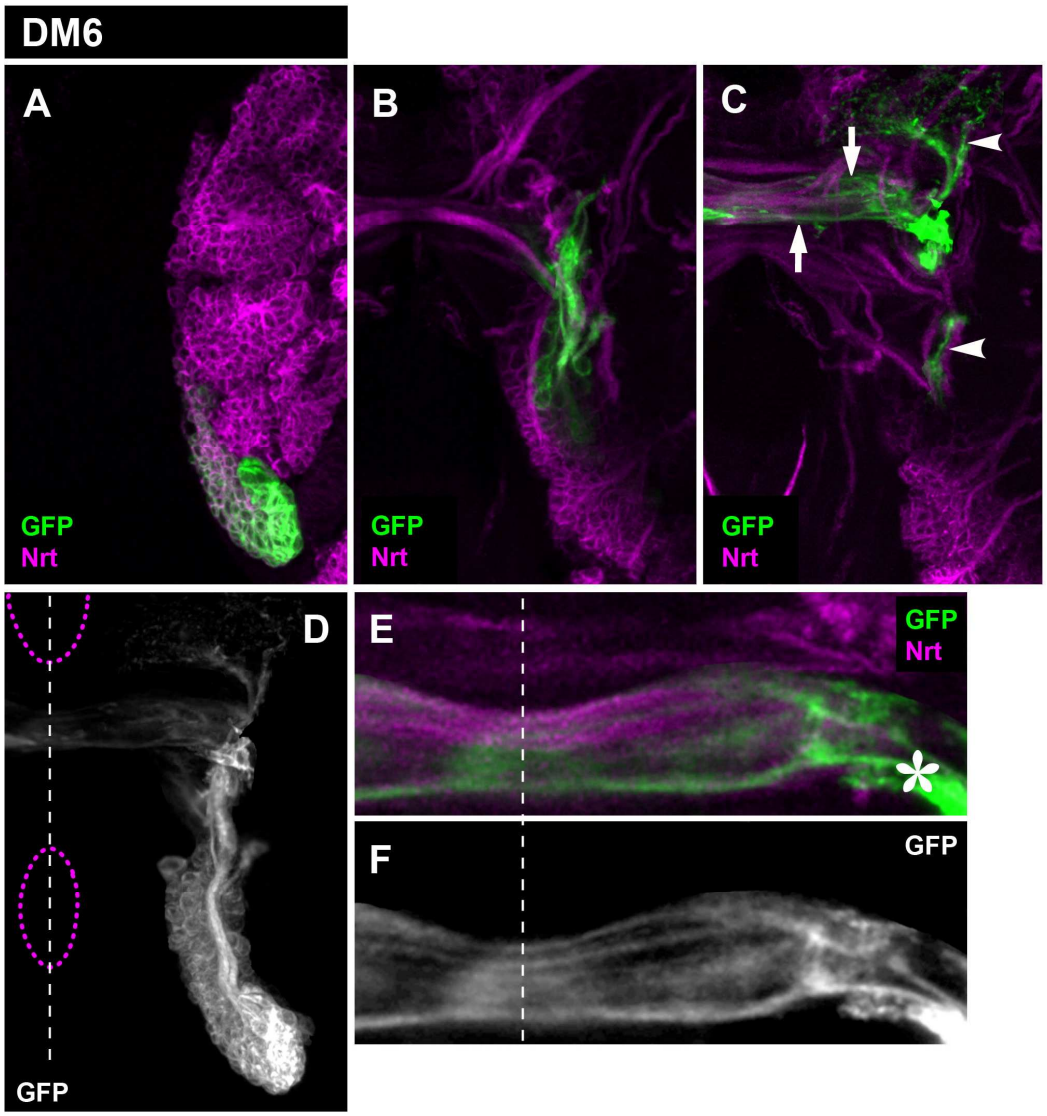

Figure 3.7 Projection pattern of the larval DM6 lineage
(A, B, C) Consecutive confocal images (from dorsal to ventral) of DM6 MARCM-labeled clone (GFP, green). Secondary neurons and their projections are stained with anti-Neurotactin (magenta). Note commissural (arrows) and longitudinal (arrowheads) projections. (D) Z-projection of the entire DM6 lineage. Brain and oesophagus outline is shown as a magenta dashed line, midline is the white dashed line. (E, F) A close-up view of the commissural projections. Note the site where DM6 axons enter the commissure (asterisk).

3.3.3 A Dll-Gal4 line labels DM neuroblast lineages in the postembryonic brain

In the late larval brain, the six DM lineages located at the dorsomedial margins of each brain hemisphere can be identified based on their overall size and position by using mosaic-based MARCM techniques. In subsequent postembryonic stages, unambiguous identification of DM lineages based only on size and position is no longer possible due to the pronounced morphological changes caused by the extensive growth of neuropil in the brain and developing optic lobes. In order to identify DM lineages and analyse the cellular phenotypes of their neural cells in pupal development, we searched for Gal4 lines that might label the DM lineages. We identified such a line carrying a Gal4 transgene insertion into the promoter of the *distal-less* gene ([123]; in the following referred to as Dll-Gal4) which revealed six large groups of cells at the dorsomedial margins of the brain hemisphere when coupled to the reporter genes UAS-CD8::GFP (membrane labeling) or UAS-H2B::RFP (nuclear labeling) (Fig. 3.8A-A", B-B").. This Gal4 line also labelled cell clusters in the optic lobes as well as scattered cells in the ventral ganglia; these were not considered further here. Based on number of labelled cells as well as on their position in the developing brain hemispheres of late larval stages, the groups of cells revealed by Dll-Gal4 were likely to be DM lineages.

