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

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Abstract

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

Introduction

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

Drosophila neural stem cells, called neuroblasts for historical reasons, are similar to vertebrate neural stem cells in many aspects of asymmetric cell division, self-renewal, and cell fate determination. Indeed, they are currently one of the best-understood models

for neural stem cell biology (reviewed in Doe, 2008; Knoblich, 2008; Brand and Livesey, 2011; Homem and Knoblich, 2012). Recent work has shown that the neuroblasts of the Drosophila brain can be divided into two classes based on their proliferation pattern, called type I and type II. Type I neuroblasts generate their neural progeny through non self-renewing ganglion mother cells (GMCs) which divide only once to produce two postmitotic neural cells, neurons or glial cells. Type II neuroblasts generate their progeny through self-renewing INPs which have features of transit amplifying cells. Since an INP undergoes several rounds of proliferative cell divisions that each result in self-renewal of the INP and in the generation of a GMC which produces two neural progeny, a marked amplification of proliferation occurs (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008; Weng et al., 2010; reviewed in Weng and Lee, 2011; Saini and Reichert, 2012; Homem and Knoblich 2012). Most of the 100 central brain neuroblasts correspond to type I and each of these generates neural lineages consisting of 100-150 neural cells. In contrast, 8 identified brain neuroblast pairs correspond to type II and, due to transit amplification of proliferation via INPs, each of these type II neuroblasts generates neural lineages consisting of an average of 500 cells.

Taken together, the 8 amplifying type II neuroblast pairs generate approximately one-fourth of the total number of neural cells in the Drosophila central brain. Thus, similar to the situation in the developing mammalian cortex, a significant proportion of the neural cells in the fly brain are generated by neural stem cells via transit amplifying INPs. However, until recently, the neural phenotypes of these INP lineage-derived cells as well as the circuitry to which they contribute were unknown. Recent studies that focussed on

the 6 medial pairs of type II neuroblasts, referred to as PAN or DM1-6 neuroblasts (see Bello et al., 2008; Bowman et al., 2008), demonstrate that five of these type II neuroblasts, DM1-5, are multipotent neuroglial progenitors that contribute both neuronal and glial cells to a highly complex multimodal neuronal integration center called the central complex (Izergina et al., 2009; Bayraktar et al., 2010; Viktorin et al., 2011; Jiang and Reichert, 2012; reviewed in Boyan and Reichert, 2011).

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

In contrast to the 6 pairs of medially located type II lineages (DM1-6), which have been characterized in detail, the remaining two, more laterally located pairs of type II

neuroblasts have not been studied further. Thus neither the proliferative properties of these type II neuroblasts, nor the phenotypes of the cells in their lineages, nor the role of these cells in the developing brain are currently known. In this report, we identify the lineages produced by the two laterally located type II neuroblasts, DL1 and DL2. We show that the DL2 lineage contains only neurons while the DL1 lineage comprises both neurons and glial cells, indicating that the DL1 neuroblast, as well as its neurogliogenic INPs, are multipotent neuroglial progenitors. We then focus on the DL1 lineage and demonstrate that the neurons in this lineage contribute to the central complex while the glial cells in this lineage contribute exclusively to the optic lobe. Immediately following their generation in the central brain during the second half of larval development, these DL1 lineage-derived glial cells migrate into the developing optic lobe, where they differentiate into three identified types of optic lobe glial cells, including the prominent glia of the inner and outer chiasm. Taken together, these findings identify the type II DL1 neuroblast as a novel multipotent neuroglial progenitor that gives rise both to central brain interneurons and to optic lobe glial cells via transit amplifying INPs.

Materials and Methods

Fly stocks, MARCM, and Flp-out analysis

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

food competition and to ensure exact staging. First and second instar larvae were distinguished according to air inflation of their tracheae, and confirmed after dissection according to the size and shape of the mouth hooks (Park et al., 2002).

Immunohistochemistry

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

Microscopy and image processing

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

using the CellCounter plugin for Fiji/ImageJ (Kurt De Vos).

Results

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

Among the total of eight transit amplifying type II neuroblasts in each brain hemisphere, six are located at the posterior medial edge, and these have been referred to as PAN or DM1-6 (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008). Because these six lineages are easier to identify, most of the previous studies of type II neuroblasts and their lineages have focused on DM1-6 (Fig. 1A, arrowheads). In addition to these six lineages, there are two further type II neuroblast lineages located more laterally in each brain hemisphere. These two more laterally located type II lineages, like the DM1-6 lineages, are selectively labeled by an *earmuff* genomic enhancer-fragment driven reporter *erm^{R09D11}-CD4-tdtomato* (Pfeiffer et al., 2008; Han et al., 2011); their location in a third larval instar brain hemisphere is shown in Fig. 1A (arrows). Secondary axon tracts (SATs) from both of these lateral lineages initially project towards each other, join, and then branch apart again (Fig. 1B-F). Confirming their identity as type II lineages, both of these lateral lineages contain Deadpan-positive mature INPs (Fig. 1G-H). These two lineages tentatively correspond to the CP2/3 lineage pair, however, based on SAT trajectory alone it has not been possible to distinguish further between the two (Pereanu and Hartenstein, 2006).

For individual identification of the two lineages in the late larval and early pupal brain, we took advantage of our finding that one of the two lineages reliably expresses *gcm-lacZ^{rA87}* (Jones et al., 1995) in a band of cells located near the outer lineage surface close

to the neuroblast while the other does not (Fig. 1G,I). (Both lateral type II lineages lie close to a type I lineage with a similar, but consistently stronger, band of *gcm-lacZ* expressing cells (Fig. 1I, Fig. 2B-C) that has been previously described by Soustelle and Giangrande (2007), who also showed that the *gcm* gene does not have a gliogenic role in this lineage.) We designate the lateral type II lineage, which does not have the proximally located band of *gcm-lacZ*-labeled cells, to be the DL1 lineage and, correspondingly, we refer to the other lateral type II lineage as the DL2 lineage. The relative positions of the DL1 and DL2 lineages show some variability in different preparations. In 87% of wandering third instar larval brain hemispheres the DL2 lineage lies dorsolateral to the DL1 lineage and in the remaining 13%, this orientation is reversed (n>30 hemispheres).

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

Although their SAT trajectories in the larval brain are very similar, the DL1 and DL2 neuroblasts give rise to very different sets of neural progeny during postembryonic development. This is manifest in mosaic-based MARCM labeling experiments using *tub-Gal4* to drive *UAS-mCD8GFP* (Lee and Luo, 1999), in which recombination is induced after larval hatching and neuroblast clones are recovered at wandering third instar. Strikingly, DL1 neuroblast clones invariably comprised a cluster of cells in the central brain as well as a large array of large cells in the adjacent developing optic lobe (Fig. 2A). (100% of the DL1 neuroblast clones recovered from sparsely labeled MARCM brains (n=26) contained a central brain cell cluster and an optic lobe cell cluster, and no

optic lobe cell clusters were found without a labeled DL1 lineage). We confirm their common origin by observing gliogenesis in young DL1 clones below (see Fig. 5). In contrast, DL2 neuroblast clones, identified by their proximal band of *gcm-lacZ*-labeled cells, only had central brain progeny and never contained optic lobe cells (Fig. 2D). (100% of the DL2 neuroblast clones recovered (n=15) contained cells in the central brain but not in the optic lobe.) These findings were independent of the relative position of DL1 and DL2 lineages, underscoring the fact that the proximal band of *gcm-lacZ*-expressing cells is an identifying characteristic of DL2 versus DL1 lineages.

The morphological features of the DL2 cell cluster in the larval central brain indicate that the cells in this lineage differentiate into typical secondary, adult-specific interneurons; their fate was not studied further in this report. The morphology of the two separate cell clusters generated in the DL1 lineage suggests that the central brain cluster corresponds to secondary, adult-specific interneurons and that the cluster of cells in the optic lobe corresponds to glial cells. The cells in the central brain manifest a tight cluster of their somata and have two prominent SATs that project dorsomedially towards the main commissural region of the brain. In contrast, the cells in the optic lobe are arranged in extensive arrays, have elaborated the elongated processes typical of glial cells, and appear to correspond to three different morphological types; their nuclei are relatively large and all express the glial marker Repo (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995), confirming their glial cell nature (see Fig. 3).

