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Early development of the *Drosophila* mushroom body: the roles of *eyeless* and *dachshund*

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SUMMARY

The mushroom body (MB) is a uniquely identifiable brain structure present in most arthropods. Functional studies have established its role in learning and memory. Here we describe the early embryonic origin of the four neuroblasts that give rise to the mushroom body and follow its morphogenesis through later embryonic stages. In the late embryo, axons of MB neurons lay down a characteristic pattern of pathways. *eyeless* (*ey*) and *dachshund* (*dac*) are expressed in the progenitor cells and neurons of the MB in the embryo and larva. In the larval brains of the hypomorphic ey^R strain, we find that beside an overall reduction of MB neurons, one MB pathway, the medial lobe, is malformed or missing. Overexpression of *eyeless* in MBs under the control of an MB-specific promoter results in a converse type of axon pathway abnormality, i.e. malformation or loss of the dorsal lobe. In contrast, loss of *dachshund* results in deformation of the dorsal lobe, whereas no lobe abnormalities can be detected following *dachshund* overexpression. These results indicate that *ey* and *dachshund* may have a role in axon pathway selection during embryogenesis.

Key words: *Drosophila*, Brain, Mushroom body, *Pax6*, *eyeless*, *dachshund*, Axon guidance, Cell proliferation

INTRODUCTION

The regulatory gene *eyeless* (*ey*)/Pax 6 is a member of the Pax class of transcription factors that contain two conserved DNA binding regions, the paired-box and the homeobox (Walther and Gruss, 1991; Quiring et al., 1994). Across species, *Pax6* genes are found to be highly conserved and expressed in eyes and central nervous system (Callaerts et al., 1997; Gehring and Ikeo, 1999). In *Drosophila*, hypomorphic *ey* alleles have been known for many years (Hoge, 1915). Two alleles, ey^R and ey^2 (Serebrovsky and Sakharov, 1925; Bridges, 1935), cause partial or complete absence of the compound eyes. Targeted expression of *ey* in various imaginal disks leads to induction of ectopic eyes (Halder et al., 1995). In vertebrates, mutations in *Pax6* lead to the *Small eye* phenotype in mouse (Hill et al., 1991) and *Aniridia* in human (Ton et al., 1991). *Pax6* can also induce ectopic eyes in *Drosophila* (Halder et al., 1995) and *Xenopus laevis* (Altmann et al., 1997; Chow et al., 1999). An enhancer of *Drosophila ey* placed upstream of a *Pax6* promoter can accurately simulate the endogenous expression of *Pax6* in mouse (Xu et al., 1999b). The above indicate that *Pax6* genes are evolutionarily conserved and important for eye development in both vertebrates and invertebrates.

In *Drosophila*, *ey* specifies eye tissue identity in the context of a coordinated system of genes, which include *dachshund* (*dac*), *eyes absent* (*eya*), *sine oculis* (*so*), *teashirt* (*tsh*) and *twin of eyeless* (*toy*) (Bonini et al., 1997; Cheyette et al., 1994; Shen and Mardon, 1997; Pan and Rubin, 1998; Czerny et al., 1999). Most of the above can induce ectopic eyes and interact with *ey* (Gehring and Ikeo, 1999; Treisman, 1999). The above gene network appears to be evolutionarily conserved in vertebrates, as the vertebrate orthologues have been found to have conserved interactions and cooperative roles during vertebrate organogenesis (Xu et al., 1997; Hammond et al., 1998; Heanue et al., 1999; Loosli et al., 1999; Ohto et al., 1999; Xu et al., 1999a).

During vertebrate neural development, *Pax-6* is expressed in large domains of the neural tube, including the dorsal telencephalon, diencephalon and cerebellum (Callaerts et al., 1997; Hanson and Van Heyningen, 1995). Mice with homozygous mutations in *Pax6* have severe defects in the eyes, face and CNS and die at birth (Callaerts et al., 1997). Defects in the nervous system of these animals indicate that *Pax6* is involved in activities such as neural precursor migration (Schmahl et al., 1993; Stoykova et al., 1996; Caric et al., 1997; Engelkamp et al., 1999), neurite extension (Mastick et al., 1997; Engelkamp et al., 1999; Kawano et al., 1999), neural proliferation and differentiation (Ericson et al., 1997; Warren and Price, 1997; Osumi et al., 1997; Gotz et al., 1998; Warren et al., 1999) and boundary formation (Stoykova et al., 1996; Grindley et al., 1997; Mastick et al., 1997; Stoykova et al., 1997; Chapouton et al., 1999; Kioussi et al., 1999); however, due to the complex nature of the vertebrate nervous system, a simple picture of the function of *Pax6* has not emerged.