To confirm that the Dll-Gal4 labelled cell groups at the dorsomedial brain midline were indeed the DM lineages, a more detailed cellular and molecular analysis of the labelled cells was carried out. A characteristic hallmark of DM lineages is the fact that they contain multiple mitotically active cells located at variable distance from the parental neuroblast [116, 120, 121]. By using an anti-PH3 antibody to detect mitotic DNA (via the phospho-histone 3 epitope) in larval brain expressing the reporter genes under Dll-Gal4 control, labelled cell clusters did display the expected pattern of multiple mitotic cells throughout the cluster (Fig. 3.8B-B", C-C"). A second diagnostic feature of DM lineages is the fact that both the DM neuroblast and a few closely associated cells the intermediate progenitors lack nuclear Prospero expression at interphase, whereas the intermediate progenitors typically show asymmetric cortical distribution of Prospero protein at mitosis [116, 120, 121]. To investigate if this was a characteristic of the Dll-Gal4 labelled cell clusters, we co-immunolabelled these cell clusters with anti-Prospero with anti-PH3 antibodies. In all cases, Dll-Gal4 labelled cell clusters had the expected immunostaining patterns. The neuroblasts and

closely associated cells were Prospero-negative and mitotically active (PH3-positive), intermediate progenitors showed cortical Prospero crescents (Fig. 3.8C-C"). Based on these findings, we conclude that the six cell clusters labelled by the Dll-Gal4 reporter line in the developing central brain correspond to the DM neuroblast lineages.

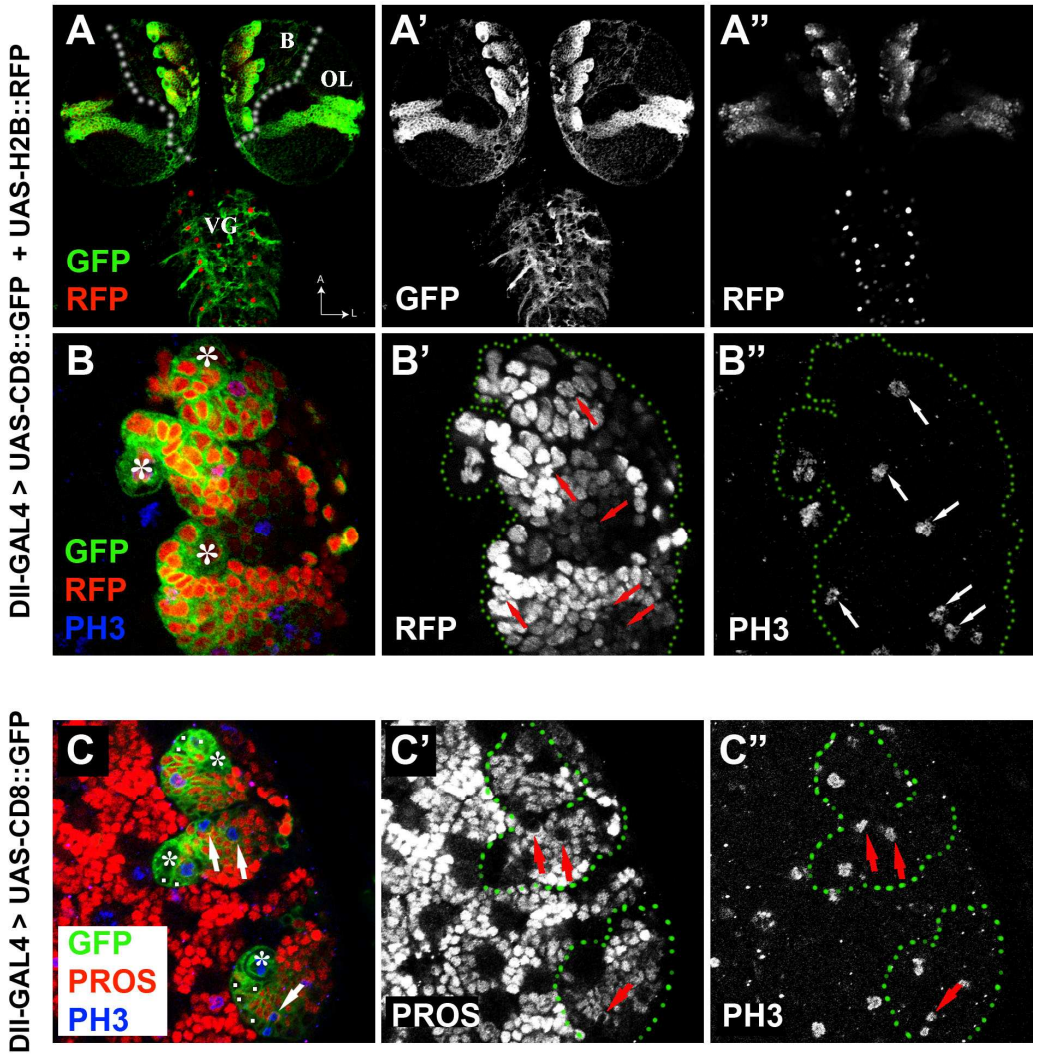

Figure 3.8 Specific labeling of DM lineages by Dll-Gal4
Confocal images of third instar larval nervous system expressing mCD8::GFP (green) and H2B::RFP (red in A,B) under the control of Dll-GAL4 and immunostained with anti phospho-histone3 (PH3, blue) and anti-prospero (PROS, red in C). Panels on the left column show merged views of indicidual channels presented in grey scale on the middle and right culumns for clarity. Green dotted lines indicate the contours of the GFP-labeled domains.
A-A": dorsal view of a larval CNS at low magnification reveals restricted expression of the reporter genes in the medial part of the brain hemispheres (B) in the optic lobes (OL) and a few cells in the ventral ganglia (VG).

B-B": Higher magnification of the area boxed in A. Shown are three DM neuroblasts (asterisks) and their closely associated progeny cells. Scattered cells undergoing mitosis are observed throughout the cell clusters (arrows).
C-C": Three DM lineages marked by Dll-GAL4 expression lack expression of Prospero in the neuroblasts and their surrounding daughter IP cells (C: asterisks and white dots respectively). IP cells undergoing mitosis (arrows) are marked by PH3 staining (C"; arrows). They show a crescent localization of of prospero (C'; arrows) in contrast to the nuclear localization of the protein in most post-mitotic cells visible in the field.

Taken together, these reporter line-based studies provide cellular and molecular data that confirm the identification of DM lineages based on anatomical criteria. Moreover, they provide a labeling method for DM lineages in early pupal stages.

