Development 139, 2510-2522 (2012) doi:10.1242/dev.077883

Origin of *Drosophila* mushroom body neuroblasts and generation of divergent embryonic lineages

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SUMMARY

Key to understanding the mechanisms that underlie the specification of divergent cell types in the brain is knowledge about the neurectodermal origin and lineages of their stem cells. Here, we focus on the origin and embryonic development of the four neuroblasts (NBs) per hemisphere in *Drosophila* that give rise to the mushroom bodies (MBs), which are central brain structures essential for olfactory learning and memory. We show that these MBNBs originate from a single field of proneural gene expression within a specific mitotic domain of procephalic neuroectoderm, and that Notch signaling is not needed for their formation. Subsequently, each MBNB occupies a distinct position in the developing MB cortex and expresses a specific combination of transcription factors by which they are individually identifiable in the brain NB map. During embryonic development each MBNB generates an individual cell lineage comprising different numbers of neurons, including intrinsic γ-neurons and various types of non-intrinsic neurons that do not contribute to the MB neuropil. This contrasts with the postembryonic phase of MBNB development during which they have been shown to produce identical populations of intrinsic neurons. We show that different neuron types are produced in a lineage-specific temporal order and that neuron numbers are regulated by differential mitotic activity of the MBNBs. Finally, we demonstrate that γ-neuron axonal outgrowth and spatiotemporal innervation of the MB lobes follows a lineage-specific mode. The MBNBs are the first stem cells of the *Drosophila* CNS for which the origin and complete cell lineages have been determined.

KEY WORDS: Brain development, Mushroom body, Neuroblast lineage, γ-neuron, *dachshund* (*dac*), *eyeless* (*ey*), *Retinal homeobox* (*Rx*)

INTRODUCTION

The *Drosophila* brain arises from ~100 neural stem cells (on either side), called neuroblasts (NBs), that derive from the early procephalic (head) neuroectoderm. Each brain NB assumes an individual identity, as reflected by specific combinations of gene expression (Urbach and Technau, 2004). Embryonic NBs divide asymmetrically to produce ganglion mother cells, which divide once to give rise to two postmitotic progeny cells (Pearson and Doe, 2004; Skeath and Thor, 2003). Neurons born in the embryo control larval behavior and may become reorganized during metamorphosis to contribute to the adult neural circuitry (Lee et al., 1999; Marin et al., 2005; Technau and Heisenberg, 1982; Truman et al., 2004). After a period of mitotic quiescence, embryonic brain NBs (now called postembryonic NBs) become reactivated in the larva to add large numbers of adult-specific neural cells (Ito and Hotta, 1992; Pereanu and Hartenstein, 2006). About 100 postembryonic cell lineages (per hemisphere) have been recognized in the larval brain (e.g. Cardona et al., 2010; Kumar et al., 2009; Lai et al., 2008; Larsen et al., 2009; Lichtneckert et al., 2007; Pereanu and Hartenstein, 2006; Yu et al., 2009; Yu et al., 2010), suggesting that almost all embryonic brain NBs become reactivated postembryonically. However, the assignment to individually identified NBs (Urbach and Technau, 2003a) and an analysis of the embryonic components of these brain lineages are still pending. This is a major challenge because knowledge about the identities and origin of the individual NBs and the composition of their entire lineages is crucial for studying the molecular mechanisms that underlie the spatiotemporal specification of cell fates.

In this study we investigate the embryonic development of the mushroom bodies (MBs), a pair of prominent central brain structures that play an essential role in olfactory learning and memory (Davis, 2011; Heisenberg, 2003). In the adult fly, each MB comprises more than 2000 intrinsic neurons (Technau and Heisenberg, 1982) called Kenyon cells, which extend dendrites into the calyx and axons to form the peduncle and five distinct lobes (the vertical α and α′ lobes and the medial β, β′ and γ lobes) (Armstrong et al., 1998; Ito et al., 1997; Kurusu et al., 2002; Strausfeld et al., 2003; Tanaka et al., 2008; Yang et al., 1995). The Kenyon cells are produced by four mushroom body neuroblasts (MBNBs) per hemisphere that continuously divide from the embryonic to the late pupal stage (Fig. 1C) (Ito and Hotta, 1992; Prokop and Technau, 1994; Stocker et al., 1995; Truman and Bate, 1988). So far, the lineages of the four MBNBs have been traced and analyzed during their postembryonic development when they generate the cells required for the adult MBs. It has been shown that the clonal units generated by the MBNBs during larval and pupal stages are indistinguishable from each other (Ito et al., 1997). Each MBNB sequentially generates three types of Kenyon cells (γ-neurons until the mid-third instar larval stage, α′/β′-neurons at the late larval stage, and α/β-neurons during the pupal stage), which send axons into specific lobes of the mature MB (Armstrong et al., 1998; Crittenden et al., 1998; Lee et al., 1999; Ito et al., 1997; Yu and Lee, 2007; Zhu et al., 2003). The structure of the larval MB is simpler than that of the adult MB in that the lobe system consists of one medial and one vertical lobe, both innervated by γ-neurons (Fig. 1A-C) (Kurusu et al., 2002; Noveen et al., 2000; Tettamanti et al., 1997). However, the basic design of the

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Accepted 7 May 2012

larval olfactory system is similar to that of the adult (Rameakers et al., 2005), and early larvae already exhibit appetitive olfactory learning that relies on a small subset of embryonic-born Kenyon cells (Pauls et al., 2010).

Here, we clarify the neuroectodermal origin of the embryonic MBNBs and, based on the expression of an NB-specific combinatorial code of transcription factors, assign them to identified progenitors in our brain NB map (Urbach and Technau, 2003a). We show that each embryonic MBNB generates an individual cell lineage consisting of different numbers of neurons. Among these neurons are intrinsic γ-neurons (forming the larval MB neuropil) and non-intrinsic interneurons that make connections with the contralateral brain hemisphere and ventral nerve cord (VNC). The different types of neurons develop in a fixed temporal order. The lineage-specific numbers of γ-neurons are not regulated by programmed cell death but by MBNB-dependent mitotic activity in the last third of embryogenesis. Additionally, we show that the onset of γ-neuron axonal outgrowth and spatiotemporal innervation of MB lobes is distinct for each MBNB lineage. Taken together, these data uncover the existence of non-intrinsic neurons in MB lineages and indicate that, in the embryo, different aspects of MB development are controlled in a lineage-specific manner, which contrasts with postembryonic stages when the four MBNBs produce identical clonal subunits of intrinsic Kenyon cells.

MATERIALS AND METHODS

Fly stocks

Fly strains used were: Oregon R (wild type), ubi-GFPnls, UAS-mCD8::GFP, *seven up*-lacZ, OK107-Gal4, *Notch*^{ts1} (all Bloomington Stock Center) and repo4.3-Gal4 (kind gift from C. Klämbt, Münster, Germany).

Time-lapse microscopy

Embryos were placed on a glue-coated coverslip in dorsolateral orientation and covered with Voltalef oil (10S). Using a Leica SP2 confocal microscope, images were captured as 512×512 pixel *z*-stacks every minute, and *z*-stacks (increment of 1 μm) were processed using Leica software.

DiI labeling of NB clones and ablation experiments

Labeling of NB clones was performed as described previously (Bossing et al., 1994), except that embryos were orientated as described above. The position of a labeled cell was determined by counting cell rows within mitotic domain B (δB) from the dorsal midline in a ventral direction. Lipophilic dyes were dissolved in vegetable oil [DiI (5 mg/ml), CM-DiI (3 mg/ml), DiD (10 mg/ml); Molecular Probes]. Most NB lineages were labeled with CM-DiI, which yielded the strongest stainings. The behavior of marked cells (i.e. delamination, mitosis) was inspected 60 minutes after labeling.

For ablation experiments, MBNB clones were DiI labeled in OK107>CD8::GFP [called *eyeless* (*ey*)>GFP] embryos. Labeled cell(s) (NB or early progeny, distinguished by cell diameter) were removed by suction into a pulled and bevelled glass capillary (tip diameter ~2 μm) by brief application of low pressure. At stage (st) 17 or first larval instar (L1), the CNS was dissected and NB clones were documented in PBS with a Leica SP2 confocal microscope. Imaging was performed using Leica software and Adobe Photoshop. Drawings were prepared from confocal *z*-stacks and documented using Adobe Illustrator. For cell counts in a *z*-stack, cells were marked and counted using the Landmarks function of Amira 4.0 (Visage Imaging). For 3D reconstructions, digitized confocal sections (increment 1 μm) of DiI-labeled lineages were imported into Amira. DiI and GFP patterns were reconstructed using a combination of the Threshold function and manual labeling.

DiI labeling of single postmitotic cells

Dissected brains from st17 (or L1) *ey*>GFP embryos were fixed in 5:1 PBS:formaldehyde for 12 minutes, washed in PBS, glued onto a polylysine-coated coverslip, and covered with PBS. *ey*>GFP-positive γ-neurons were identified under an Olympus fluorescence microscope equipped with a 60× water-immersion objective. Single γ-neurons were labeled using a glass capillary filled with 1 mg/ml DiI/ethanol that was brought into contact with a cell body and a depolarizing current applied for up to 1 minute [according to Rickert et al. (Rickert et al., 2011)]. Brains were again fixed, and labeled single cells documented as described above.

EdU experiments

ey>GFP embryos were injected between st13 and st17 with 2 mM EdU/PBS and after 1.5 or 6 hours fixed for 15 minutes in 4% paraformaldehyde in PBS. EdU was detected according to the manufacturer's instructions (Click-iT EdU Imaging Kit, Invitrogen), and thereafter embryos were stained with GFP antibody as described below. Student's two-tailed *t*-test was performed for statistical significance.