Like all other type II neuroblasts, the DL1 neuroblast generates its progeny through INPs

that act as transient amplifying cells. To determine if these INPs can give rise to mixed sublineages containing both neuronal cells of the central brain and glial cells of the optic lobe, MARCM labeled INP clones were induced at larval hatching and recovered in late third instar brains. In all cases (n=20), these INP clones contained both glial cells in the optic lobe as well as neuronal cells in the central brain implying that INP sublineages contain cells of mixed fate. INP clones that contained glial cells but not neuronal cells were never seen. Closer inspection of the glial cells in these INP clones indicates that three different glial cell types can be generated; INP clones containing one, two, or all three of these glial cell types together with neuronal cells expressing the neuronal marker Elav were recovered (Fig. 3). This in turn implies that INPs have multipotent progenitor potential, at least for those clones labeled in out experiments, i.e. induced at larval hatching, or glial fate may not be entirely determined by lineage.

Based on their morphology and location in the third instar larval brain, we hypothesize that two of these glial cell types correspond to the outer chiasm glia (Xg^o, frequently referred to as medulla glia), and inner chiasm glia (Xg^i), both large glial cells that wrap the axon tracts of the two optic chiasmata (Tix et al., 1997; Chotard and Salecker, 2007; Hofmeyer et al., 2008; Edwards and Meinertzhagen, 2010; Hartenstein, 2011; Edwards et al., 2012). Xg^o were readily identified in late wandering third instar larvae and early pupae as the proximal most of three adjacent, dorso-ventral rows of glial nuclei, with distinctly larger nuclei than the two distal rows (compare Fig. 4A-D). The two distal rows of glial nuclei correspond to the epithelial and marginal glial cells of the lamina, in between which the R1-R6 photoreceptor axons terminate (Winberg et al., 1992; Poeck et

al., 2001). We confirmed our recognition of these glial cells in MARCM clones of the eye disc that contain photoreceptor axons terminating between epithelial and marginal glial cells, as well as with two Gal4 drivers known to be expressed in Xg^o glial cells, *R25A01-Gal4* (Edwards and Meinertzhagen, 2012) and *mz97-Gal4* (Poeck et al., 2001). The single row of Xg^i glial cells with intercalated nuclei was easily recognizable from late third instar on, based on their very large oblong nuclei and their position in the center of the developing optic lobe where no other glial cells are present (Tix et al., 1997; Edwards and Meinertzhagen, 2012). We confirmed our recognition of Xg^i glial cells using *mz97-Gal4* expression (Poeck et al., 2001). The characteristic arrangements of Xg^o and Xg^i glial cells is most clearly visible in slowly moving, late third instar larvae close to pupariation and beyond. The third glial cell type that is derived from the DL1 neuroblast lies closest to the central brain and does not correspond to a known glial cell type at wandering third instar, but is hypothesized to develop into the cortex glia-like cells that are associated with adult DL1 clones (described in Fig. 9).

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

To confirm the glial cell types generated by the DL1 lineage, we first sought to identify molecular markers that would allow us to follow the development of the DL1 lineage cells through pupal stages and into the adult. To this end, we screened diverse Gal4 collections for drivers that might be appropriate for Flp-out based lineage-tracing experiments specific to DL lineages (see Materials and Methods). In these Flp-out experiments, screened Gal4 drivers were used in combination with *actin>>Gal4* (Pignoni and Zipursky, 1997), *UAS-mCD8::GFP* (Lee and Luo, 1999), and two copies of *UAS-flp*

to visualize both current and lineage-traced expression of a given Gal4 driver. Alternatively, to see the current expression with UAS-mCD8GFP separate from the flp-out history of expression, *act>>nlacZ* was used as Flp-out reporter (Struhl and Basler, 1993).

In lineage tracing experiments of this type, the *R38H02-Gal4* line, which represents a neurotactin enhancer fragment (Jenett et al., 2011), allowed stochastic, but very specific labeling of DL1 progeny throughout postembryonic development. In approximately 12% of the late larval brain hemispheres recovered, *R38H02-Gal4* Flp-out clones target the DL1 lineage (n=31 DL1 lineages from 236 hemispheres) and reveal both the neuronal cell cluster in the central brain and the glial cells in the optic lobe, in many cases with little other brain expression (Fig. 4). Importantly, the DL1 lineage cell clusters labeled by *R38H02-Gal4* Flp-out and those labeled by MARCM clonal methods are very similar (compare Fig. 2A, 4A). In MARCM, labeling of only the DL1 lineage is achieved by adjusting the time of heat shock-driven clone induction such that only one or very few lineages are labeled in any one brain (see Material and Methods). In *R38H02-Gal4* Flp-out, induction of DL1 clones is a feature of the *R38H02* enhancer fragment in combination with the Flp-out stock used. (In this sense, the *R38H02-Gal4* Flp-out used behaves analogous to the direct *ey*-Flp and *repo*-Flp fusion constructs that have been used to generate eye disc and glial cell clones (Newsome et al., 2000; Silies et al., 2007). All *R38H02-Gal4* Flp-out clones that comprised an optic lobe glial array could be identified unambiguously as DL1 by the same means as the randomly induced MARCM clones, namely as the lateral type II lineage that shares an SAT with the other lateral type II

lineage expressing *gcm-lacZ* (n=22 clones). This result confirms that *R38H02-Gal4* Flp-out labels DL1 and not a similar lineage that may also produce optic lobe glial cells, but may not be easily recovered by our MARCM conditions. The DL1 lineages labeled by *R38H02-Gal4* Flp-out (in wandering third larval instar brains) have a slightly higher number of glial cells in the optic lobe (32±6) than corresponding MARCM labeled DL1 lineages (27±4), but are otherwise indistinguishable from MARCM clones. Moreover, labeled INP-like sublineages appear as well at high frequency in *R38H02-Gal4* Flp-out experiments, indicating that the *R38H02* enhancer fragment can also be active in DL1 lineal INPs.

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

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

From the late third larval instar onward through pupal development and in the adult (compare Fig. 8,9), the putative DL1- derived glial cells are located in the optic lobe and, are clearly separated from the DL1 derived neuronal cells in the brain hemisphere.

However, since we hypothesize that both the glial cells and the neuronal cells are lineal progeny of the same brain neuroblast, the glial cells like the neuronal cells in the DL1 lineage are likely to be generated in the central brain. This, in turn, implies that the DL1 glial cells (or their intermediate progenitors) translocate from their site of origin in the central brain into the nascent optic lobes during larval development.

To investigate this, we again used *R38H02-Gal4* Flp-out labeling as well as MARCM clonal labeling to study the spatiotemporal development of DL1-derived glial cells during larval stages. Our experiments indicate that the initiation of gliogenesis in the DL1 lineage occurs in the late second larval instar (Fig. 5, Fig. S1, Table 1). At 61h AEL (after egg laying), corresponding to the late second instar, we recovered MARCM labeled DL1 neuroblast clones (n=7) and INP clones (n=5), and found that the neuroblast clones did not contain Repo-positive glial cells whereas the INP clones did (Fig. 5A,B). (Note that MARCM recombination in a type II neuroblast stochastically labels only one of its two daughter cells - either the neuroblast, or the INP, and their respective progeny.) The presence of Repo-positive glial cells in INP clones but not in their sister neuroblast clones at 61 h AEL indicates that at that time, gliogenesis has been initiated in the first INPs but not yet in the subsequently generated INPs contained within a labeled neuroblast lineage. This sets the time of onset of gliogenesis in the DL1 lineage at around 61 h AEL, or approximately 5-10 hours prior to the L2/L3 transition.