3.3.3 DM neurons form widespread arborizations and innervate the developing central complex in the pupal brain

During pupal development, the relatively simple secondary axon tracts generated by adult-specific neurons in the larval brain arborize and undergo final morphogenesis [6-8, 11]. Given the multiplicity of longitudinal and commissural components that characterize the secondary axon tracts of the DM lineages during larval development, it might be expected that DM lineages will form multiple and widespread arborizations during pupal development. Moreover, since all six DM lineages project a subset of their fiber bundles into the larval DPC1 commissure, it seems likely that some of these arborizations will contribute to the central complex neuropil during pupal development. (The DPC1 commissure is thought to represent part of the primordium of the central complex [11]).

To investigate this, Dll-Gal4 based MARCM labeling of DM neuroblast clones was induced at early first instar larval stage. Labelled DM clones were recovered at 24h after puparium formation. (This time point for clone recovery was chosen because major neuropil structures of the future adult brain were already identifiable.) In contrast to the larval DM lineages, each of which was easily identifiable in the larval brain, the recovered MARCM labelled pupal DM lineages could not be unambiguously individually identified since lineage-related neurons sometimes changed position and no longer formed compact groups during pupal development. In consequence, we could not assign individual arborization patterns with confidence to any one of the six pupal DM lineages. Nevertheless, two features of the adult-specific DM neurons characterized all of the MARCM labelled DM lineages in early

pupal development. First all DM lineages formed multiple axonal projections and widespread arborizations in the brain. Second, all DM lineages projected a subset of their axon fascicles into the central complex neuropil. Two examples of the type of adult-specific arborizations formed by DM lineages in the early pupal brain are shown in figures 3.9 and 3.10.

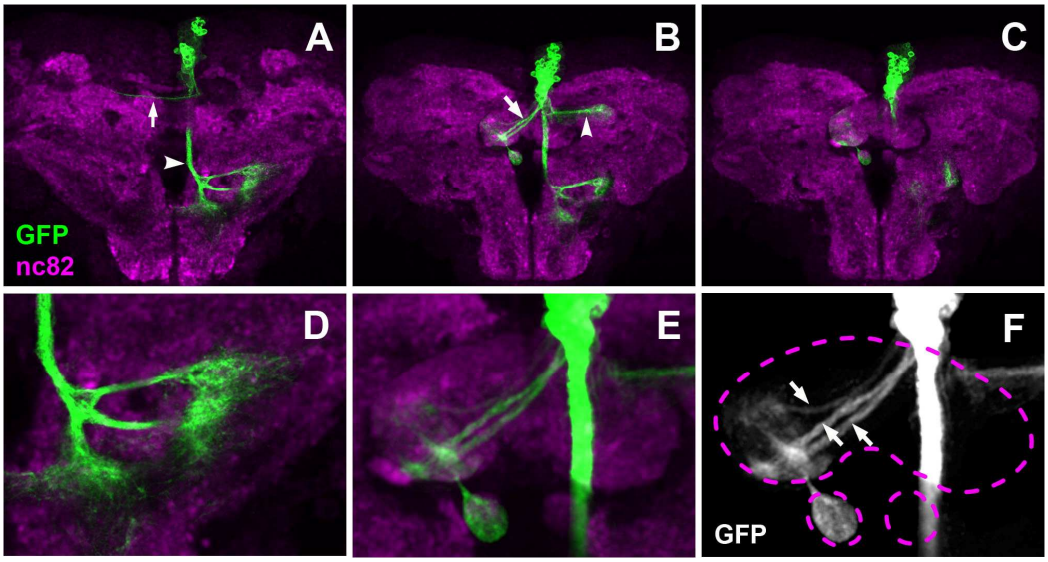

Figure 3.9 Example of a first DM lineage projection pattern in the pupal brain at 24h after puparium formation
(A, B, C) Consecutive confocal Z-projections of a DM MARCM-labeled clone (GFP, green). Brain neuropil is labeled with anti-nc82 (magenta). Note the contralateral (arrows) and ipsilateral (arrowheads) projections. (D) A close-up view of the ipsilateral arborisation in the posterior part of the brain. (E, F) A close-up view of the projections into the developing fan-shaped body and the contralateral nodulus. Note two major fascicles and one minor fascicle (arrows) entering the fan-shaped body and forming columnar arborisations.

Figure 3.9 shows a MARCM labelled DM lineage (probably a DM1 lineage) in which the cell bodies formed a discrete cluster positioned dorsally and close to the brain midline (Fig. 3.9A-C). From this cell body cluster, a major ipsilateral descending fascicle was evident as a thick axon bundle which descended near the midline into a more ventral region of the neuropil where it branched and formed arborizations (Fig. 3.9A, B, D). The DM cell body cluster also gave rise to two major commissural fascicles which projected in parallel across the midline via the protocerebral bridge and entered the fan-shaped body of the central complex from the posterior aspect (Fig. 3.9B, E, F). Within the fan shaped body, these fascicles formed arborizations in two

contralateral domains, and they also projected to and arborized in the contralateral nodulus. Moreover in the protocerebral bridge, they formed a restricted domain of arbors (data not shown). In addition to these major descending and commissural fascicles, several minor axon bundles were observed emanating from the DM neuron cell bodies; one of these formed a T-shaped bifurcation and projected ipsilaterally and contralaterally into in the dorsal neuropil (Fig. 3.9A, B).