Inactivating Notch signaling

To inactivate Notch signaling, 1-hour collections of *Notch*^{ts1} embryos were raised for 3 hours 20 minutes at 22°C (until st7), then shifted for 3.5 hours at 29°C (until st11/12), again cultured at 22°C until st17, and fixed.

Immunohistochemistry and in situ hybridization

Embryos were immunostained according to previously published protocols (Urbach et al., 2003). Primary antibodies used were: rabbit anti-β-Gal (1:2000, Promega), chicken anti-β-Gal (1:1000, Abcam), mouse anti-Dac [1:250, Developmental Studies Hybridoma Bank (DSHB)], sheep anti-DIG (1:1000, Roche), guinea pig anti-Dpn (1:1000, kind gift from J. Skeath, St Louis, MO, USA), rabbit anti-Ey (1:1000, kind gift from U. Walldorf, Saarbrücken, Germany), rat anti-Ey (1:5000, kind gift from P. Callaerts, Leuven, Belgium), rabbit anti-Mira (1:500, kind gift from J. Knoblich, Vienna, Austria), mouse anti-Pros (1:100, DSHB), rabbit anti-PH3 (1:500, Millipore), rabbit anti-Rx (1:1000, kind gift from U. Walldorf), mouse anti-GFP (1:125, Roche) and chicken anti-GFP (1:500, Millipore). Secondary antibodies coupled to Alexa 488, Alexa 568, Alexa 647, Dylite 549 and AP (all 1:500) were obtained from Dianova.

Image stacks were collected using a Leica SP2 confocal microscope and were processed using Leica confocal software and Adobe Photoshop. In situ hybridizations were performed as described previously (Urbach et al., 2006). The *achaete* RNA probe was synthesized using *Xho*I-linearized pBluescript SK(–) (BDGP) as a template with T3 RNA polymerase, and the *scute* RNA probe using *Nco*I-linearized pGEM-T Easy (Promega) as a template with SP6 RNA polymerase. The *lethal of scute* RNA probe was synthesized using EST clone *lsc* RE59335 [Berkeley Drosophila Genome Project (BDGP)] cloned in *Pst*I-linearized pFLC1 (BDGP) as a template with T3 RNA polymerase, and the *earmuff* RNA probe using EST clone *erm* GH14092 (BDGP) cloned in *Eco*RI-linearized pOT2 (BDGP) as a template with SP6 RNA polymerase. All probes were DIG labeled according to the manufacturer's protocol (Roche). Images were acquired using a Zeiss Axioplan microscope and processed with Adobe Photoshop.

RESULTS

MBNBs derive from mitotic domain B of procephalic neuroectoderm

Since MARCM and FLP/FRT methods fail to label entire NB lineages in the embryo (see Discussion) we made use of the DiI labeling technique (Bossing and Technau, 1994) to examine the embryonic development of MBNBs (Fig. 1A-D). To map the origin of the four MBNBs in the early procephalic neuroectoderm (NE) we employed the stereotypical pattern of 'mitotic domains' (Foe, 1989) as morphological landmarks (Fig. 2A). In ubi-GFPnls embryos (which ubiquitously express nuclear GFP) mitotically active and inactive domains can be clearly distinguished (Fig. 2A,B). Mitotic domain B (δB) is known to give rise to a large part of the dorso-posterior protocerebrum (Urbach et al., 2003). Accordingly, we obtained MBNB lineages only upon labeling of NE cells in δB (Fig. 2). Because 4D microscopy revealed that the cellular pattern of δB is largely invariant by stage (st) 7 (Fig. 2A-B″), we systematically DiI labeled individual δB cells to map this

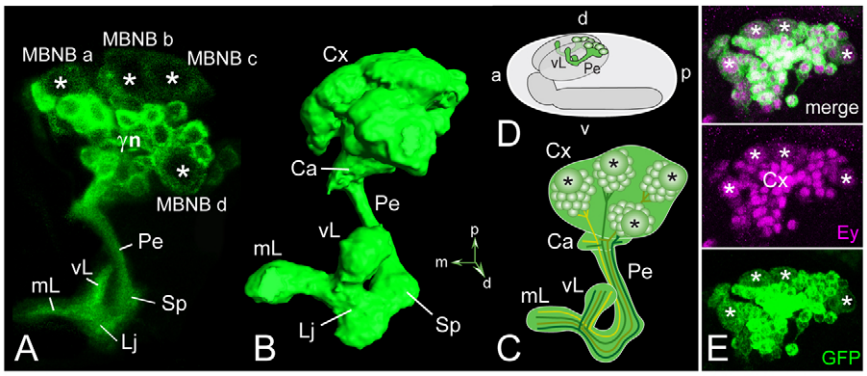

Fig. 1. Organization of the mushroom body in the *Drosophila* late embryo/early larva. (A-C) Shown is the mushroom body (MB) in the right brain hemisphere in dorsal view. (**A**) Composite confocal image of OK107(*ey*)>CD8::GFP visualizes the morphology of the whole MB. CD8::GFP is detected in γ-neurons (γn) and the four mushroom body neuroblasts (MBNBs; asterisks in A,C,E) in the late st17 embryo. (**B**) Three-dimensional reconstruction of the MB in the early L1 based on OK107(*ey*)>CD8::GFP expression. (**C**) Scheme illustrates the four clonal subunits of the larval MB. γ-neurons form axonal tracts running through the peduncle (Pe) into the medial (mL) and vertical (vL) lobe and dendritic branches in the calyx (Ca). (**D**) MBs in a lateral view within the CNS of the late embryo. (**E**) Co-expression of Ey and OK107(*ey*)>CD8::GFP in all cell bodies of the late st17 MB cortex (Cx). a, anterior; p, posterior, d, dorsal; v, ventral; m, medial; Lj, lobe junction; Sp, spur.

domain more precisely. We identified a group of 10-12 cells from which the four MBNBs emerge. These are located within the ventral half of δB (Fig. 2B′,B″), unlike previously reported (Robertson et al., 2003).

Each MBNB develops from a distinct neuroectodermal progenitor cell

The set of four MBNBs could principally result from two different scenarios: they could evolve through symmetric divisions of a common precursor (either an NE cell or NB; Fig. 2E, scenarios 1 and 2), as for example in the holometabolous honey bee and in hemimetabolous insects (reviewed by Urbach and Technau, 2003b); or, alternatively, each MBNB could develop from a different NE progenitor cell (Fig. 2E, scenario 3). To test these possibilities we DiI labeled single NE cells in δB in OK107>CD8::GFP embryos and followed in vivo the early steps (i.e. delamination, mitosis) in the development of each MBNB. The domain of OK107-Gal4 expression in the posterior protocerebrum reflects the MB-specific component of *eyeless* (*ey*) expression (hereafter termed *ey*>GFP) (Fig. 1E) (Adachi et al., 2003). Colocalization of DiI and *ey*>GFP was therefore used to identify MBNBs and their cell clones. In all cases (n=119), a single DiI-labeled δB cell delaminated to produce only one of the four MBNBs, which then asymmetrically divided to generate daughter cells. Therefore, we can rule out the possibility that the set of four MBNBs arises by division of a common precursor cell (Fig. 2E, scenarios 1 and 2).

MBNBs can develop from adjacent neuroectodermal progenitors within the same proneural domain

The NE cell group in the ventral half of δB from which the four MBNBs evolve co-expresses the proneural genes *achaete*, *scute* and *lethal of scute*, indicating that they belong to the same proneural domain (Fig. 2C). All cells maintain high proneural gene expression levels during the short time window in which MBNBs and other protocerebral NBs emerge from this domain. Since most, or perhaps all, cells of this proneural domain adopt an NB fate (see also Urbach et al., 2003), it is likely that MBNBs and other brain NBs develop from neighboring δB cells. To provide direct evidence for this, we labeled adjacent NE cells within the ventral half of δB with DiI (or DiD). The labeled pairs of NE cells either produced an MBNB and another (protocerebral) NB clone (n=4) or two MBNB clones (n=3; Fig. 2D,D′). These findings indicate that MBNBs derive from neighboring NE cells and suggest that among cells within this proneural domain, lateral inhibition, mediated by Notch signaling, is less efficient or lacking. This is supported by our observations in the temperature-sensitive $Notch^{ts}$ mutant: upon blocking Notch signaling during st8-11, when brain NBs develop, the number, size and position of Ey-positive MBNBs were normal, whereas the number of other brain NBs seemed to be increased as judged by the expression of the NB-specific marker Deadpan (143.8±7.2 cells per hemisphere in $Notch^{ts}$; 129.0±8.0 cells in wild type; n=13 each; P<0.0001).