Gliogenesis in the DL1 lineage continues rapidly during development around the L2/L3 (second larval instar/third larval instar) molt. At 66-68 h AEL, shortly before the molt,

all DL1 neuroblast clones (7 MARCM clones, 5 *R38H02-Gal4* Flp-out clones) contained 1-3 Repo-positive cells (Fig. 5C-D; Fig. S1A-B) and in some cases, one of these Repo-positive cells had already extended a cytoplasmic process and appeared to translocate towards the optic lobe (data not shown). In MARCM and *R38H02-Gal4* Flp-out labeled neuroblast clones recovered at 72h AEL, shortly after the L2/L3 molt, the number of Repo-positive cells had increased and most of these glial cells appeared to be migrating out of the central brain and into the optic lobe (Fig. 5E-H, Fig. S1C-D). Throughout the early L3 stage, numerous new glial cells were formed and migrated out rapidly, as seen by the large increase in glial cell numbers from L2 to L3 (Table 1). All of these migrating glial cells entered the optic lobe between the posterior borders of the IPC (inner proliferation center), the structure that abuts the central brain and forms part of the optic lobe neuroblasts (see Egger et al., 2007 for posterior view). However, the number and spatial arrangement of the glial cells seen migrating through the IPC was variable in different preparations (compare Fig. 5F-H). At 84-85 hours AEL, many migrating glial cells had passed between and beyond the IPC, and had begun to spread out laterally along the distal surface of the IPC (Fig. 5I-J, Fig. S1E-F). All of these migrating glial cells appeared to be interconnected by (*mCD8-GFP*-labeled) cellular processes, some of which also extended to the cluster of DL1-derived neuronal cells in the brain hemisphere (orange arrows in Fig. 5F,H,J and Fig. S1D,F). The processes that connected glial cells with neuronal cells in the lineage were observed in all brains up to 90 h AEL; afterwards, these processes began to detach, and were not detectable at 108 h AEL and beyond (data not shown).

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

Since we did not observe any glial cells within the neuronal DL1-derived cell cluster located in the central brain of wandering third instar larva, gliogenesis presumably ceases during the second half of the third larval instar. To determine this more precisely we used *R38H02-Gal4*-based lineage tracing to quantify the number of glial cells that had already migrated and the number of glial cells that were still located in the central brain at different time points (Table 1). At 84h and 90 hours AEL, the number of glial cells migrating or already in the optic lobe had increased to almost the same number found in wandering third instar larvae, and only single glial cells remained in the central brain cluster. From 96h AEL onward no new nascent glia were found within the central brain cell body cluster; the number of labeled glial cells in the optic lobe then remained the

same until the adult (MARCM clones again had a slightly lower number of glial cells compared to *R38H02-Gal4* based lineage tracing).

Soon after migrating into the optic lobe, the chiasm glia started to extend their processes around axon tracts. Between 102 and 106h AEL, outer chiasm glial cells were beginning to extend processes around nascent axon tracts between the proximal medulla and the lobula primordia (Fig. 6A,B). Inner chiasm glial cells started wrapping inner chiasm axon tracts between 114 and 120 h AEL (Fig. 6C,D). In contrast, the glia of the lateral cell body rind were seen to envelop the first cortex cell bodies only in the second half of pupariation (data not shown).

The gliogenesis period in DL1 begins slightly earlier than that of other type II lineage-derived glial cells such as the central complex glial cells generated by DM1-3 (Viktorin et al., 2011). Like most other glial cells, the DL1-derived optic lobe glia initially express *gcm-lacZ* shortly before expressing Repo (Fig. 7A), and this expression of *gcm-lacZ* gradually diminishes. Thus, at wandering third instar, *gcm- lacZ* is almost absent from DL1-derived optic lobe glia, while the later formed central complex glia as well as many surrounding optic lobe glia of different origin, such as medulla neuropile glia and the lamina glia from the glia precursor center, still express it (Fig. 7C-D).

Taken together, these results indicate that gliogenesis in the DL1 lineage begins at the end of L2 and is most prolific during the first half of L3, with most glia being generated within a few hours after the L2/L3 molt. Their cell division rate is low, indicating that

DL1-derived glial cells are primarily generated by differentiation of neuroblast progeny, rather than by mitotic proliferation of newly formed glial cells. Moreover, the spatiotemporal features of DL1 gliogenesis imply that once the glial cells are generated, they rapidly migrate out of the central brain and into the developing optic lobe and begin to wrap around axon tracts.

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

Given their excellent correspondence, we used *R38H02-Gal4* Flp-out clonal labeling together with MARCM clonal labeling to follow the lineal fate of the two DL1 cell clusters during metamorphosis in the pupa and in the mature brain of the adult. Fig. 8 shows labeled DL1 clones in pupae at 10h and 24h APF (after puparium formation) co-immunolabeled with the glial cell specific marker anti-Repo and antibodies against neural cell adhesion molecules BP106/anti-Nrt and BP104/anti-Nrg to label secondary axon tracts. At both time points, the DL1 lineage is clearly composed of the two cell clusters, one in the central brain and the other in the optic lobe (Fig. 8A,E).

The most distal layer of glial cells corresponds to outer chiasm glia (Xg^o). At the pupal stages examined, their cell bodies are aligned in a curved row that extends from dorsal to ventral along the distal edge of the medulla, immediately adjacent to two rows of lamina glia called epithelial glia and marginal glia (Fig. 8B-D). Outer chiasm glial cells have three principal processes; one extends distally into the lamina (Fig. 8H), one extends caudally and distally along the edge of the lamina and at the border to the medulla (Fig.

8I), and the longest process follows axon tracts that run from the distal to the proximal medulla that are labeled by anti-Nrg but not anti-Nrt antibodies (Fig. 8B,F,G). The more proximal layer of glial cells corresponds to inner chiasm glia (Xg^i). Their nuclei are also aligned in a curved row that lies between the medulla and the lobula and runs parallel to the row of outer chiasm glia (Fig. 8C-D,G-H). These glial cells extend processes distally and proximally, wrapping the thick axon bundles of the inner optic chiasm (Fig. 8G-H), as well as caudally into the developing lobula complex (Fig. 8D,I). A third glial cell type is located at the border between optic lobe and central brain and extends thin processes along axon tracts that interconnect optic lobe and central brain in the pupa (LCBRg; Fig. 8C,G-H). We tentatively refer to this type of glia as LCBRg since they subsequently acquire features of cortex glia of the lateral cell body rind (LCBR) found in the adult (see Fig. 9).

In the adult brain, the DL1 lineage cells have completed their differentiation and assumed their mature morphology and position in the brain (Fig. 9). The neuronal cells in the mature central brain have differentiated into two subclusters of interneurons, one of which arborizes in the superior protocerebrum and in two lamina of the fan-shaped body of the central complex, while the other arborizes more diffusely in the ventrolateral protocerebrum (Fig. 9A). The DL1-derived Repo-positive glial cells in the mature optic lobe have differentiated into three clearly distinguishable optic lobe glial cell types, all of which form elaborate processes. Fig. 9B-H show examples of isolated cells of the three types from DL1 INP clones and DL1 neuroblast clones. In addition to the outer chiasm (Fig. 9B) and the inner chiasm glial cells (Fig. 9C-E), which correspond to optic lobe

tract glia (Edwards and Meinertzhagen, 2010), the third type of DL1 lineage glial cell in the adult optic lobe has developed into cortex glia-like cells that are located in the lateral cell body rind (LCBR). DL1-derived LCBR glial cells had variable morphologies in the adult; Fig. 9E-F shows an example of an LCBR glial cell with its nucleus located at the edge of the medulla and lobula neuropiles, that wraps around several nuclei in the overlying cortex and extends one process into the space between medulla and lobula neuropiles at the level of the inner chiasm glia. Fig. 9G-H shows two examples of glial cells that are located deeper in the lateral cell body rind, wrapping around many nuclei of the LCBR. One of them extends a short process into the central brain along an axon tract that interconnects the optic lobe and central brain.