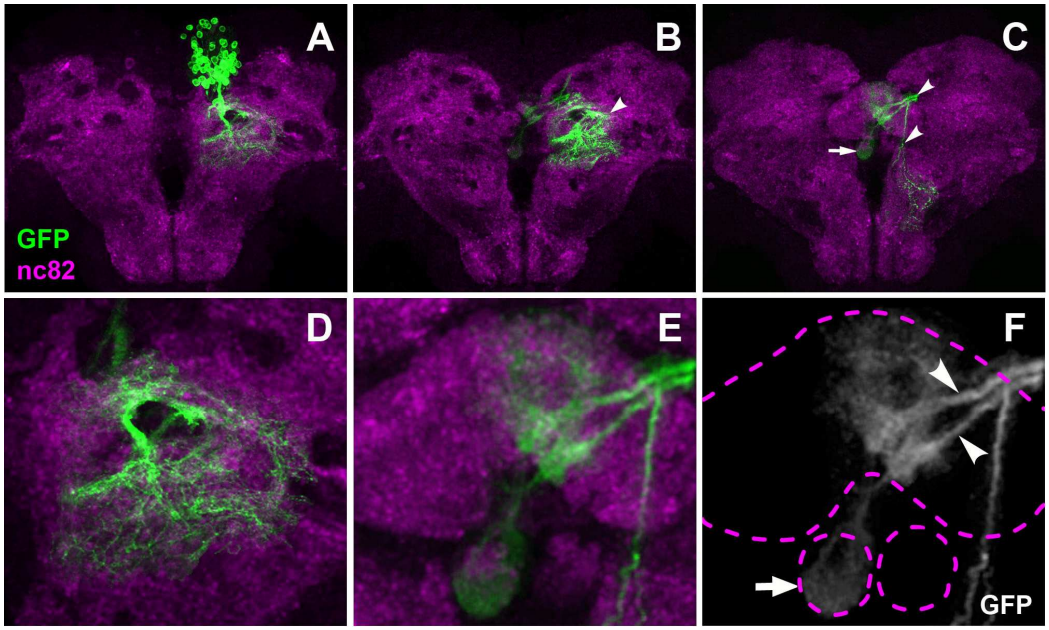

Figure 3.10 Example of a second DM lineage projection pattern in the pupal brain at 24h after puparium formation
(A, B, C) Consecutive confocal Z-projections of a DM MARCM-labeled clone (GFP, green). Brain neuropil is labeled with anti-nc82 (magenta). Note the contralateral (arrows) and ipsilateral (arrowheads) projections. (D) A close-up view of the major ipsilateral arborisation. (E, F) A close-up view of the projections into the developing fan-shaped body and contralateral nodulus. Note two fascicles (arrows in F) entering the fan-shaped body and forming columnar arborisations.

Figure 3.10 shows a MARCM labelled DM lineage (probably a DM3 lineage) in which the cell bodies formed a cluster positioned dorsally and close to the midline but located more posteriorly in the brain than the previous DM lineage (Fig. 3.10A). From this cell body cluster, a short major axon bundle descended ipsilaterally and formed a large zone of arborizations in the dorsal brain neuropil (Fig. 3.10A, B, D). The DM cell body cluster also gave rise to two major fascicles which projected towards the midline and entered the fan-shaped body of the central complex from the posterior aspect (Fig. 3.10B, E, F). Within the fan-shaped body, these two fascicles formed arborizations in two ipsilateral domains, and they also projected to and

arborized in the contralateral nodulus. Moreover in the protocerebral bridge, they formed a restricted domain of arbors (data not shown). In addition to these three major commissural, one minor axon bundle was observed; this descending axon bundles projected along the ipsilateral midline into posterior brain neuropil where it formed arborizations (Fig. 3.10C).

For a more comprehensive characterization of the neuroanatomy of individual DM1-6 lineages in pupal development, lineage-specific labeling techniques will be required. For this, more selective Gal4 lines that subdivide the six DM neuroblasts into individuals will be needed. However, based on the ensemble of MARCM labelled pupal DM lineages that were recovered and analysed in this study, one common feature of all pupal DM lineages appears to be that a subset of the neurons in each of these lineages contributes to the developing central complex neuropil.

3.4 Discussion

In this report we have studied the postembryonic development of the neural cells generated by the DM lineages in the larval and early pupal brain. We report three main findings. First, DM lineages comprise both adult-specific neurons and glia; DM neuroblasts thus have features of neuroglioblasts. Second, during larval development, adult-specific neurons form complex secondary axon tracts composed of separate commissural and longitudinal fascicles. Third, during pupal development the commissural fascicles arborize in and contribute to the central complex neuropil. In the following, we discuss the major implications of these three findings.

3.4.1 DM neuroblasts are multipotent neuroglial progenitors

In *Drosophila*, as in other insects, glial cells fall into three classes, surface glia, cortex glia and neuropil glia, each of which is represented in the larval brain [3]. The glial cells of the early larval brain, also referred to as primary glia, arise from a small number of embryonically active neuroglioblasts. Glial numbers in the brain increase during larval development and this increase in cell number is due, at least in part to mitotic divisions of glial cells, however, the bulk of added (secondary) glial cells has been postulated to stem from the proliferation of unidentified secondary neuroglioblasts [28]. However, the identity of these postulated multipotent precursors in the postembryonic brain was unknown.

A recent developmental analysis by Awasaki et al. [27] has shown that among the different types of glial cells in the *Drosophila* brain, only the perineurial surface glia and the neuropil glia are extensively generated during postembryonic development, whereas most of the subperineurial surface glia and cortex glial cells are thought to have their origin in embryogenesis. Moreover, this analysis has provided evidence that perineurial glial precursors are distributed around the brain surface, whereas neuropil (ensheathing and astrocyte-like) glial cells are derived from specific proliferation centers within the brain. However, the progenitors of these

postembryonically generated glial cells as well as the mode of postembryonic glial proliferation still remained elusive. Here we identify the first postembryonic neuroglioblasts in the *Drosophila* brain. (Embryonic neuroglioblasts have been described previously in the ventral thoracic ganglia [71]). Indeed, all six DM lineages generate a set of glial cells with anatomical features of neuropil glia in addition to numerous neuronal cells during postembryonic development.

DM neuroblasts proliferate through asymmetric division that involves intermediate progenitors with transit-amplifying cell features ([116]; [120]; [121]). This implies that DM lineage-derived glia, unlike any other glial cell type in *Drosophila*, are generated by amplifying intermediate progenitors. However, an exact clonal analysis of the relationship between glial cells and intermediate progenitors in DM lineages will be required to validate this notion. There is some evidence that DM derived glial are generated early in the lineage. If this is indeed the case, it is conceivable that these glial cells might be important for the differentiation of the subsequently generated neuronal cells in these lineages. For example, the extended processes of DM-derived glia located near the emergent secondary axon tracts or associated with the larval brain commissure might be important in guiding axons of DM-derived neurons (which subsequently contribute to central complex neuropil) across the midline.