Embryonic MBNBs express distinct combinations of transcription factors

In the established brain NB map the identity of the MBNBs remained unclear as we were unable to detect four *dachshund* (*dac*)/*ey*-co-expressing MBNBs emerging from Dac/Ey-co-expressing NE (Urbach and Technau, 2003a), as had been previously suggested (Noveen et al., 2000). In order to identify the MBNBs, we made use of a combination of marker genes, namely *dac*/*ey*/*seven up* (*svp*)-lacZ (see also Kurusu et al., 2000; Noveen et al., 2000) and *Retinal homeobox* (*Rx*), that are specifically expressed in MBNBs of st17 embryos (Fig. 3), as well as of morphological criteria (i.e. the larger size of MBNBs between st13 and st17, the position relative to each other and other NBs). Applying these criteria, we traced back the fate of each individual MBNB from st17 to st9 and identified Pcv9, Pcd4, Pcd9 and Pcd2 [compare NB maps in Urbach et al. (Urbach et al., 2003) and Urbach and Technau (Urbach and Technau, 2003a)] to represent the four MBNBs (hereafter MBNBa, b, c and d, respectively) (Fig. 3A-J). We observed that during the morphogenetic rearrangements of the brain between st11 and st17, the set of MBNBs undergoes a rotation and is translocated into the most posterior brain region (Fig. 3A,D,G). Furthermore, we found that Dac, Ey and Rx are differentially expressed in individual MBNBs: Dac is never expressed in MBNBd; before st13, Rx is not expressed in MBNBc and Ey not in MBNBb/c. Thus, in the early developmental period,

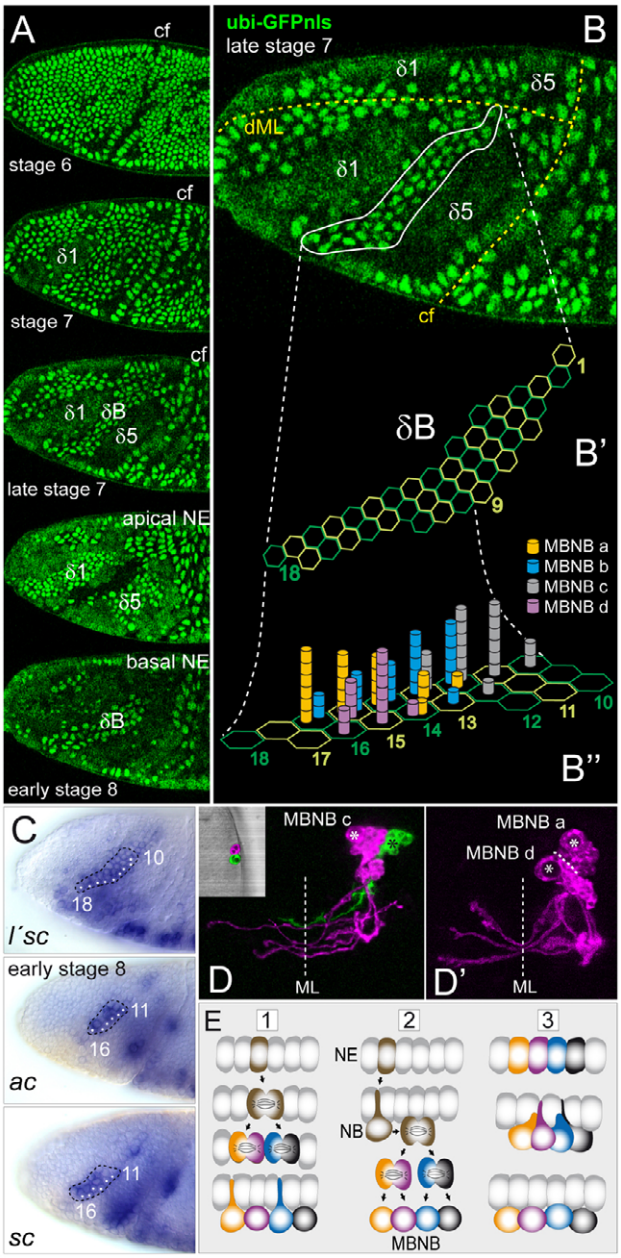

Fig. 2. Individual MBNBs develop from defined neuroectoderm precursor cell subsets in a common proneural cluster within the ventral half of δB. (**A**) Domains δ1, δ5 and δB in ubi-GFPnls *Drosophila* embryos. Nuclei of mitotically inactive δB cells [basally in the neuroectoderm (NE) layer] persistently express GFP. (**B-B″**) Late st7 embryo shown in A. Yellow dashed lines indicate the dorsal midline (dML) and cephalic furrow (cf); white line encircles δB consisting of 40±5 NE cells (n=5) organized in 18 cell rows along the dorsoventral axis, each between one and three cells broad, as schematically summarized in B′. (B″) MBNBs develop from NE cells in the ventral half of δB (rows 10-16). Diagram shows the distribution of MBNB clones derived from individually DiI-labeled δB cells (each cylinder represents one case; MBNBa, n=19; MBNBb, n=19; MBNBc, n=18; MBNBd, n=12). Four partly overlapping subgroups of δB cells are distinguishable according to MBNB fate (indicated by different colors). (**C**) Initial expression of *lethal of scute* (*l'sc*) encompasses the entire ventral δB (rows 10-18, as indicated by white dots in the encircled area, and some more dorsal δB cells), whereas initial *achaete* (*ac*) and *scute* (*sc*) expression, more specifically, is co-detected in the 10-12 δB cells (in rows 11-16) from which the MBNBs develop. (A-C) Anterior is left, dorsal to top. (**D**) MBNBc clone and another protocerebral NB clone (at late st17) obtained after labeling of two neighboring δB cells (in row 13) with DiI (magenta) and DiD (green) (see inset). (**D′**) MBNBa and MBNBd clones obtained after DiI labeling of two adjacent NE cells in rows 14/15. White asterisks mark NBs; ML, midline. (**E**) Three different scenarios by which MBNBs could develop in the early neuroectoderm. See main text for details.

each MBNB expresses a unique combination of these regulatory genes. This, and the morphological criteria, allow one to trace MBNBs individually throughout embryonic development, and, for the first time, establishes the link between a set of identified brain NBs (Urbach and Technau, 2003a) and their corresponding cell lineages.

Embryonic MBNBs produce neurons that are intrinsic and non-intrinsic to the MB

DiI labelings of the four MBNB cell clones in *ey*>GFP embryos show that each MBNB and its progeny occupies a distinct area within the (GFP-positive) MB cortex (Fig. 4). Single-cell DiI labelings of postmitotic neurons at late st17 reveal that axons of γ-neurons project through the peduncle and bifurcate to innervate the medial and vertical MB lobe, and first dendritic neurites innervate the developing calyx (Fig. 5A; see Fig. 8K,M,P). However, in each MBNB lineage we found that part of the daughter cell somata is located outside the *ey*>GFP domain (Fig. 4B-E″). Remarkably, unlike the MB-intrinsic γ-neurons, these *ey*>GFP-negative neurons do not send axons through the peduncle into the lobes; instead, they project into the contralateral brain hemisphere and/or into the terminal abdominal neuromere (Fig. 4B-E″; Fig. 5C,D; supplementary material Fig. S1). Since these neurons do not form part of the MB neuropils, we designated them as non-intrinsic neurons (ni-neurons). Moreover, there are indications of a further neuron subtype, at least among the progeny of MBNBa. The somata of these neurons are located in the periphery of the *ey*>GFP-positive MB cortex, between those of the γ- and ni-neurons. In contrast to the γ-neurons, their axons exit the peduncle in the 'spur' region and do not innervate the lobes (Fig. 5B,D), and are therefore termed type 2 non-intrinsic neurons (ni-type2-neurons). Thus, embryonic MBNBs produce distinct neuron types with heterogeneous molecular and morphological properties: with regard to somata position, Ey expression, axonal and dendritic arborization patterns, we distinguish between γ-, ni- and ni-type2-neurons, the latter sharing features of both subtypes.

Embryonic MBNBs do not generate glial cells

To clarify whether embryonic MBNBs generate glia cells, we produced DiI-labeled MBNB clones in embryos expressing nuclear GFP in a glia-specific pattern (*repo*>nGFP, which reliably reproduces the pattern of brain glial cells as confirmed in anti-Repo/*repo*>nGFP double labelings; supplementary material Fig. S2). We observe that DiI-labeled clones of all four MBNBs (at late st17 or in early L1) never overlap with *repo*>nGFP (n=3 for each MBNB clone; Fig. 5E; supplementary material Fig. S2), indicating that embryonic MBNBs do not produce glial cells.