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

Discussion

In this report, we focus on the two lateral type II neuroblast lineages of the central brain in which amplification of proliferation is mediated through transit amplifying INPs (reviewed in Boyan and Reichert, 2011; Brand and Livesey, 2011; Homem and Knoblich, 2012). While one of these lineages, DL2, generates exclusively interneurons of the central brain, the other, DL1, generates both central brain interneurons and glial cells of

the optic lobe. Thus, while DL2 functions as a neuroblast, DL1 has neuroglioblast function. Like the five other type II neuroglioblasts, DM1-5, the interneurons generated by DL1 contribute to the central complex neuropile (Izergina et al., 2009; Viktorin et al., 2011). However, in contrast to DM1-5, the glial cells generated by DL1 do not contribute to the central complex. Indeed, they do not contribute to the central brain at all. Although they are generated in the central brain, they rapidly migrate out into the developing optic lobes, where they subsequently differentiate into outer chiasm glia, inner chiasm glia and cortex glia. This is the first example of a central brain lineage that gives rise to cells of the optic lobes and is at the same time the first identification of the developmental origin of optic lobe chiasm glia.

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

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

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

1996; Chotard and Salecker, 2007). The developmental mechanisms which may integrate DL1-derived glial cells with glial cells of other origin into seemingly homogeneous arrays of chiasm glia are currently unknown.

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

Together with DM1-5, DL1 is the sixth identified neuroglioblast that generates neuronal and glial cells during postembryonic development of the central brain. Thus, all of the postembryonically acting neuroglioblasts in the brain identified to date are type II neural stem cells that amplify their proliferation through INPs. The neuronal and glial cells of the DL1 lineage do not originate directly from the DL1 neuroblast, rather they are generated via INPs, multipotent secondary progenitors with features of transit amplifying cells that can give rise to both neuronal and glial cells (Viktorin et al., 2011; this report).

Remarkably, in mammalian brain development, many neuronal and glial cells also originate from transit amplifying intermediate progenitors and not directly from neural stem cells (see Kriegstein and Alvarez-Buylla, 2009). Indeed, in the mammalian cortex, the majority of neural cell-generating proliferative divisions occur through intermediate progenitors at all stages of development, implying that the major role of cortical neural stem cells is to generate intermediate progenitors (Kowalczyk et al., 2009; Lui et al., 2011). The intriguing parallels between the INP-generating type II neural stem cell lineages in the Drosophila brain and the intermediate progenitor-generating neural stem cell lineages in the mammalian brain suggest that comparable lineage types might be present in other developing brains and, hence, represent a common and phylogenetically conserved feature in the development of complex brain architecture (Boyan and Reichert, 2011).

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Note added in proof

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

Figure legends

Fig. 1. The two lateral type II lineages DL1 and DL2 are distinguished by expression of *gcm-lacZ^{rA87}*.

(A-I) Hemisphere of a *gcm-lacZ^{rA87}*/+ ; *ermR09D11-CD4-tdtomato/+* transgenic larva at wandering third instar, labeled with anti-Dpn (green) and anti-beta-galactosidase (magenta); CD4-tdtomato expression is shown in white in (A-F). (A) Maximum intensity projection of confocal stacks of CD4-tomato expression. Arrowheads point to medial type II lineages named DM1-6, arrows point to lateral type II lineages DL1 and DL2. (B-E) Single confocal slices following the secondary axon tracts (SATs) of DL1 and DL2 by CD4-tomato expression. The SATs fuse (B-D), then each lineage branches in the same manner (E-F). (G-I) Single confocal slice of Dpn-expressing INPs (green, green arrows) and *gcm-lacZ* expressing cells (magenta, magenta arrows) close to the neuroblasts of the DL1 and DL2 lineages (dotted outlines, white arrows). (H-I) Closeup of Dpn (H) and *gcm-lacZ* (I) expression within lineages DL1 and DL2. Both lineages have Dpn-positive INPs (green arrows in G) , but only DL2 has a band of *gcm-lacZ*-expressing cells in the

proximal part of the lineage (magenta arrows in H). Magenta arrowhead in (I) points to the strongly *gcm-lacZ*-expressing lineage from Soustelle et al. (2007) that is not a type II lineage. CB, central brain. OL, optic lobe. Scale bars, 20 μm.

Fig. 2. DL1, but not DL2, neuroblasts generate a large array of optic lobe glia

tub-Gal4 MARCM clones induced at hatching and recovered at wandering third instar of DL1 (A-C), and DL2 (D-F). DL1 is associated with a large array of optic lobe glia (orange dotted outline in A), but no *gcm-lacZ* expression in the neuronal part of the lineage (B,C). The DL2 lineage is not associated with any other cells apart from the neuronal lineage itself, but has *gcm-lacZ* expressing cells in the neuronal part of the lineage (F). Large panels (A,D) are maximum intensity projections of the whole clone, small panels (B-C, E-F) are single confocal slices at the level of *gcm-lacZ* expression, taken from the inset in (A,D); brightness and contrast is adjusted to different levels in insets to visualize a large variation in fluorescence intensities between neuronal part of the lineage and axon tracts. CB, central brain. OL, optic lobe. Scale bars, 20 μm.

Fig. 3. INP lineages are mixed neuronal and glial

(A-C) Three examples of DL1 INP MARCM clones induced at hatching and recovered at wandering third instar; all three clones contain both neurons (white insets, enhanced contrast) and glial cells. Glial cell types (for discussion see Fig. 5) and numbers of glia vary between different INPs. Panels (A-C, A^i-B^i) show maximum intensity projections. (C^i) and insets in lower panels show single confocal sections with anti-Repo staining in glial nuclei (A^i, B^i), and anti-Elav staining in neuronal nuclei (C^i) but not glial nuclei

(C^{ii}). Hemispheres are outlined in white dotted lines. Dorsal is to the top, medial to the right. Scale bars, 20 µm.

Fig. 4. R38H02-Gal4 give rise to DL1 Flp-out clones

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

Fig. 5. Time line of glia formation and their migration into the optic lobe.

(A-J) Maximum intensity projections of *tub-Gal4* MARCM clones (white) in larval brains. Clones were induced at hatching and recovered at the stages and time points indicated in the top right corner of each panel. Hemispheres are outlined (dotted lines). (D,F,H,J) as well as insets in (B) are either magnified single confocal slices or maximum intensity projections of few slices to show Repo+ glial cells (magenta) within the clones shown in the panels above, regions outlined in white. Anti-Fas3 (green in A-C) or anti-Dpn (green in I,J), together with anti-Repo, identify DL1 lineages and the optic lobe

proliferative epithelia (IPC, inner proliferation center); dotted lines in (D,F,H,J) delineate the border between optic lobe (OL) and central brain (CB). - (A,B) Two clones from 61 hour AEL L2 larval brains; the DL1/2 neuroblast clone (A) has no Repo+ cell yet, but the DL1 INP clone (B) consists of one Repo+ glial cell in addition to four other cells. (C,D) DL1 neuroblast clone in an L2 larval brain shortly before the L2/L3 molt; the clone contains one Repo+ cell that has not yet migrated away from the lineage (inset). (E-H) Two DL1 neuroblast clones from L3 larval brain shortly after the L2/L3 molt with several Repo+ cells migrating away from the base of the lineage between the two posterior ends of the IPC epithelia into the optic lobe. The glial cells remain physically connected to the lineage (orange arrowheads). (I-J) At 85 hours AEL, more glial cells have formed and the glia have spread inwards and sideways beneath the inner surface of the IPCs. Scale bars, 20 μm.

Fig. 6. Chiasm glia differentiate during the second half of third instar

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

Fig. 7. *gcm-lacZ* expression reflects the early formation of DL1-derived optic lobe glia

(A-A^{ii}) In early third instar, all Repo-positive cells (green in A'') in DL1 MARCM clones (white) also express *gcm-lacZ* (magenta in A^{i}), but several cells per clone express only *gcm-lacZ* but not (yet) Repo (orange arrows). By 120 hours AEL, in late wandering third instar larvae, *gcm-lacZ* expression is lost (C^{ii}), or almost lost (D^{ii}) from all outer and inner chiasm glia (dotted outlines in C-D^{iv}), which distinguishes these glia clearly from the surrounding glia in the optic lobe, as well as from the central complex glia (D^{iii}) that originate from medial type II lineages at a later time. The DL1 INP clone in B-D^{iii} (yellow) is the same clone as in Fig. 4A. Scale bars, 20 μm.