3.4.2 DM lineage neurons form complex secondary axon projections

During postembryonic development, secondary, adult-specific neurons generated by reactivated neuroblasts produce secondary lineages and axons of a given secondary lineage fasciculate with each other to form a discrete and generally unbranched secondary axon tract within the brain cortex and neuropil ([3]; [11]). In contrast, in the case of DM secondary lineages, discrete albeit short secondary axon tracts were visible in the cortex but were rarely observed in the neuropil. Rather, within the neuropil, the axons of any given DM lineage split into multiple axon fascicles which projected to very different parts of the brain as commissural and longitudinal axon

bundles. Thus, at the anatomical level, the DM lineage already appears to be subdivided into different neuronal subgroups with different outgrowth behavior during the larval stages.

The multiplicity of axonal bundles that emerge from a given DM lineage had features which are more reminiscent of a secondary axon tract system comprising the axon tracts of several lineages than a single secondary axon tract. This may be an indirect result of the fact that DM lineages contain 3-5 times more secondary neurons than do conventional neuroblast lineages, which, hence, would generate a 3-5 times more axons than conventional lineages resulting in an excessively large secondary axon tract in the neuropil if branching did not occur. While the underlying mechanisms are currently not known, it is also possible that this anatomical complexity is related to the particular mode of neurogenesis in the DM lineages which involves amplifying intermediate progenitors. Thus, a given intermediate progenitor might produce a neural progeny which develop a common type of axonal projection pattern, whereas progeny subsets derived from different intermediate progeny might develop different axonal projection types.

3.4.3 DM lineages contribute to the developing central complex

Like other adult-specific neurons, DM secondary neurons differentiate during the pupal period, when they evolve into the long tracts that characterize the adult brain and send out proximal as well as terminal arborizations that form synapse-rich neuropil circuitry. During the early pupal differentiation period, some of the neuropil structures that specifically characterize the adult brain become visible. Among these are the principle components of the central complex. The central complex, one of the most prominent neuropil structures in the adult brain, is located centrally between the two hemispheres and consists of four substructures. These are the protocerebral bridge, the fan-shaped body, the ellipsoid body, and the noduli, all of which are interconnected by sets of columnar interneurons that form regular projection patterns

[86]; [78]; [79]. A remarkable and common feature of DM lineages is that a subset of the neurons in each lineage contributes to the developing central complex neuropil.

In the late larval brain, a distinction of the four substructures of the adult central complex is difficult. However a putative midline neuropil primordium of the central complex has been described in the late larval brain in the form of a large commissural neuropil domain, consisting of the DPC1 and trCM and probably part of the DPC2 commissures [11]. All DM lineages project a subset of their axon bundles into the DPC1 during larval development. Based on their axonal projections (and the position of their cell body clusters in the central brain), we tentatively assign the DM1-6 lineages to the DPMm1/2, DPMpm1, DPMpm2, CM4, and CM5, CM1/2 of Pereanu and Hartenstein (2006) [11]. Interestingly, in the DPC1, these commissural bundles defasciculated into smaller lattice-like projections similar to those observed in the developing central complex of the grasshopper, an insect with direct development [124].

From the first day of pupal development onward, the major components of the fan shaped body, ellipsoid body, noduli and protocerebral bridge can be clearly recognized. In the early pupal brain, DM derived commissural neurons have already contributed to the arbors in the fan-shaped body, nodulus and protocerebral bridge. Although we were not able to identify them individually, it is very likely that the central complex-innervating DM neurons in the pupal brain are the same neurons that projected commissural axon bundles into the DPC1 commissure of the larval brain. This observation supports the notion that the DPC1 is indeed part of the central complex primordium.

A noteworthy feature of the DM derived projections in the fan-shaped body is the fact that two major fascicles are formed which project in parallel into the fan shaped body and form two separate arborization domains. This may reflect a contribution of a given DM lineage to a pair of the eight modular subdomains (“staves”) that make up the fan-shaped body ([88]; [86]). A similar contribution of the DM derived neurons to two sections (“glomeruli”) of the protocerebral bridge is also likely, however, single cell resolution will be required to resolve this.

Most neurons of the central complex belong to one of two categories: large-field elements or small-field elements-forming ([86]). A large-field neuron typically arborizes in only a single substructure and links it to one or two central brain regions outside the central complex. Small-field neurons, as a rule, connect small columnar domains of several substructures, and the majority of small-field cells are intrinsic to the central complex. In view of the specific arborization pattern of the DM derived neurons in the fan shaped body, noduli and protocerebral bridge, we hypothesize that most of these neurons represent columnar small field elements of the central complex. However, it should be noted that only a subset of the neurons in any given DM lineage are likely to innervate the central complex; all DM lineages form longitudinal projections to other parts of the brain. Thus, unlike the lineages that give rise to the mushroom body intrinsic neurons, the neuronal progeny of the DM lineages are not dedicated to a single neuropil center. Rather the unusually large number of neurons in these lineages appears to contribute to multiple, spatially separated neuropil areas in the developing brain.

4 DISCUSSION

A black cat crossing your path signifies that the animal is going somewhere.
Groucho Marx

4.1 Amplification of proliferation in *Drosophila* postembryonic brain.

Our findings provide cellular and molecular evidence for a new mode of neurogenesis in the larval brain of *Drosophila*. In the canonical model for postembryonic neurogenesis exemplified by the non-DM lineages of the brain and the lineages of the ventral ganglia, NBs divide asymmetrically in a stem cell mode to self-renew and generate a GMC that divides once to produce two post-mitotic cells that differentiate. In this process, cell fate determinants Prospero and Miranda segregated asymmetrically from the parent NB into the GMC. In the GMC Prospero acquires nuclear localization, forcing the cell to exit the cell cycle. Prospero also remains nuclear in the GMC's postmitotic progeny.