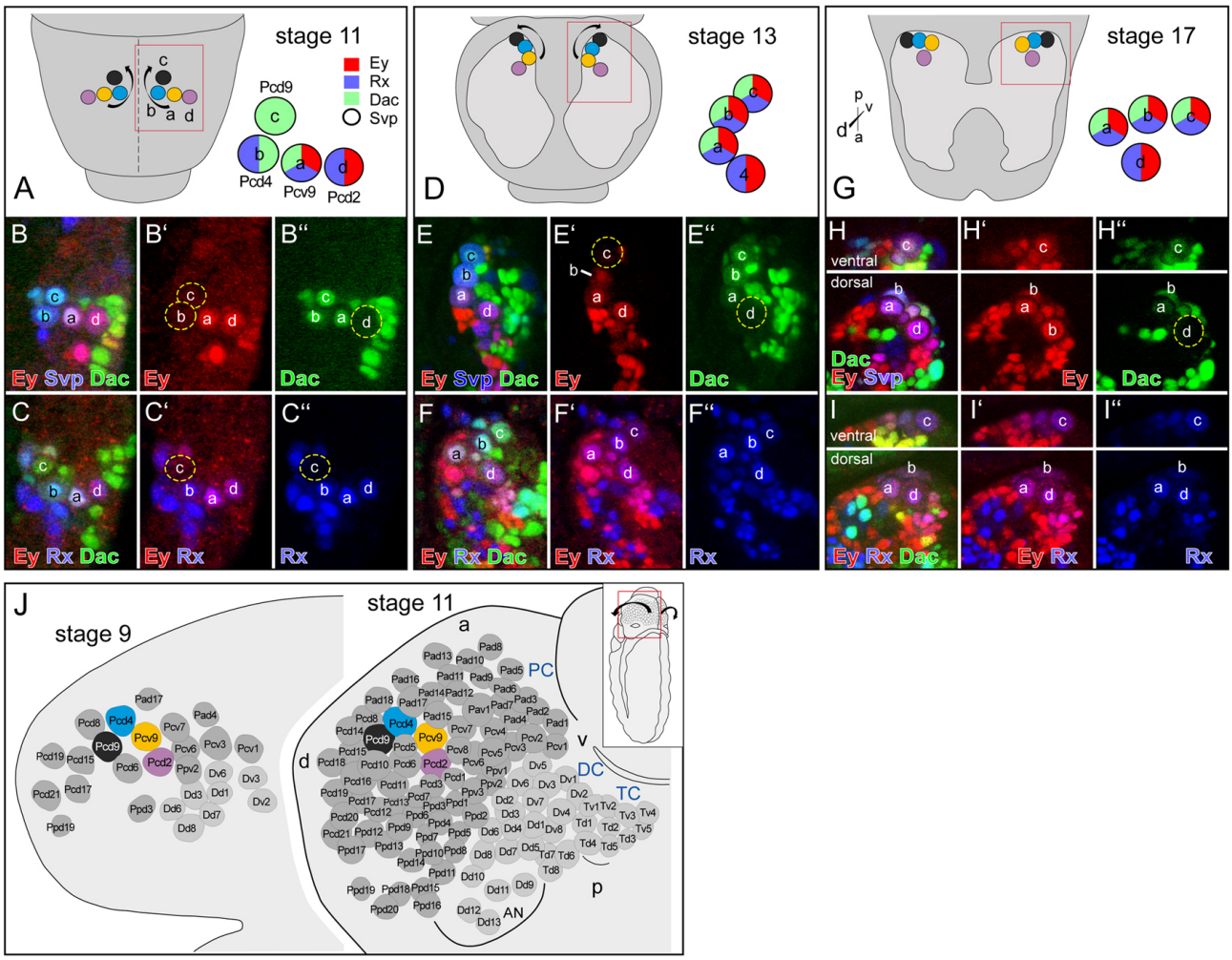

Fig. 3. Differential expression of Ey, Dac and Rx allows the fate of individual MBNBs to be followed throughout embryogenesis. (**A**,**D**,**G**) (Left) Schematic dorsal view of the head and brain (light gray in D,G) in *Drosophila* embryos at different developmental stages. Arrows indicate morphogenetic movements (rotation) that translocate the set of MBNBs (a, b, c and d, indicated in different colors) into the most posterior positions of the brain cortex. Red boxes indicate areas shown in B-I. (Right) Summary of gene expression in individual MBNBs. (**B-C″**,**E-F″**,**H-I″**) Composite confocal images of stainings for Ey, Dac and *svp*-lacZ (B,E,H), or Ey, Dac and Rx (C,F,I). (B) Early st11. Note that MBNBd (Pdc2) is Dac negative [encircled in B″; in contrast to our previous observations from histochemical stainings (Urbach and Technau, 2003a)] and that only two MBNBs express Ey, and only MBNBa co-expresses Ey/Dac [in contrast to observations by Noveen et al. (Noveen et al., 2000)]. (C) Late st11. (E) Early st13. (F) Late st13; MBNBs become identifiable also by size and position. (E′,F′) Ey becomes detectable in MBNBb/c and Rx in MBNBc (F″). (**J**) Schematic presentations of flat preparations (see inset) of the left side of the head illustrating brain NB patterns at st9 and st11 (for details, see Urbach et al., 2003), including the four identified MBNBs, which belong to the earliest-born brain NBs. TC, DC and PC are trito-, deuto- and protocerebrum, respectively; AN, antennal appendage.

Each embryonic MBNB creates a specific cell lineage

As shown above, MBNBs generate different neuron types, raising the question of whether the four embryonic MBNB clones contain a virtually identical set of neurons, as has been shown for the postembryonic MBNB clones (Ito et al., 1997; Lee et al., 1999). Remarkably, our data indicate significant differences between the embryonic lineages of all four MBNBs. As summarized in Table 1 and Fig. 4 for MBNB clones at mid/late st17 (and in supplementary material Fig. S1 for early L1), these differences concern the total cell number, the composition of neuronal types, and their axonal projection patterns: (1) whereas γ-neurons and, to a lesser degree, ni-neurons are produced by all four MBNBs, we identified ni-type2-neurons only in the lineage of MBNBa; (2) cell numbers differ considerably between the individual types of MBNB clones (from 29.2±1.9 to 44.6±6.0 neurons for MBNBc and MBNBd clones, respectively), which (3) is largely correlated with differences in the number of γ-neurons; and (4) each MBNB clone produces a distinct number of contralateral axon fascicles from ni-neurons (between 1 and 4) that arborize in specific target regions in the contralateral brain hemisphere (including a distinct area close to the MB lobes); only the MBNBb lineage exhibits a ni-neuron axon fascicle that descends into the ipsilateral VNC (Fig. 4; supplementary material Fig. S1). Thus, as opposed to the postembryonic period of development, during the embryonic period each MBNB creates a unique lineage of characteristic shape and size and containing distinct combinations of neuron types.

As mentioned above, the four MBNBs emerge from a group of 10-12 neuroectodermal cells in δB that are part of the same proneural domain. In order to see whether heterogeneity already

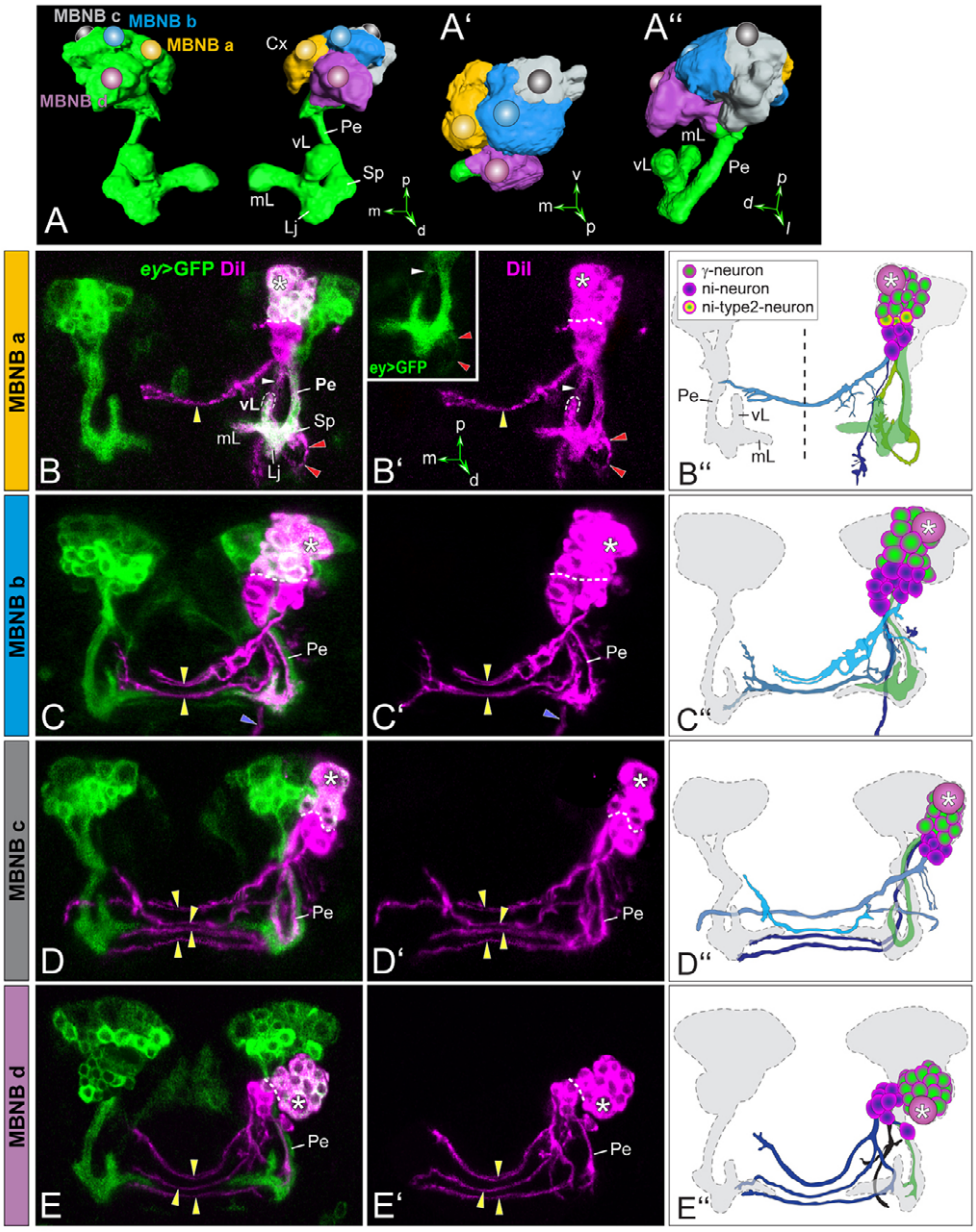

Fig. 4. Each MBNB generates an individual cell lineage. (**A-A″**) Three-dimensional reconstructions of *ey*>GFP-positive MB cortex and neuropil in a newly hatched L1. Different colors indicate the cortex area occupied by the γ-neurons of each MBNB (based on DiI labelings); dorsal (A), posterior (A′) and lateral (A″) views. (**B**,**B′**,**C**,**C′**,**D**,**D′**,**E**,**E′**) Combined confocal images (dorsal view) of representative DiI-labeled MBNB clones in *ey*>GFP brains at mid/late st17. White asterisks indicate MBNBs and the white dashed line indicates the border between GFP-negative and GFP-positive progeny cells. Yellow arrowheads indicate contralateral axonal fascicles and blue arrowheads indicate the axonal fascicles of MBNBb ni-neurons descending into the ventral nerve cord (VNC). Red and white arrowheads (B,B′ and inset) mark weakly GFP-positive axonal and dendritic projections, respectively, of ni-type2-neurons from MBNBa. (**B″**,**C″**,**D″**,**E″**) Illustrations of clones in B-E based on reconstruction of confocal stacks. Non-intrinsic axonal projections are colored from ventral to dorsal in black, dark-, mid- and light-blue. Mid-green indicates γ-neuron axonal projections into the MB and light green indicates axonal projections of the ni-type2-neurons. Other abbreviations are as Fig. 1.

exists among cells of this group with respect to the various MBNB fates we mapped these cells more precisely. We identified four (partly overlapping) subgroups of four to six cells that have the ability to generate MBNBs of distinct identities (Fig. 2B″). Thus, there are clear positional preferences for particular MBNB fates along the dorsoventral and anteroposterior axes of δB, suggesting that MBNB identities are specified by positional information within δB.