Fig. 8. Morphology of DL1-derived neuronal and glial cells during metamorphosis

(A-D) tub-Gal4 MARCM clones from two 10 h APF pupae in frontal view (A-C, dorsal to the top, medial to the right) and horizontal view (D, rostral to the top, medial to the right), counterstained with anti-Repo (magenta) and anti-Nrt (white in B-D). (A) Maximum intensity projection of the entire clone. The glial and neuronal cell clusters are outlined in white and orange dotted lines, respectively. Insets show single confocal slices through nuclei of the three glial cell types to indicate their positions. (B,C) Single confocal slices from the clone in (A) at the level of the glial cell bodies and processes of the outer chiasm glia (Xg^{o}; B-C), inner chiasm glia (Xg^{i}, C) and the most proximal glia labeled LCBRg (lateral cell body rind glia, C) that are assumed to be identical to the proximal most glial type in the adult lateral cell body rind (compare with Fig. 9E-H).

The horizontal view in (D) shows three processes of an Xg^i cell, as well as the rows of epithelial, marginal, and outer chiasm glia (inset). (E-I) Maximum intensity projection (E; insets and outlines as in (A)) and single confocal slices (F-I) of an *R38H02-Gal4* Flp-out clone at 24 hours APF, counterstained with anti-Repo (magenta) and anti-Nrg (white in F-I). White arrows point to glial nuclei, green arrows to glial processes. The longest processes of Xg^o run from distal to proximal along the anterior surface of the medulla neuropile (F), at the level of the medulla neuropile glia nuclei (F, mng). The three parallel processes shown in (F) (green arrow) belong to a single outer chiasm glial cell. All Xg^o processes (green arrows in F-I) wrap around outer chiasm axon tracts that are labeled by anti-Nrg (F-H) but not anti-Nrt (B). Beneath and parallel to the layer of outer chiasm glia lie the thicker axon bundles of inner optic chiasm that are labeled by both anti-Nrt and anti-Nrg (C,G,H). Xg^i wrap these axon bundles along their whole length (G,H), and project additional processes into the developing lobula complex (D,I, green arrow) that come to lie between the lobula and lobula plate in the adult (compare with Fig. 9D). Scale bars, 20 μm.

Fig. 9. Morphology of DL1-derived neuronal and glial cells in the adult

tub-Gal4, UAS-mCD8GFP labeled MARCM clones of DL1 (A,E-F) and DL1 INPs (B-D,G-H) in adult brains to reveal the morphology of DL1-derived secondary axon tracts (A), outer chiasm glia (B, Xg^o), inner chiasm glia (C-E, Xg^i), and two examples of cortex glia in the lateral cell body rind (E-F and G-H, LCBRg). All panels show frontal views, dorsal is to the top, medial is to the right. The brains were counterstained with anti-Repo (magenta) and either nc82 (white in C-E) to reveal neuropile structures, or anti-Nrg

(magenta in B, white in G) to reveal axon tracts. (A) Maximum intensity projection of an isolated adult DL1 neuroblast clone. The neuronal part of the clone is composed of two cell clusters (orange dotted outlines); the dorsal cluster arborizes in the intermediate-medial region of the superior protocerebrum (short orange arrow) and projects to two layers of the fan-shaped body (FB, long orange arrows). The ventral cluster arborizes broadly in the posterior ventrolateral protocerebrum (orange arrowhead). Two glial cell nuclei of the DL1 neuroblast clone are shown in insets. (B) Adult morphology of two Xg^{o} cells; the inset shows the top cell in a maximum intensity projection (green arrow), the bottom cell is shown in a single slice at the level of the nucleus (long white arrow) that is located within the single row of Xg^{o} nuclei (short white arrow) at the edge of the medulla, adjacent to the lamina (La). (C,D) Adult morphology of four Xg^{i} cells; the inset in (C) shows the top two cells in a maximum intensity projection (green arrows), and the bottom two cells in a single slice at the level of their nuclei (white arrows) that lie within the row of Xg^{i} nuclei (short white arrow). (D) Apart from their proximodistal orientation, the Xg^{i} cells also extend processes posteriorly between the lobula plate (LoP) and lobula (Lo), and the lobula plate (LoP) and medulla (Me) (green arrows). (E-H) Two cortex glial cells with different morphology in the lateral cell body rind (LCBR), the region of neuronal cell bodies between the optic lobe and central brain. The LCBR glial cell (LCBRg) in (E,F) has its cell body at the dorsal edge of the lobula and medulla (long white arrow), wraps around several adjacent Repo-negative cell bodies in the cortex (yellow arrows), and extends a diffuse process (long green arrow in F) with fine branches into the space between medulla and lobula at the level of the Xg^{i} cells (short white arrows in E). The LCBR glial cells in (G,H) wraps around many more Repo-negative cell bodies

in the LCBR (yellow arrows in G), and extend short processes into the central brain (green arrow in G) along axon tracts that interconnect the central brain and optic lobe (orange arrow in G). (H) Maximum intensity projection of one LCBRg cell from (G); Repo channel included (magenta) to visualize its position between central brain (CB) and optic lobe (OL). Scale bars, 20 μm.

Table legends

Table 1. Timing of gliogenesis in DL1 neuroglioblast lineages.

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

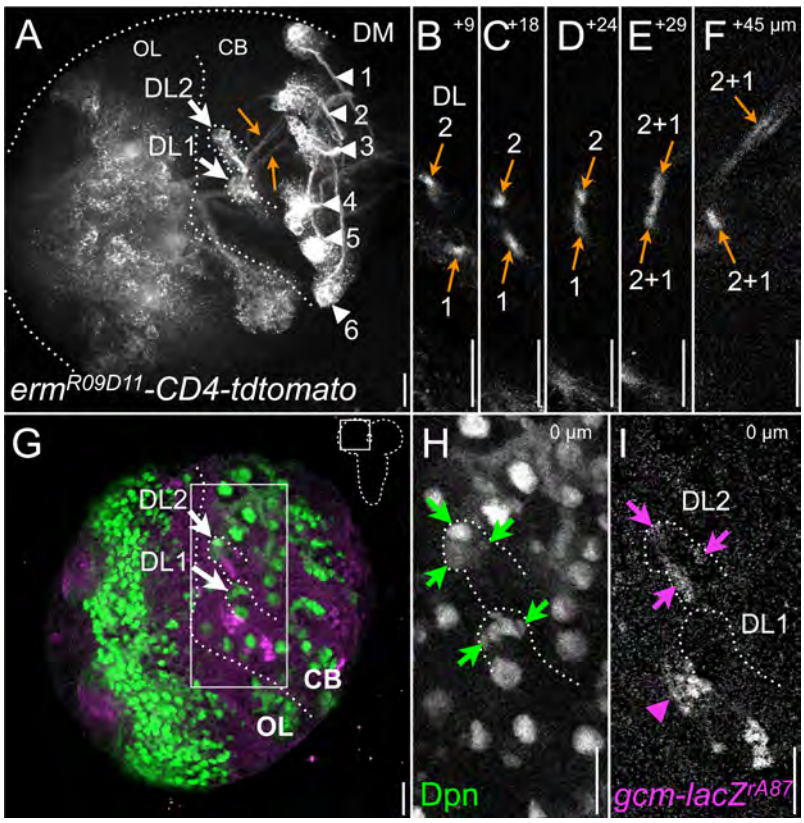
Supplementary figure legends

Fig. S1. Time line of glial cell formation and division in R38H02-Gal4 Flp-out clones.