The data presented here demonstrate a novel model for neurogenesis exemplified by the DM NBs, which divide asymmetrically in a stem cell mode to self-renew and generate daughter cells (intermediate progenitors). In this process, they do not segregate the cell fate determinant Prospero into the IP cells, but segregate Miranda. IPs are of the size of GMCs, but divide repeatedly and asymmetrically in a neuroblast-like manner, segregating the cell fate determinants Prospero and Miranda during mitosis. The daughter cell that receives the Prospero and Miranda determinants is fated to become a differentiating GMC-like cell, whereas the IP retains its ability to divide several more times.

This novel model postulates that DM NBs produce exclusively IPs and not GMCs. The alternative notion, that the NB sometimes produces an IP and sometimes a GMC, is unlikely given that Prospero is never detected in the NB and, thus, cannot be segregated to one of its daughter cells as would be required for GMC generation. The model also posits that GMCs are produced by IPs through (functionally) asymmetrical divisions that result in one daughter cell becoming a GMC while the other daughter cell self-renews as an IP. Alternative scenarios, such as one in which IPs first divide symmetrically to expand in numbers and then adopt a GMC fate to generate differentiating neurons, are unlikely, given the spatiotemporal pattern of Prospero/Miranda expression and the stable ratio of IPs versus GMCs observed in DM NB clones throughout larval development.

The experimental findings that support this novel model have implications for our understanding of neural stem cells and proliferation control. These are discussed in the following text.

The NBs of the developing central brain and ventral ganglia divide asymmetrically in a stem cell mode in which the larger NB self renews and the smaller daughter cell differentiates into a different cell type, usually a GMC (reviewed by [31, 32, 92, 94, 95, 115]). This asymmetric division of the parent NB has been thought to be tightly coupled with the asymmetric segregation of cell fate determinants, and central among these molecular determinants is the transcription factor Prospero, which is required in GMCs to inhibit self-renewal and to promote differentiation [31, 42, 43, 98-101]. Our findings indicate that the asymmetric segregation of Prospero does not occur in all dividing brain NBs. Indeed, in the DM NBs the lack of asymmetric segregation of Prospero to the IPs may be a key element in imparting (transient) NB-like features to these proliferating cells.

It has been assumed that only cells of a certain critical size show NB-like proliferative properties. The small size of the GMC would be a key factor promoting cell cycle exit and differentiation of its progeny (see [92]). This simple link between cell size and self renewing/terminal division is also called into question by our findings, since IPs are comparable in size to GMCs and yet they possess a very distinct mitotic potential. Our findings indicate that even though IP cells are of the same size as their progeny, Prospero and Miranda are partitioned to only one of their daughter cells. Thus, the morphologically symmetric cell division of a NB-derived daughter cell does not necessarily engender equal portioning of differentiation factors into both resulting cells.

The only repeatedly dividing progenitor cell type identified to date in the central nervous system of *Drosophila* is the NB. Our studies identify the IP cell as a second progenitor type with the capacity to undergo multiple rounds of divisions. This characteristic is coupled with several cellular and molecular features that are shared with NBs. Among these are the specific expression patterns of Prospero, Miranda and CycE during mitosis as well as the ability to asymmetrically segregate Prospero and Miranda during cell division. The number of divisions that IPs typically carry out is currently not known with precision. Our observations based on quantification of cell

number in multicellular clones suggest an average of three-to five divisions as a conservative estimate. If, as assumed by our model, each IP cell division results in the generation of one GMC-like daughter cell, this estimate would predict a three- to five-fold amplification of the number of neuronal progeny in DM lineages compared with other lineages of the central brain and ventral ganglia. This prediction is in reasonable accordance with the amplified cell numbers observed in NB clones of DM versus non-DM lineages. The ultimate fate of the IPs is currently not known. The fact that almost all intermediate precursor-derived multicellular clones are composed exclusively of postmitotic neurons suggests that, after multiple divisions, these cells are either eliminated by programmed cell death, or terminally divide and differentiate.

Although the DM NBs do not express and segregate Prospero to their daughter intermediate precursors, these daughter cells do express Prospero in a cortical and polarized manner during mitosis. The off/on state of Prospero must be kept under tight control for a controlled amplification of proliferation achieved in DM lineages since complete mutational loss of Prospero in brain clones leads to uncontrolled proliferative activity and brain tumor formation [104-106, 114]. Indeed, our observations on the DM lineages imply that deregulated IPs that fail to express Prospero might be an important source of tumor cells in the brain. Interestingly, region-specific action of tumor suppressor genes in the larval brain has been previously reported using somatic cell clones [105].

4.2 DM neuroblasts are multipotent progenitors

In *Drosophila*, as in other insects, glial cells fall into three classes, surface glia, cortex glia and neuropil glia, each of which is represented in the larval brain [3]. In *Drosophila* embryo, all glial cells originate either from glioblasts, or from multipotent precursors which can produce both neurons and glia. Glial numbers in the brain increase during larval development and this increase in cell number is due, at least in part to mitotic divisions of glial cells, however, the bulk of added (secondary) glial cells has been postulated to stem from the proliferation of unidentified secondary neuroglioblasts [28]. Only glioblasts have been described in postembryonic development (Soustelle, Giangrande 2008; Awasaki 2008). The identity of multipotent precursors in the postembryonic brain was unknown.

We show here that DM neuroblasts are multipotent and produce both neurons and glia. DM neuroblasts are therefore exceptional in that they represent the only currently known neuroglioblasts in the postembryonic brain. Besides DM lineages, we also examined more than a 100 of non-DM (canonical) neuroblast lineages and didn’t find glial cells in the progeny, which means that DM neuroblasts are most probably the only postembryonic multipotent neural progenitors.

4.3 Novel type of gliogenesis in DM lineages

In the *Drosophila* embryonic CNS glial cells are known to be generated either from glioblasts (GB), which produce exclusively glia (e.g. embryonic anterior glioblast, Jacobs et al, 1989) or from multipotent precursors, neuroglioblasts (NGB) (Fig 1-6). There are two types of neuroglioblasts known to date. For the first type of the neuroglioblasts (e.g. NGB6-4T and NGB5-6) it has been demonstrated that the early bifurcation of the glial versus neuronal sublineages takes place during the first division after embryonic delamination from the neuroectoderm. *gcm* is expressed asymmetrically in the glial sublineage and decision is made on the level of the neuroglioblast (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). The second type of neuroglioblasts proliferation (e.g. embryonic NB1-1A) involves Notch acting upstream of a *gcm.* NB1-1A first produces neurogenic ganglion mother cell that gives rise to a pair of neurons. During next three divisions NB1-1A produces three ganglion mother cells each of which divide asymmetrically producing sibling neuron and glial cell. In this case, *gcm* acts as an effector of Notch signalling during sibling cell fate specification [62].