Generation of distinct neuron types occurs in an MBNB-specific temporal sequence

As ni-neurons lie furthest from the MBNB of origin, and ni-type2-neurons lie between the clusters of ni-neurons and γ-neurons, this suggests that ni-neurons are born first, followed by ni-type2-neurons and/or γ-neurons (Fig. 4B-E″). To test this hypothesis, we DiI labeled neuroectodermal progenitor cells in *ey*>GFP embryos by st7, mechanically ablated the MBNB at defined time points between st11 and st14, and analyzed the composition of the clone at st17. Ablation of MBNBb by early st13, or later, resulted in clones with normal numbers of ni-neurons but with entirely absent or strongly reduced *ey*>GFP-positive γ-neurons (Fig. 6C,C′,D; *n*=4). Upon NB ablation prior to st13, γ-neurons are absent and, in addition, the number of ni-neurons is diminished (data not shown). These data indicate that, by st13, MBNBb undergoes a switch from the production of ni-neurons to γ-neurons. Comparable results were obtained for the lineages of MBNBc and MBNBd, albeit the switch in the production of the two neuron subtypes seemed to occur slightly earlier, between st12 and st13 and by st11, respectively (Fig. 6B,B′,D,F; supplementary material Fig. S3; *n*=3 in each case). Similarly, ablation of MBNBa by st12, or later, resulted in clones consisting of normal numbers of ni- and ni-type2-neurons, but a complete or partial loss of γ-neurons (data not shown; *n*=3). However, MBNBa ablation at earlier time points (st11-12) yielded a reduced number of ni-type2-neurons but not of ni-neurons (Fig. 6A,A′,D; *n*=3). These data suggest that MBNBa produces ni-neurons first, switches to ni-type2-neurons by st11, and to γ-

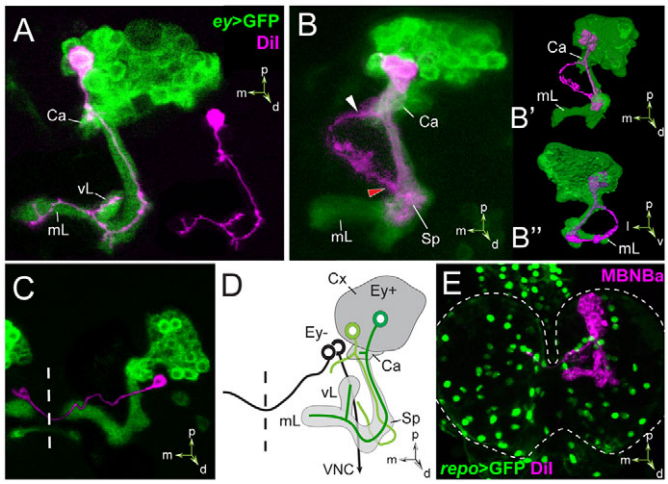

Fig. 5. MBNBs produce different neuron types. (A-C) Composite confocal stacks of iontophoretically DiI-labeled single cells in *ey*>GFP brains of newly hatched L1. (**A**) γ-neuron from MBNBa. (**B**) About four (simultaneously labeled) ni-type2-neurons from MBNBa showing axonal projections that exit in the spur (Sp) region (red arrowhead) of the peduncle and dendrite-like projections leaving the calyx (Ca; white arrowhead). (**B′**,**B″**) Three-dimensional reconstruction of (B) in dorsal (B′) and ventral (B″) view. (**C**) Contralaterally projecting ni-neuron from MBNBb, presumably being part of the contralateral fascicle indicated in light blue in Fig. 4C. Dashed line indicates midline. (**D**) Scheme summarizes the different neuron types emerging from MBNBs: ni-neurons (black), ni-type2-neuron (light green), γ-neuron (dark green). (**E**) DiI-labeled MBNBa clone in early L1 in a *repo4.3*>nuclear-GFP background, indicating no overlap between the MBNB clone and glial cells. Other abbreviations are as Fig. 1.

neurons by st12. To confirm that ni-neurons are the first-born daughter cells, followed by ni-type2-neurons and/or γ-neurons in the developing lineages of MBNBa and MBNBd, we ablated by st12 the early-born daughter cells instead of the NB. In the resulting st17 clones of MBNBa, the number of ni-neurons was strongly or entirely reduced, whereas that of ni-type2-neurons was only slightly reduced and the number of γ-neurons was unaffected (Fig. 6A,A″,E; *n*=2); similarly, in clones of MBNBd, the number of ni-neurons was strongly diminished (Fig. 6B,B″,E; *n*=2).

Collectively, our ablation experiments demonstrate that distinct neuron types are produced in subsequent time windows in an MBNB-specific manner: all four MBNBs generate ni-neurons exclusively in the early time window and γ-neurons in the late time window; only MBNBa additionally generates ni-type2-neurons in an intermediate time window (Fig. 6F).

The lineage-dependent number of γ-neurons is not regulated by programmed cell death but by MBNB-specific mitotic activity

Differences in the size of the four MBNB clones are largely due to different numbers of γ-neurons (Table 1). To examine whether γ-neuron numbers are diminished by programmed cell death, we counted the number of Ey^+ γ-neurons in the MB cortex of *H99* mutant embryos, in which apoptosis is prevented (White et al., 1994). The total number of Ey^+ cells in the late st17 MB cortex was unaltered in *H99* (93.0±7.0; *n*=12) as compared with wild type (93.0±7.0; *n*=14; *P*=0.89). This indicates that apoptosis does not regulate clone-specific γ-neuron number.

To investigate whether MBNB lineages exhibit different proliferation patterns, we performed EdU-incorporation experiments. Applying short EdU pulses (90 minutes) at various time points during st11-17, we found that all MBNBs proliferate throughout embryogenesis, confirming previous reports (Prokop and Technau, 1994; Truman and Bate, 1988). However, during st14-17 the number of EdU-labeled ganglion mother cells (GMCs) and γ-neurons (Fig. 7A,B), as well as of PH3/Prospero/Ey-expressing mitotic GMCs (Fig. 7C), was significantly higher in the MBNBd clone than in the MBNBc clone, suggesting a clone-specific regulation of the magnitude of cell proliferation. We excluded the possibility that this is due to the existence of transit-amplifying intermediate neural progenitors in MBNB lineages, as cells with a corresponding molecular profile [e.g. expression of *earmuff*, nuclear Deadpan, cytoplasmic Prospero/Miranda (Bello et al., 2008; Bowman et al., 2008; Weng et al., 2010)] were not detected in the developing MB cortex, although we observed them in other brain regions (data not shown). Therefore, we conclude that mitotic activity of the MBNBs themselves (i.e. the speed of their cell cycle) regulates the lineage-specific numbers of γ-neurons.

The onset of γ-neuron axonal outgrowth and innervation of MB lobes is specific for each MBNB lineage

To explore whether the four embryonic MBNB clones participate equally in the formation of MB substructures, as is the case in postembryonic stages (Ito et al., 1997; Lee et al., 1999), we assayed the development of axonal projections into the peduncle and lobes and of γ-neuron dendritic projections in the calyx (Fig. 8). We performed these experiments in *ey*>GFP embryos and used GFP expression in the developing MB neuropils as a reference to estimate at different stages the contribution of individual DiI-labeled MBNB clones (*n*=67) or single progeny cells (*n*=148). Our observations suggest the following model according to which the embryonic MB neuropils are formed (summarized in Fig. 8S): by early/mid st17, axonal endings of MBNBa γ-neurons reach the area

Table 1. Comparison of clone size (total cell number), number of individual cell types and axonal projections of ni-neurons in the different MBNB lineages at mid/late st17 and early L1 (2-3 hours after larval hatching)

	Mid/late st17				Early L1				ni-neuron axonal projections	
Neuroblast lineage	Total cell number	ni-neurons	γ-neurons	ni-type2-neurons	Total cell number	ni-neurons	γ-neurons	ni-type2-neurons	Contralateral	Ipsilateral
MBNBa	34.7±3.6 (*n*=14)	8.1±1.5	22.6±4.1	4.0±1.0	42.3±7.5 (*n*=3)	7.3±1.5	31.0±7.2	4.0±1.0	1	1 (DC?)
MBNBb	32.9±3.7 (*n*=8)	15.3±3.7	17.6±2.7	–	39.3±2.7 (*n*=6)	13.5±1.0	25.8±3.3	–	2	1 (VNC)
MBNBc	29.2±1.9 (*n*=6)	10.8±1.0	18.3±1.0	–	36.2±1.9 (*n*=6)	10.5±1.9	25.7±1.4	–	4	–
MBNBd	44.6±6.0 (*n*=8)	8.8±1.8	35.9±6.1	–	48.0±1.7 (*n*=3)	7.0±0.0	41.0±1.7	–	3	1 (DC?)