(A-F) In DL1 clones generated by *R38H02-Gal4* Flp-out (white, outlined by orange dotted lines), the timing of development of Repo-positive (magenta) glial cells and their

translocation into the optic lobe (OL, border indicated by white dotted lines) are identical to that seen in MARCM clones (compare with Fig. 5). (A-B) Late second instar larval DL1 clone, recovered at 68 hours AEL, coincides with *R09D11-CD4-tdTomato* expression (green) in DL lineages and contains one Repo-positive cell (magenta in B). (C-D) In early third instar at 72 h AEL, DL1-derived cells are found outside of the DL1 lineage and inside the optic lobe. The axon tract of the DL1 clone coincides exactly with that of *R09D11-CD4-tdTomato* expression (green, green arrowhead in D), which confirms the identity of the lineage. (E-F) At 84 hours AEL, more Repo-positive cells have formed and spread laterally inside the optic lobe. The one pH3-positive, dividing glial cell in this clone is shown in green. The migrating glial cells are connected to each other and to the neuronal part of the lineage by cytoplasmic processes (orange arrows in D,F). Scale bars, 20 μm.

Fig. 1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OL
CB
DM
DL2
DL1
1
2
3
4
5
6
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C +18
D +24
E +29
F +45 µm
DL
2
1
2+1
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DL1
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DL2
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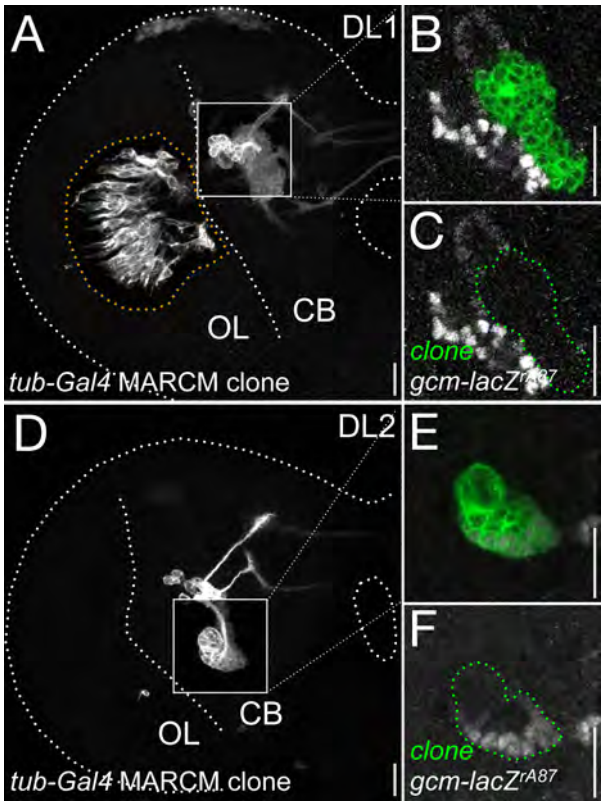
Fig. 2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clone
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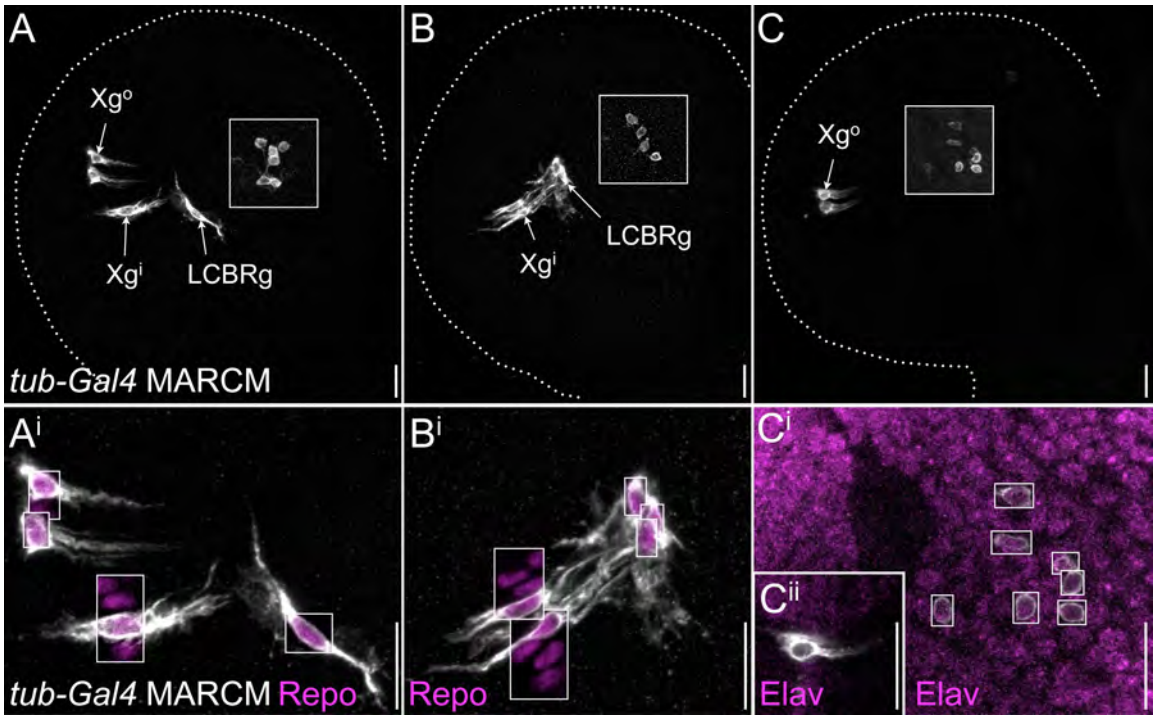
Fig. 3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Xg^o
Xg^i
LCBRg
tub-Gal4 MARCM
B
LCBRg
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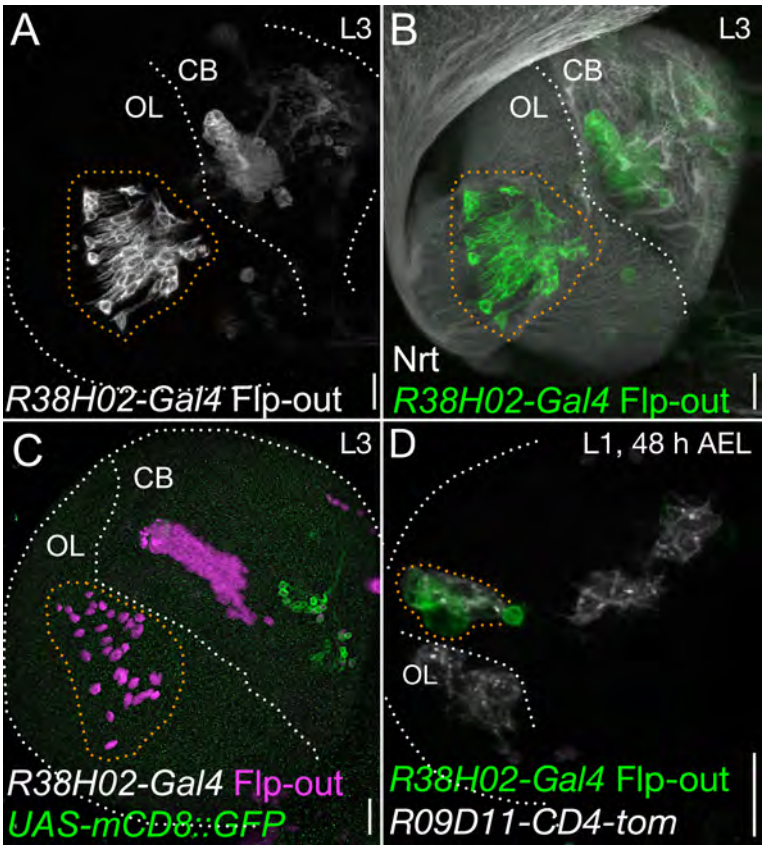
B^i
Repo
C^i
C^ii
Elav
Elav