DM neuroblasts proliferate through asymmetric division that involves intermediate progenitors with transit-amplifying cell features ([116]; [120]; [121]). This implies that DM lineage-derived glia, unlike any other glial cell type in *Drosophila*, are generated by amplifying intermediate progenitors. However, an exact clonal analysis of the relationship between glial cells and intermediate progenitors in DM lineages will be required to validate this notion.

4.4 DM lineages generate a subset of neuropile glia

A recent developmental analysis by Awasaki et al. [27] demonstrated that there are five types of glial cells present in the adult brain of *Drosophila*: cortex, subperineurial and perineurial surface glia, ensheathing and astrocyte-like neuropile glia. It was also shown that distinct adult-specific glial types derive from different precursors and that most adult perineurial, ensheathing and astrocyte-like glia are produced after embryogenesis. Perineurial glial cells are made locally on the brain surface. In contrast, the wide-spread ensheathing and astrocyte-like glia derive from specific brain regions. However, the progenitors of postembryonically generated glial cells as well as the mode of postembryonic glial proliferation still remained elusive [27].

The DM originated glial cells were generally found clustered in the vicinity of the larval interhemispheric commissure. Moreover, their glial processes were often closely associated with the secondary axon tract of the DM lineages. Noteworthy that Repo-positive surface and cortex glia located immediately adjacent to the DM clones were never GFP-labeled, implying the DM glia were not of the surface (or cortex) glial type. Thus, our findings suggest that DM glia is a neuropil glia.

Two types of adult-specific neuropil glia are known to date. The first type of neuropil glial cells has fibrous lamellar morphology and preferentially ensheaths the neuropils or their subcompartments, surrounds neural bundles. These cells were called ensheathing glia. The other type of neuropil glial cells has dendritic morphology and apparently fills the interior of the neuropils, therefore was called astrocyte-like glia [27].

DM glial cells are closely associated with the DM axonal bundle, outlining the larval neuropil compartments, but never sending processes inside. Based on the morphology of DM-derived glia we suppose that this is an ensheathing subtype. However, more accurate analysis on a single-cell resolution is needed to make this suggestion a solid data.

4.5 DM lineages contribute to the developing central complex

A recently published neuroblast lineage atlas of developing adult brain in the late larva subdivides each brain hemisphere into approximately 100 clonal lineages, each represented by a fasciculated neurite bundle that forms an invariant pattern in the neuropil [11]. Therefore, the question arises how does each family of clonally related neurons contribute to the formation of the adult neural circuits?

During the early pupal differentiation period, some of the neuropil structures that specifically characterize the adult brain become visible. Among these are the principle components of the central complex (CCX). The central complex, one of the most prominent neuropil structures in the adult brain, is located centrally between the two hemispheres and consists of four substructures. These are the protocerebral bridge, the fan-shaped body, the ellipsoid body, and the noduli [85, 86]. None of these structures are visible in the developing larval brain even at the latest stages. We demonstrated that at the late larval 3rd instar each DM lineage sends two or more of their axonal bundles into the DPC1 commissure where they defasciculate upon entering and form a specific meshwork-like fascicle arrangement. In the previous study this notably big commissure was referred as a probable central complex primordium (classification of Pereanu and Hartenstein, 2006 [11]). Our data supports this idea and suggests that the specific meshwork-like arrangements are possibly developing columnar and tangential elements of the adult fan-shaped body.

From the first day of pupal development onward, the four major components of the central complex, the fan shaped body, the ellipsoid body, the noduli and the protocerebral bridge, can clearly be recognized. All central complex substructures are interconnected by sets of interneurons that form regular patterns of projections [78, 79, 86]. Most neurons of the central complex belong to one of two categories with respect to their projections. Large-field neurons typically arborize in only a single substructure (often sending projections along their whole length) and link it to one or two central brain regions outside the CCX. Small-field neurons, as a rule, connect small domains of substructures. The majority of small-field cells are intrinsic to the CCX. Some, however, project to the accessory areas (Hanesch, 1989). A remarkable and common feature of DM lineages is that a subset of the neurons in each lineage

contributes to the developing central complex neuropil. We demonstrated that DM lineages project into three of the central complex substructures: the protocerebral bridge, the fan-shaped body and the noduli. No projections to accessory areas were observed. We suggest that either DM neurons are generally small-field neurons, or projections to accessory areas were not yet developed at the time of experiment (24h after puparium formation).

Various types of small-field neurons were described in an outstanding study of Hanesch et al, 1989. Noteworthy are two of them: the first is the so-called horizontal fiber system type (HFS), which interconnects protocerebral bridge, and the fan-shaped body with accessory areas in a specific manner. The second type is a vertical fiber system type (VFS), which interconnects protocerebral bridge, fan-shaped body and noduli (Fig. 4.1).