DC, deutocerebrum; VNC, ventral nerve cord.

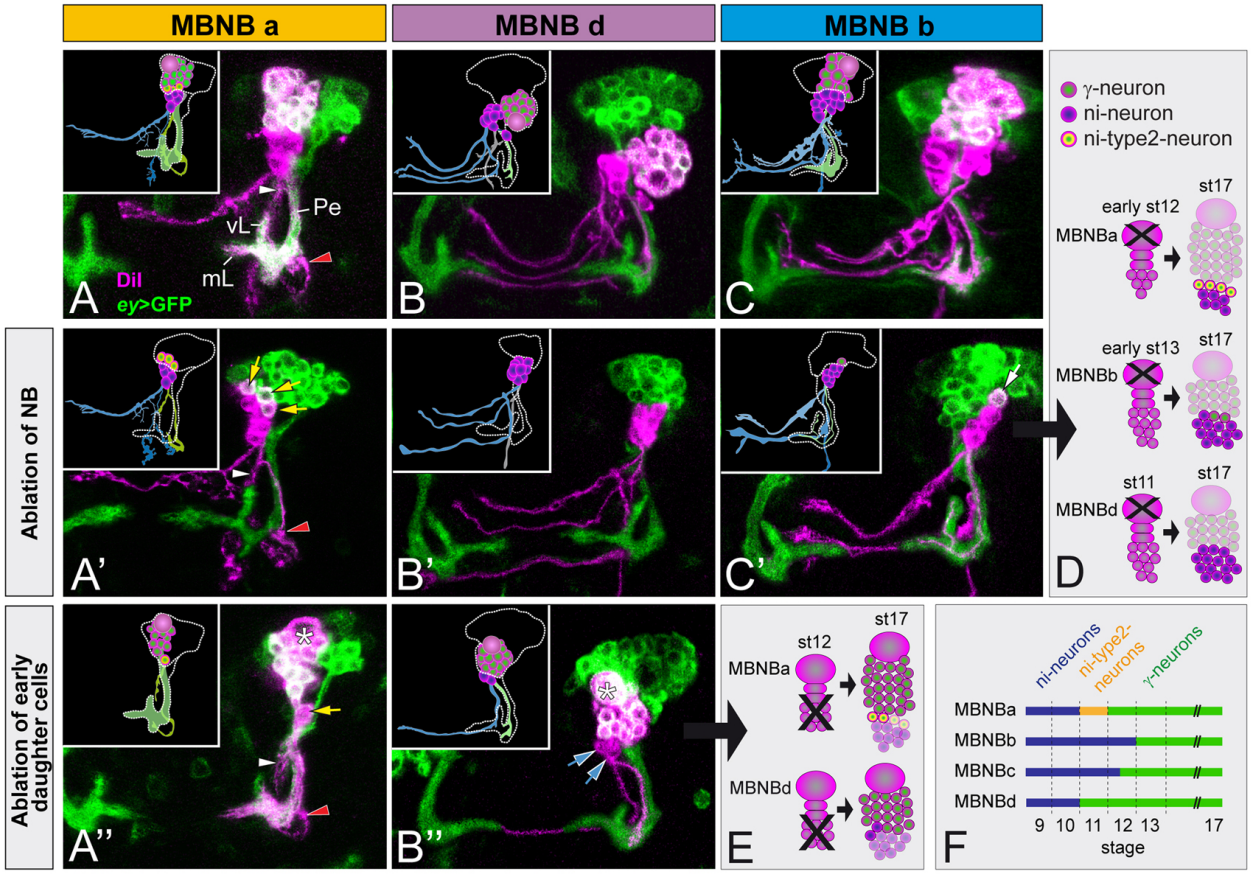

Fig. 6. Embryonic MBNBs produce distinct neuron types in subsequent time windows. (**A-C**) Normal DiI-labeled MBNBa, b and d clones (magenta) in *ey*>GFP *Drosophila* embryos at late st17 (as illustrated in insets). (**A′-C′**,**D**) Corresponding clones at late st17 after ablation of the NB at early st12 (A′), early st13 (B′) or st11 (C′), as summarized in D. (A′-C′) ni-neurons are unaffected, as indicated by their normally elaborated axonal projections. (A′,B′) MB is not innervated, indicating that γ-neurons are missing. Axons of *ey*>GFP-positive ni-type2-neurons from MBNBa (somata are indicated with yellow arrows in A′) project along the peduncle and exit in the spur (red arrowhead). (C′) ni-neurons are unaffected, but only one γ-neuron has remained, making projections into the MB neuropils (white arrow). (**A″**,**B″**,**E**) MBNB clones after ablation of the early daughter cells by st12, as summarized in E. (A″) MBNB (asterisk), γ-neurons with projections into the MB, and one or two *ey*>GFP-positive ni-type2-neurons (yellow arrow) with dendritic (white arrowhead) and axonal (red arrowhead) projections remained. ni-neurons are missing. (B″) MBNB and γ-neurons, projecting axons into the peduncle, remained; two ni-neurons (blue arrows) elaborate one contralateral tract. (**F**) Summary of the specific temporal sequence in which each MBNB produces distinct neuron types. See text for details.

of the prospective lobe junction and then simultaneously form the initial fiber tracts of the medial and vertical lobe (Fig. 8A-B′). Slightly later, they are followed by γ-neuron axons from MBNBb and MBNBc clones. Whereas MBNBb γ-neuron axons enter both lobes simultaneously (Fig. 8D,D′,E,E′,M), those from MBNBc grow into the vertical lobe first and later (by late st17) into the medial lobe (Fig. 8G,G′,H,H′,O,P). By contrast, axonal outgrowth of γ-neurons from MBNBd is significantly delayed and first axons do not invade the lobes before larval hatching (Fig. 8I,I′,J,J′,Q,R). Similarly, the embryonic calyx is built up only by γ-neuron dendrites from MBNBa-c (Fig. 8K-P), whereas those from MBNBd do not appear before L1 (Fig. 8R,S). We also recognized clonal differences in the spatial distribution of axons within the late embryonic peduncle and lobes. Whereas γ-neuron axons from MBNBa are evenly distributed within the peduncle and broadly innervate the two lobes (Fig. 8B,B′), probably reflecting the early outgrowth of these γ-neurons, those from MBNBb and MBNBc (and MBNBd in the early L1) form dense fascicles within the peduncle and are restricted to particular areas of the lobes (Fig. 8E,E,G′,G,I′,I).

Taken together, γ-neuron axons from MBNBa-c clones innervate the embryonic peduncle, lobes and calyx in a lineage-dependent spatiotemporal manner. Intriguingly, axonal growth of γ-neurons (but not of ni-neurons) from MBNBd lags behind significantly, although MBNBd generates γ-neurons as early as the other MBNBs and its lineage finally contains the greatest number of embryonic γ-neurons (Table 1).

γ-neurons from the MBNBa clone are not instructive for axonal guidance of γ-neurons from other MBNB clones

The sequential axonal growth of embryonic γ-neurons suggests that γ-neurons from the MBNBa clone might pioneer the primary axonal scaffold of the peduncle and lobes and might be necessary for proper guidance of γ-neurons from other MBNB clones. To test this, we mechanically ablated the NB in DiI-labeled MBNBa clones at st11/12, before it has started to generate γ-neurons, and investigated the morphology of the *ey*>GFP-expressing peduncle and lobes formed by γ-neurons from the remaining MBNB clones. We observed that, in the absence of γ-neurons from MBNBa, the

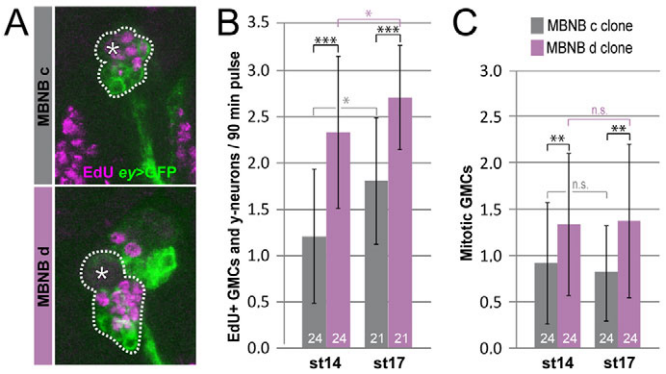

Fig. 7. Lineage-specific differences in cell proliferation in the MBNBc and MBNBd clones. (**A**) EdU/*ey*>GFP-positive cells in the MBNBc (8.7±1.3; *n*=25) and MBNBd (13.6±2.2; *n*=25) lineage after a 6-hour EdU pulse between st14 and st17. White asterisks indicate MBNBs. (**B**) The number of EdU/*ey*>GFP-positive ganglion mother cells (GMCs) and γ-neurons is significantly higher in the MBNBd than in the MBNBc lineage after a 90-minute EdU pulse at st14 (MBNBc, 1.2±0.7; MBNBd, 2.3±0.8; *P*<0.0001) and st17 (MBNBc, 1.8±0.6; MBNBd, 2.7±0.6; *P*<0.0001). (**C**) Correspondingly, the number of mitotic (PH3/Pros/*ey*>GFP-expressing) GMCs is higher in the MBNBd than in the MBNBc lineage at st14 (MBNBc, 0.9±0.7; MBNBd, 1.3±0.8; *P*=0.0091) and st17 (MBNBc, 0.8±0.5; MBNBd, 1.4±0.8; *P*=0.0022), whereas for each type of lineage the numbers remain almost constant between these stages. White numbers in columns indicate the number of MBNB lineages scored at the different stages; error bars indicate s.d.; *, *P*<0.05; **, *P*<0.01; ***, *P*<0.0001; n.s., not significant.

principal morphology of peduncle and both lobes appears normal (*n*=3; Fig. 6A′). This suggests that even though γ-neuron axons from MBNBa might pioneer the axonal scaffold of the peduncle and lobes, they do not seem to be instructive for the outgrowth and guidance of the following γ-neuron axons from other MBNB lineages. Analogous experiments showed that ablation of γ-neurons from MBNBb or MBNBc also has no obvious influence on the axonal differentiation of γ-neurons from the remaining MBNB lineages (data not shown).