Fig. 4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L3
CB
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R38H02-Gal4 Flp-out
B
L3
CB
OL
Nrt
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C
L3
CB
OL
R38H02-Gal4 Flp-out
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D
L1, 48 h AEL
OL
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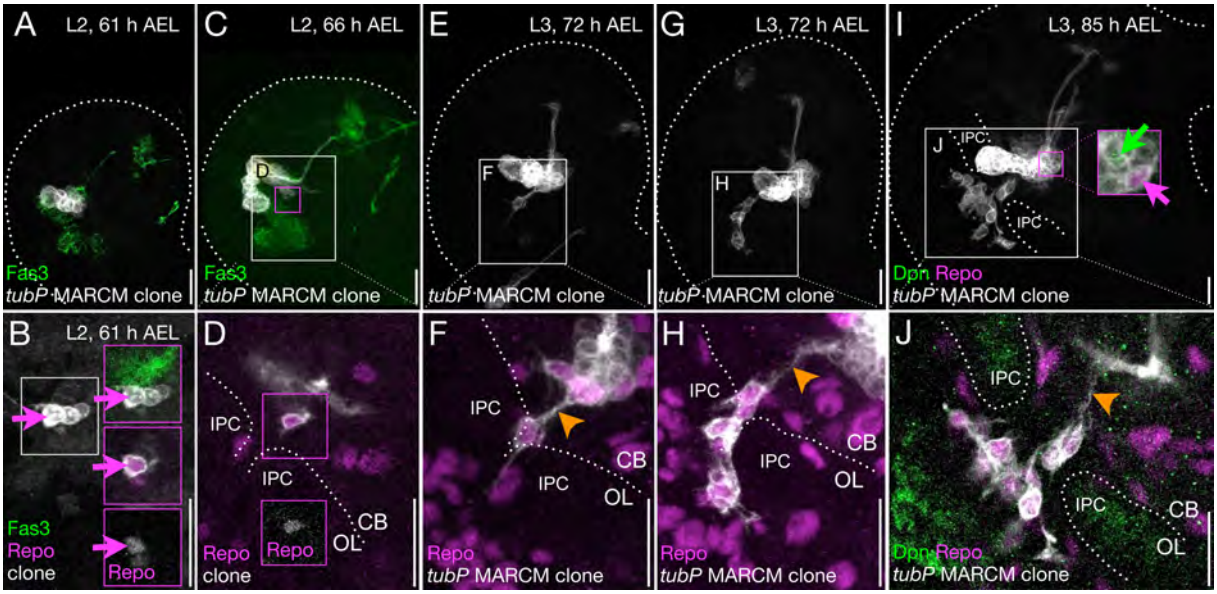
R09D11-CD4-tom

Fig. 5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Fas3
tubP MARCM clone
C
L2, 66 h AEL
D
Fas3
tubP MARCM clone
E
L3, 72 h AEL
F
tubP MARCM clone
G
L3, 72 h AEL
H
tubP MARCM clone
I
L3, 85 h AEL
J
IPC
IPC
Dpn Repo
tubP MARCM clone
B
L2, 61 h AEL
Fas3
Repo
clone
Repo
D
IPC
IPC
CB
OL
Repo
clone
Repo
F
IPC
CB
IPC
OL
Repo
tubP MARCM clone
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IPC
CB
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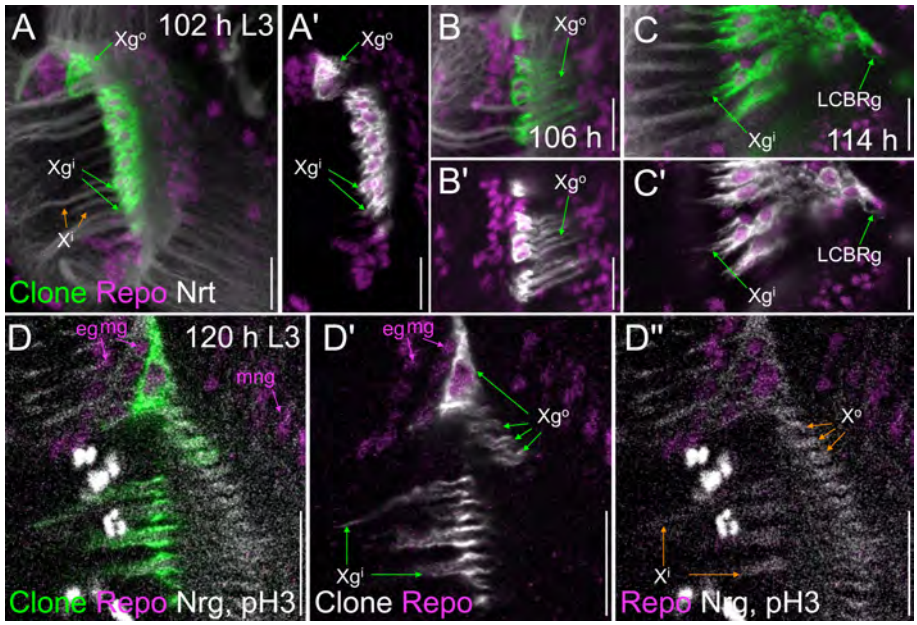
Dpn Repo
tubP MARCM clone

Fig. 6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Xg°
Xgⁱ
Xⁱ
Clone Repo Nrt
A'
Xg°
Xgⁱ
B
Xg°
106 h
B'
Xg°
C
LCBRg
Xgⁱ
114 h
C'
LCBRg
Xgⁱ
D
eg mg
120 h L3
mng
Clone Repo Nrg, pH3
D'
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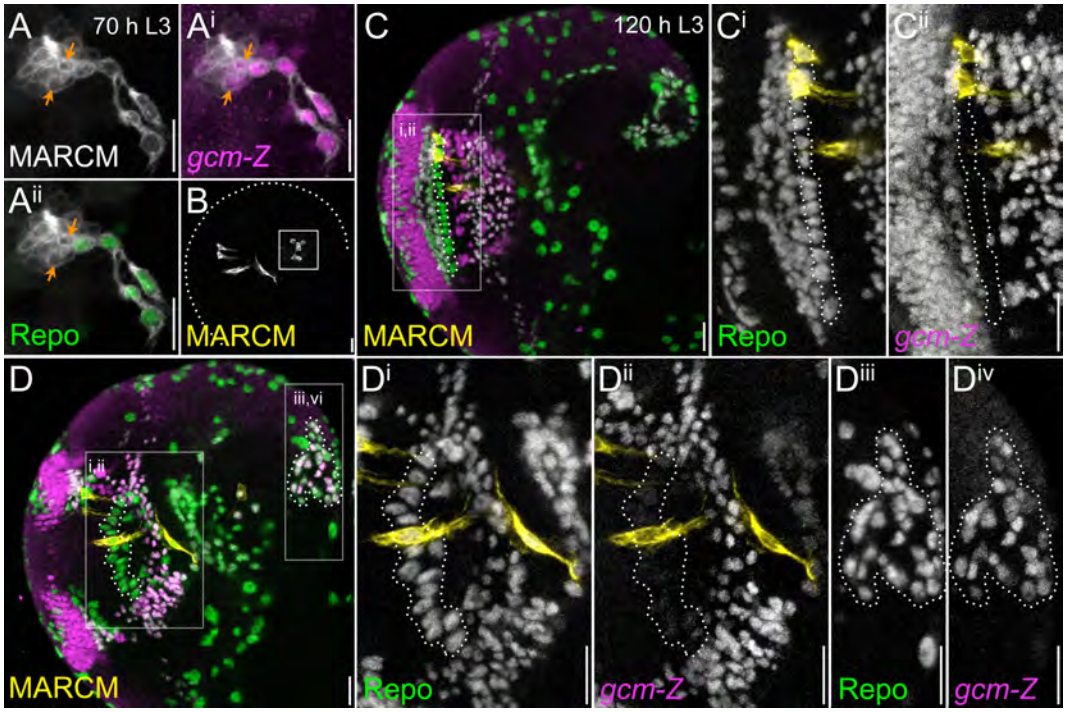
Xg°
Xgⁱ
Clone Repo
D''
X°
Xⁱ
Repo Nrg, pH3