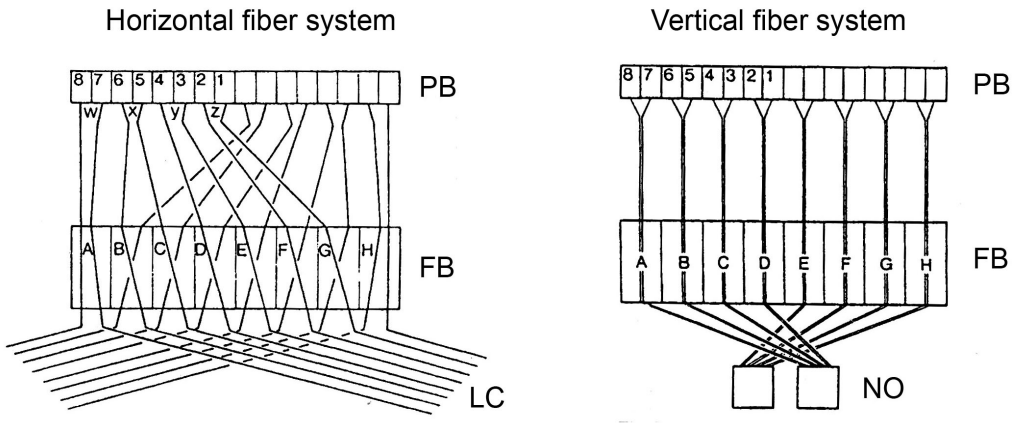

Fig. 4.1 Vertical and horizontal fiber systems of the central complex. Diagrams showing the arrangement of the set of VFS and HFS neurons. Protocerebral bridge (pb), fan-shaped body (fb), noduli (no) (modified after Hanesch et al., 1989).

The DM-originated neurons should logically consist of VFS neurons because DM lineages form arborisations in protocerebral bridge, fan-shaped body and noduli. But visually, the projection patterns of central complex-contributing fascicles of DM lineages recapitulate the projection mode of the HFS neurons, even though the connection with accessory areas was not demonstrated in our study, and HFS neurons do not project into the noduli. Thus, I think that DM lineages partially consist of HFS

neurons. Further analysis with single-cell resolution will support or disprove this notion.

It is generally believed that the neurons of central complex form arborisations in three or less substructures of central and lateral complexes (for example, fan-shaped body, noduli and bulb) [86]. If DM lineages are indeed consist of the HFS neurons then another interesting conclusion emerges: either DM lineages consist of more than one subtype of neurons or DM lineage-originated neurons represent a novel subtype of cells which arborizes in more than three areas of central and lateral complexes.

5 EXPERIMENTAL PROCEDURES

5.1 Fly strains and genetics

All *Drosophila* stocks were reared and maintained on standard yeast-cornmeal-agar medium and all experiments were performed at 25°. Unless otherwise stated fly stocks carrying transgenes and recombinant chromosomes were obtained from the Bloomington stock center and assembled using standard genetics. To generate positively marked MARCM clones:

1) y,w,hsFLP; FRT40A, tubP-GAL80^{LL10}/CyO,ActGFP^{JMR1}; tubP-GAL4^{LL7}, UAS-mCD8::GFP^{LL6}/TM6,Tb,Hu were mated to w; FRT40A, UAS-mCD8::GFP^{LL5} (standard cell lineage labelling with membrane-tethered GFP) or UASp-cnnGFP$^{26.1}$; FRT40A, UAS-cLacZ$^{Bg4-1-2}$ (for additional labeling of centrosomes), or w; FRT40A, UAS-cLacZ$^{Bg4-1-2}$; UAS-tauGFP$^{12/2/3}$ (gift of A Brand), for live imaging.

2) ;FRTG13, tub-Gal80, hs-Flp/(CyO,ActGFP^{JMR1}) were mated to ;FRTG13, Dll-Gal4, UAS-mCD8::GFP/(CyO,ActGFP^{JMR1}).

The distal-less Gal4 line: ;Dll-Gal4/ CyO; UAS-mCD8-GFP, UAS-H2B-mRFP1.

Generation of MARCM clones and larval staging was performed as previously described [104] for this sub-section.

5.2 Immunohistochemistry and live imaging

Nervous systems were dissected from larvae and 24h old pupae, fixed and immunostained as previously described [111]. Primary antibodies were as follows: rabbit anti-PH3 (1:400; Upstate, Charlottesville, Virginia, USA), mouse anti-MIRA (1:50; gift of P Overton), rabbit anti-MIRA (1:200; gift of YN Jan), mouse anti-PROS (1:10; Developmental Study Hybridoma Bank (DSHB), Iowa City, Iowa, USA); mouse anti-ELAV (1:30; DSHB) rat anti-ELAV (1:30; DSHB), mouse anti-CYCE (1:50; gift of H Richardson), mouse anti-Repo (1:5; Developmental Study Hybridoma Bank DSHB), rabbit anti-Repo (1:10000, gift from V. Rodrigues), mouse anti-Neurotactin (1:10; DSHB), rabbit anti-GFP (1:1000; Molecular Probes), mouse anti-nc82 (1:20; DSHB), Alexa Fluor-conjugated secondary antibodies (Molecular Probes) were used at 1:200.

For live imaging, larval brains were dissected in Schneider's *Drosophila* Medium with 10% fetal bovine serum and mounted in 400-5 mineral oil (Sigma Diagnostic, Inc. St

Louis, MO, USA) between a glass coverslip and a gas-permeable plastic foil (bioFOLIE 25, In Vitro System and Services, GmbH, Gottingen, Germany).

5.3 Microscopy and image processing

Fluorescently stained nervous systems were imaged using a Leica TCS SP scanning confocal microscope using 40X and 63X oil-immersion objective. Z stacks were collected with optical sections at 1 µm intervals. Scanned pictures were visualized in ImageJ[111] and analyzed. For the illustration of findings the most representative scans were chosen and processed (not related non-DM lineages were excluded using the "lasso" tool). Pictures are presented as 'thick-section' merges projected as a flat image using ImageJ [111]. Figures were assembled using Adobe Illustrator and Photoshop. Clone/lineage sizes were determined from confocal Z stacks of sections, spaced by 1 µm. Using ImageJ, cells were marked section-by-section and counted. Typically, 20–50 nervous systems per staining/genotypes/larval stages were examined using 63× oil-immersion objective. Only well isolated clones were recorded from the surface-located NB to the earliest born neurons close to the neuropil. Sample sizes, means and standard deviations for all histograms are indicated in the text and figure legends.

For time lapse, Z stacks made of 1 µm thick slices were collected at 4 minute intervals. Movies were processed and assembled using house made ImageJ plug-ins. Briefly, the sample motions were corrected in X and Y dimensions by manual reference point tracking. A single slice was arbitrarily selected per time point, allowing both some Z dimension drifting correction and the follow up of the most interesting cells within the sample.

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