DISCUSSION

Applying the DiI labeling technique in combination with molecular markers and cell ablation experiments, we analyzed the embryonic development of a prominent central brain structure, the MBs. This approach allowed us, for the first time, to precisely map the origin of the MB in the procephalic NE, to elucidate the mode of MBNB formation, and to trace the spatiotemporal development and composition of the entire embryonic lineages of the four MBNBs (Fig. 9). Since cell numbers in the DiI-labeled MBNB clones (on average 29-45 cells at st17 and 36-48 cells in L1) differ drastically from those reported previously in flip-out clones (~14 cells at late st16 and ~8 cells in L1) (Larsen et al., 2009), the latter appear to encompass only part of an embryonic lineage. It is likely that this part represents the late-born cells, as recombination in an NB depends on a critical level of heat shock flippase, which does not become enriched before the early-born part of a lineage has developed (Larsen et al., 2009). Also, MARCM fails to disclose entire NB lineages in the late embryo, as clonal reporter expression additionally relies on the loss of the GAL80 repressor after recombination, which seems to persist throughout embryonic development (Luo, 2005).

Embryonic MBNBs generate individual cell lineages consisting of different neuron types that develop in a fixed temporal order

MBNBs proliferate persistently throughout embryogenesis, in contrast to all other brain NBs (except one in the deutocerebrum), which enter a phase of mitotic quiescence in the late embryo (this study) (Ito and Hotta, 1992; Prokop and Technau, 1994; Stocker et al., 1995). Accordingly, all four embryonic MBNB clones are significantly larger than those of other early NBs in the brain (T.K. and R.U., unpublished data) and VNC (Bossing et al., 1996; Schmidt et al., 1997). Whereas the postembryonic MBNB lineages form identical subunits of the adult MBs (Ito et al., 1997), we observe that during embryonic development each MBNB creates a distinct lineage with its own number and type of intrinsic and non-intrinsic interneurons, which are generated in a fixed temporal order (Fig. 9). These findings imply genetic regulation of temporal cell fates in a lineage-specific manner. Further analyses will need to show whether the ‘temporal gene cascade’ *hunchback-Krüppel-nubbin-castor-grainy head* (e.g. Isshiki et al., 2001; Jacob et al., 2008) and/or Chinmo, a determinant for temporal neuronal identities during postembryonic MB development (Zhu et al., 2006) and/or other cues (Baumgardt et al., 2009; Tsuji et al., 2008) confer temporal cell fates to MBNB progenies during the embryonic period. Considering the higher diversity of neuronal cell types, the underlying regulatory mechanisms are presumably more complex compared with the postembryonic period, in which only three types of MB-intrinsic neurons are generated (Armstrong et al., 1998; Lee et al., 1999; Yu and Lee, 2007). Interestingly, as recently reported (Yu et al., 2010), the embryonic NB that generates the antero-dorsal projection neurons (adPN lineage) in the antennal lobe, in contrast to the larval period, alters temporal identity following each division.

Ey has been shown to regulate neuron numbers and Ey and Dac to control axonal differentiation and targeting of MB neurons (Kurusu et al., 2000; Martini et al., 2000; Martini and Davis, 2005; Noveen et al., 2000). We find that *ey*, *dac* and *Rx* are differentially expressed in MBNBs and daughter cells during embryonic neurogenesis, suggesting specific functions in certain neurons or neuron subtypes. It remains to be seen whether, for example, absence of Dac accounts for the delayed differentiation of γ-neurons in the MBNBd lineage. Moreover, Ey expression, which commences in the MBNBb/c lineages when γ-neurons are produced and becomes restricted to this cell type in the MBNBa/d lineages (whereas it is downregulated quickly in ni-neurons), might be crucial for γ-neuron differentiation and its absence required for proper ni-neuron differentiation.

Our clonal analysis reveals that ~95 γ-neurons develop in the embryo (~125 by 2-3 hours after larval hatching), which corresponds to the number of Kenyon cells (~100) observed in adult brain of MB-specific Gal4 strains after hydroxyurea ablation of MBNBs in the newly hatched larva (Armstrong et al., 1998). This suggests that most or all of the embryonic γ-neurons survive to adulthood, although they become dramatically reorganized during pupal development (Boulanger et al., 2011; Lee et al., 1999; Technau and Heisenberg, 1982; Zheng et al., 2003). Accordingly, γ-neurons do not undergo programmed cell death in the embryo (this study), nor, presumably, during postembryonic stages (Technau and Heisenberg, 1982). This is remarkable because ~30-40% of embryonic brain neurons die around the time of larval hatching (Larsen et al., 2009). Interestingly, appetitive olfactory learning in L1 relies on a set of ~100 embryonic-born γ-neurons (included in the 201y-Gal4