Fig. 7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70 h L3
MARCM
Ai
gcm-Z
Aii
Repo
B
MARCM
C
120 h L3
i,ii
MARCM
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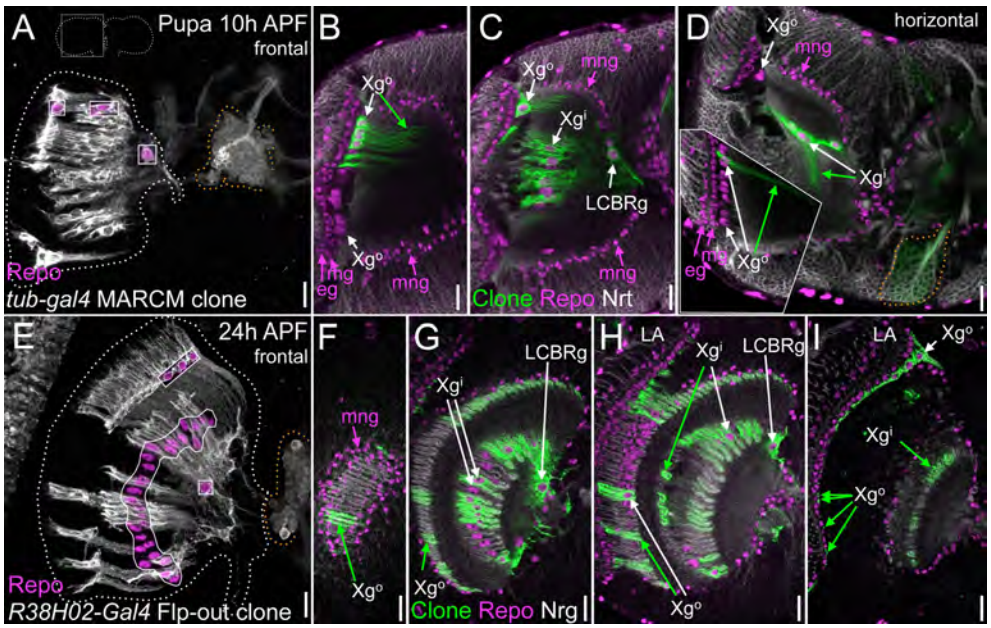
Repo
Cii
gcm-Z
D
iii,vi
i,ii
MARCM
Di
Repo
Dii
gcm-Z
Diii
Repo
Div
gcm-Z

Fig. 8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Pupa 10h APF
frontal
Repo
tub-gal4 MARCM clone
B
Xg°
mng
eg
mng
C
Xg°
mng
Xgⁱ
LCBRg
Clone Repo Nrt
mng
D
Xg°
mng
horizontal
Xgⁱ
eg
mng
Xg°
E
24h APF
frontal
Repo
R38H02-Gal4 Flp-out clone
F
mng
Xg°
G
LCBRg
Xgⁱ
Xg°
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LA
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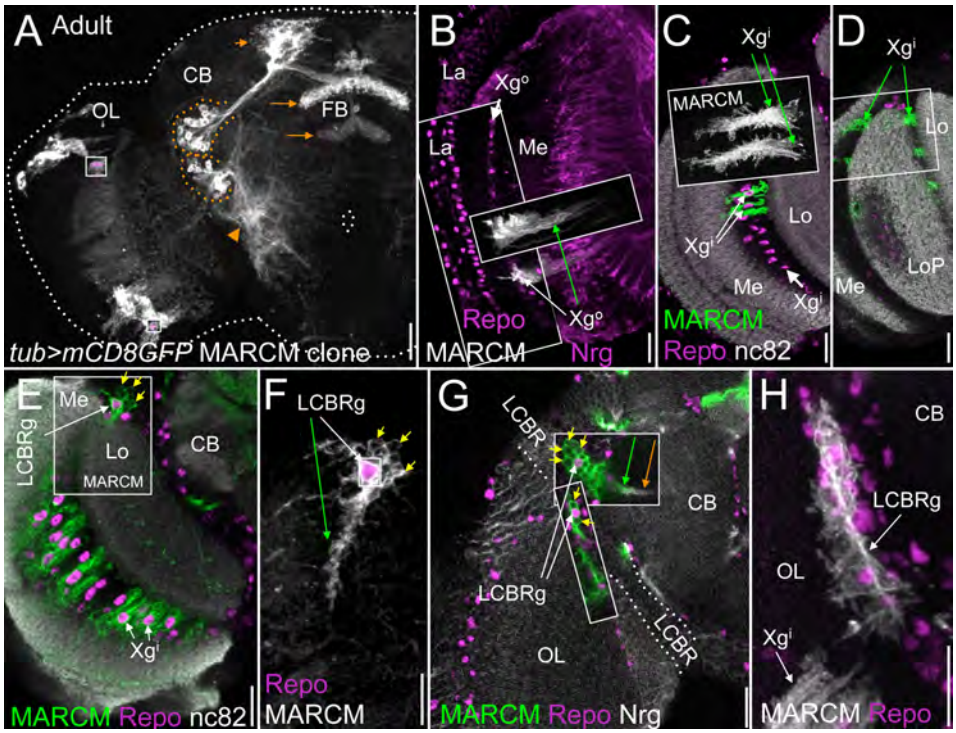
LCBRg
Xg°
I
LA
Xg°
Xgⁱ
Xg°

Fig. 9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OL
FB
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B
La
Xg°
La
Me
Repo
Xg°
MARCM
Nrg
C
Xgⁱ
MARCM
Lo
Xgⁱ
Me
Xgⁱ
MARCM
Repo nc82
D
Xgⁱ
Lo
LoP
Me
E
Me
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Repo
MARCM
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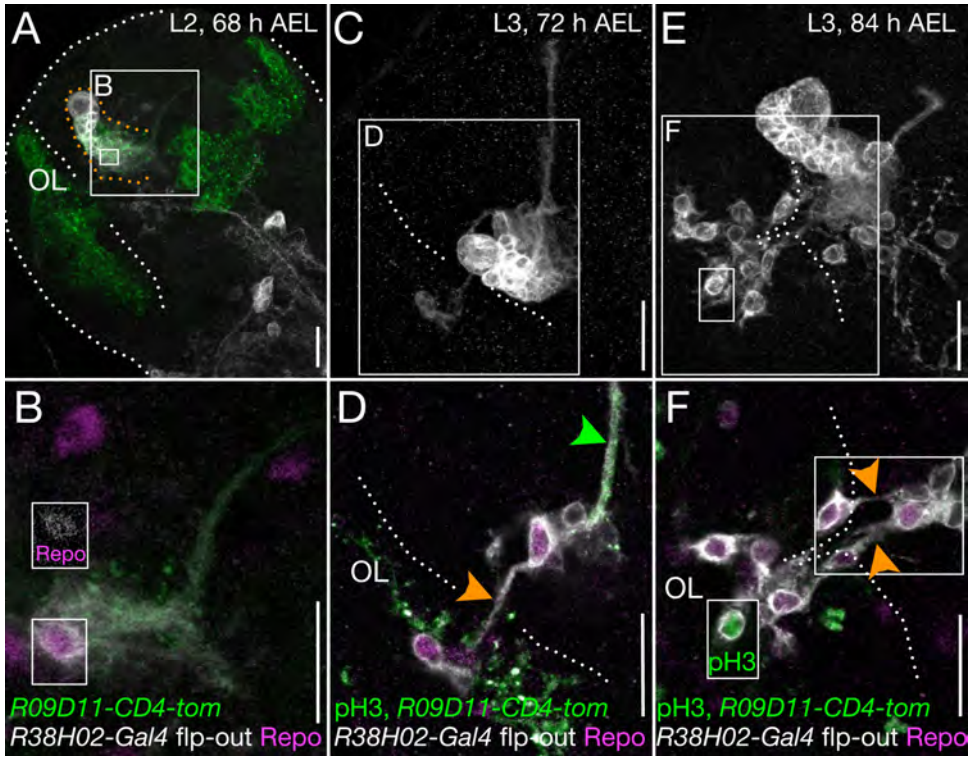
Xgⁱ
MARCM Repo

Fig. 10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OL
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L3, 72 h AEL
D
E
L3, 84 h AEL
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R38H02-Gal4 flp-out Repo
D
OL
pH3, R09D11-CD4-tom
R38H02-Gal4 flp-out Repo
F
OL
pH3
pH3, R09D11-CD4-tom
R38H02-Gal4 flp-out Repo