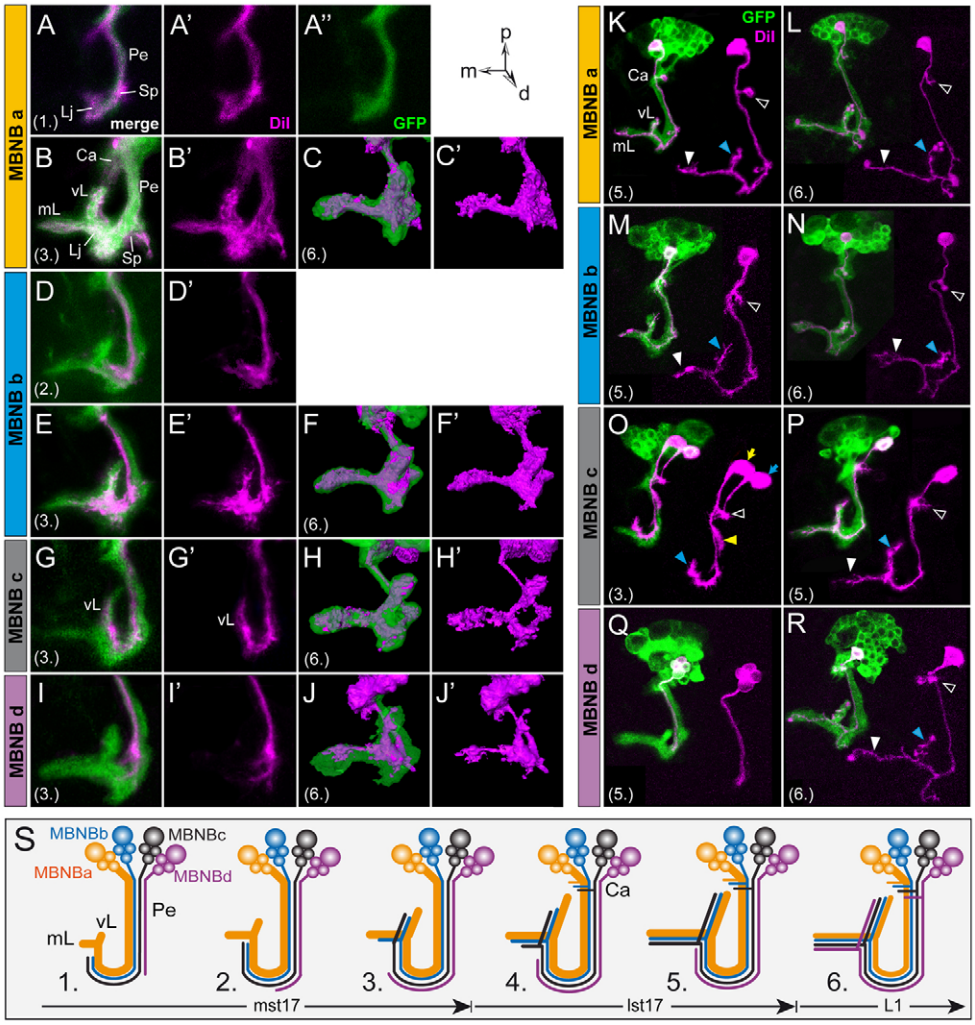

Fig. 8. γ-neurons innervate the embryonic peduncle, lobes and calyx in an MBNB lineage-dependent temporal sequence. (**A-B′**,**D-E′**,**G**,**G′**,**I**,**I′**) Combined confocal images (dorsal views) of DiI-labeled γ-neuron projections of individual MBNB clones (magenta) at different stages (1-6, as illustrated in S) in an *ey*>GFP background. Images only show the DiI signal in the developing MB neuropil substructures (Pe, vL, mL, Ca), and surrounding DiI signal from ni-neurons has been removed for clarity. Axon termini of MBNBa γ-neurons coincide with the distal border of GFP expression, whereas those of MBNBb-d terminate at more proximal positions. Note that MBNBc γ-neuron axons first innervate the vL (G,G′). (**C**,**C′**,**F**,**F′**,**H**,**H′**,**J**,**J′**) Three-dimensional reconstructions (GFP in transparent green). γ-neurons from all MBNBs innervate both lobes in the newly hatched L1, although to different extents. (**K-R**) Single- or multi-cell DiI-labeled γ-neurons from MBNBa-d at different stages (according to S) in *ey*>GFP embryos. White and blue arrowheads indicate axonal projections into medial and vertical lobe, respectively; white open arrowheads indicate dendritic projections into the calyx. γ-neurons from MBNBa-c exhibit bifurcating axons into both lobes at late st17 (substage 5; K,M,P), and those from MBNBd 2-3 hours after larval hatching (L1, substage 6; R). (O) An MBNBc γ-neuron (blue arrow) first innervates the vertical lobe (blue arrowhead); yellow arrow indicates an MBNBb γ-neuron and yellow arrowhead the corresponding axonal growth cone. (**S**) Model summarizing the temporal order according to which γ-neurons from the four MBNB lineages contribute to embryonic MB substructures at developmental substages 1-6, as deduced from MBNB clones (*n*=2-6 for each clone and substage) and single- or multi-labeled γ-neurons (*n*=4-13 for γ-neurons from each MBNB and substage). Note that mid st17 (mst17) is subdivided into three (1-3) and late st17 (lst17) into two (4,5) substages of MB development. See text for details. Other abbreviations are as Fig. 1.

and NP1131-Gal4 lines), indicating that the underlying neuronal circuits at the level of the MBs are established during embryogenesis (Pauls et al., 2010). As we observed a primordial calyx carrying dendritic arborizations of γ-neurons already in the embryo, it is likely that embryonic-born projection neurons (providing olfactory input from the antennal lobes) participate in these initial neuronal circuits (Marin et al., 2005; Raemakers et al., 2005; Yu et al., 2010).

Although the four postembryonic MBNB clones consist of the same sets of Kenyon cells, which equally contribute to peduncle and lobes, it seems that they are not in fact identical as the dendrites of each MBNB clone occupy a specific calyx subregion (Ito and Awasaki, 2008) (supplementary material Fig. S4). Projection neurons that terminate in distinct microglomeruli in the larval calyx convey olfactory information from specific antennal lobe glomeruli (Masuda-Nakagawa et al., 2009). Thus, each MBNB clone receives a different repertoire of olfactory information, which might be correlated with the unique properties of each of the four embryonic MBNBs. This raises the question as to which embryonic lineage (a-d) each postembryonic MBNB lineage evolves from. Interestingly, we find that, for example, the most lateral subdivision of the MB cortex and calyx seems to be

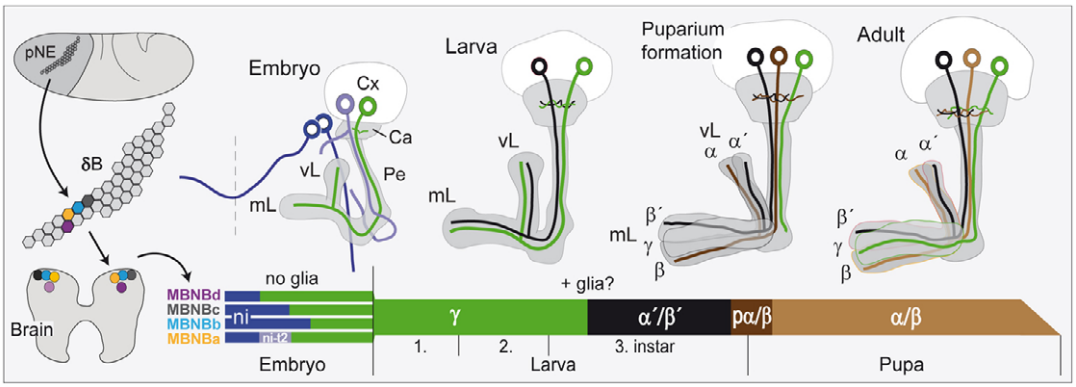

Fig. 9. Sequential generation of different subtypes of MBNB daughter cells during embryonic and postembryonic MB development. MBNBa-d derive from four individual progenitor cells within mitotic domain δB of the procephalic neuroectoderm (pNE; st7, left side). During embryonic development they generate γ-neurons and different types of ni-neurons projecting into the contralateral hemisphere or ipsilateral VNC; ni-type2-neurons (ni-t2) are generated exclusively by MBNBa. The numbers and temporal pattern of birth and the differentiation of the various types of neurons differ in a lineage-specific manner. By contrast, during postembryonic development, all MBNBs develop identical subsets of intrinsic neurons [in the temporal order: γ-, α′/β′-, pioneer αβ (pα/β)-, α/β-neurons]. Whereas embryonic MBNBs do not produce glial cells, this remains questionable for postembryonic MBNBs (see Ito et al., 1997; Lai and Lee, 2006). Axons from intrinsic MB neurons fasciculate to form the vertical (vL) and medial (mL) lobes in the embryo (only γ-neurons) and larvae (γ- and α′/β′-neurons), whereas axonal projections from γ-, α′/β′- and α/β-neurons form the five MB lobes (α, α′, β, β′, γ) that are distinguishable in pupal and adult stages [data for postembryonic stages are adapted from the literature (Ito et al., 1997; Lee et al., 1999; Yu and Lee, 2007)]. Other abbreviations are as Fig. 1.

consistently occupied by the MBNBc lineage from the embryonic to the adult stage (supplementary material Fig. S4). This further suggests that aspects of embryonic calyx topology might be retained during postembryonic development.

About 40 non-intrinsic interneurons (Table 1) are generated by the four MBNBs, most of which project into the contralateral brain hemisphere. Some of these interneurons form bouton-like structures at specific sites near the surface of the contralateral MB (supplementary material Fig. S1), reminiscent of MB-associated neurons, i.e. ‘extrinsic’ or ‘non-Kenyon cell MB-intrinsic’ neurons (Pauls et al., 2010; Tanaka et al., 2008). Therefore, ni-neurons might be involved in processing information between the higher-order olfactory systems of the two hemispheres.

Our ablation experiments suggest that γ-neurons from MBNBa are not instructive for axonal guidance of following γ-neurons from other MBNB clones. Interestingly, ni-neurons establish contralateral axonal projections before the onset of γ-neuron axogenesis. It has been shown that the Derailed receptor, which is expressed on contralateral axons of yet unidentified neurons, is necessary for proper growth of γ-neuron axons towards the midline, mediated through the Wnt5 ligand expressed in γ-neurons (Grillenzoni et al., 2007). Since contralateral fascicles of ni-neurons are in close contact with developing γ-neuron axons they might correspond to those neurons that are instructive for γ-neuron axonal growth. It is therefore tempting to speculate that ni-neurons interact with γ-neurons in two different ways: early in a developmental context and later in a functional context.

Evolutionary considerations

We show that the MBNBs originate from adjacent δB cells that belong to the same proneural domain of the procephalic NE. This peculiar mode of neurogenesis (see also Urbach et al., 2003) parallels that reported for basal arthropods. In chelicerates, the MBs originate from a proneural gene (*CsASH1*)-expressing, invaginating NE in which all cells are destined to a neural fate (Doeffinger et al., 2010). These similarities might suggest a common evolutionarily origin of a mode of neural progenitor formation that does not rely on lateral inhibition among cells of the NE.

Recently, the early gene expression profile of two potential MBNBs, the Dac-positive Pcd8 and Pcd9, was used for a molecular comparison with the MB in the annelid *Platynereis* (Tomer et al., 2010). However, since *dac* and/or *ey* are not uniquely expressed in MBNBs, the identity of the MBNBs in our NB map (Urbach and Technau, 2003a) had been unclear. Here, we show that Pcd9, but not Pcd8, belongs to the MBNBs. The gene expression profile in the other three MBNBs (Pcd2, Pcd4, Pcv9) is similar to that in Pcd9. Moreover, we detected co-expression of *Rx* and *dac*/*ey* almost exclusively in the MB. *Rx* is also expressed in the annelid MB, which further supports homology between these brain structures in insects and annelids (Tomer et al., 2010). Remarkably, the molecular fingerprint of the MB in *Platynereis* was found to be conserved in a subregion of the vertebrate forebrain, the pallium (Tomer et al., 2010). It also seems to be largely conserved in Pcd9 and, albeit less strongly, in all other *Drosophila* MBNBs. This lends support to the hypothesis that the protostome (annelid, insect) MB and the deuterostome (vertebrate) pallium evolved from a common brain center present in the bilaterian ancestor (Tomer et al., 2010).

Acknowledgements

We thank Uwe Walldorf, Patrick Callaerts, Christian Klämbt, Jürgen Knoblich, Jim Skeath, Stefan Thor, John Reinitz, the Berkeley Drosophila Genome Project, Bloomington Drosophila Stock Center, Developmental Studies Hybridoma Bank and FlyBase for providing reagents and resources; Dagmar Volland for excellent technical assistance; Ana Rogulja-Ortmann and David Jussen for critically reading the manuscript; and an anonymous reviewer for thoughtful comments.

Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft [UR1-4,2-1,3-1 to R.U.] and by a research stipend (to K.F.K.) from the Focus Program Translational Neuroscience (FTN) of the University of Mainz. Deposited in PMC for immediate release.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077883/-/DC1

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