In vertebrate embryonic development, *Dac* expression is seen in eye, ear and limb and also in a widespread region of central nervous system including telencephalon, diencephalon and mesencephalon (Hammond et al., 1998; Caubit et al., 1999; Davis et al., 1999). Recently it has been found that Dac2 regulates vertebrate muscle development in a synergistic interaction with Eya2 and Six1 (Heanue et al., 1999), reminiscent of the action of Dac in *Drosophila* eye disc development (Chen et al., 1997; Pignoni et al., 1997).

In *Drosophila*, expression of *ey* and *dac* in distinct foci of nerve cells has been reported (Quiring et al., 1994; Mardon et al., 1994), but the identity of these cells have remained unknown and their function in the CNS has not been previously described. Here we show that a major focus of *ey* and *dac* expression and function is the embryonic and larval mushroom body.

The mushroom body (MB) of the arthropod brain is a prominent neuropile compartment whose internal circuitry and connections with other brain regions are highly conserved (Strausfeld et al., 1998). Functional studies show that the MB is involved in olfactory learning and memory (for reviews see Davis, 1996; Heisenberg, 1998), courtship behavior (Ferveur et al., 1995; O'Dell et al., 1995), consolidation of short-term and long-term associative memories (McBride et al., 1999), and visual context generalization (Liu et al., 1999). In the adult *Drosophila*, the MB consists of a population of neurons (MB neurons or Kenyon cells) whose axons form a massive tract called the peduncle (Fig. 1A). Collateral branches of peduncular axons and afferent axons, in particular the antennocerebral tract that provides olfactory input from the antennae, form a dense neuropile called calyx around the proximal segment of the peduncle. At its distal tip, the peduncle splits into two main branches, one pointing dorsally (dorsal lobe), the other medially (medial lobe) (Yang et al., 1995; Ito et al., 1997; Tettamanti et al., 1997; Crittenden et al., 1998; Lee et al., 1999). In the adult, the dorsal lobe is composed of two parallel lobes called α and α′, and the medial lobe is composed of three parallel lobes called β, β′ and γ (Lee et al., 1999; Crittenden et al., 1998; Armstrong et al., 1998; Ito et al., 1997). Three types of MB neurons have been described in the adult brain, αβ, α′β′ and γ. The first type forms the α and β lobes, the second α′ and β′ lobes, and the third the γ lobe (Lee et al., 1999). In the adult, the axons of the αβ and the α′β′ neurons are branched while those of the γ neurons are not. During the first to early third instar larval stages, the only neurons that are present are those that usually give rise to the γ lobe in the pupa and the adult (Armstrong et al., 1998; Lee et al., 1999). At this stage, however, γ neurons have branched axons, one branch forming the medial lobe and the other the dorsal lobe. During metamorphosis, γ neurons lose both branches and regrow one branch that will form the γ-lobe of the pupal and adult MB, extending only into the medial lobe (Lee et al., 1999).

Previous studies have reported that the MB neurons are produced by four neuroblasts (MBNBs) that occupy a characteristic position on the vertex of the late embryonic and larval brain hemispheres (Ito and Hotta, 1992; Prokop and Technau, 1991; Truman and Bate, 1988). The origin and early embryonic development of the MBNBs have not been studied before. The peduncle and lobes of the MB can already be recognized during late embryonic stages (Tettamanti et al., 1997; Crittenden et al., 1998; Nassif et al., 1998), due to the presence of a substantial number of MB neurons. In this paper we describe the embryonic development of the MB, and show that *ey* and *dac* are expressed in the embryonic MB. Hypomorphic alleles of *ey*, and overexpression of *ey*, both result in structural defects in the MBs. In the hypomorphic ey^R strain, beside an overall reduction of MB neurons, the medial lobe is malformed or missing. Overexpression of *ey* in MBs under the control of an MB-specific promoter results in dorsal lobe malformations. Loss of the dorsal lobe is also observed in larvae lacking *dac*. These results indicate that *ey* and *dac* may have roles in axon pathway selection during embryogenesis.

MATERIALS AND METHODS

Drosophila stocks

The fly stocks were gal4 enhancer trap line 238Y (Yang et al., 1995), various enhancer trap lines expressing lacz in MBs (gift of R. Davis), UAS-*ey* (Halder et al., 1995), UAS-tau (Ito et al., 1997), UAS-*dac* (Shen and Mardon, 1997), hsgal4 (Bloomington Center), *dac*4 (null allele of *dac*; Mardon et al., 1994), ey^R and ey^2 (hypomorphic alleles of *ey*, Quiring et al., 1994) and hs-N_{intra} (hs-Notch intracellular) (Struhl et al., 1993). Due to reversion to normal eye size, ey^2 and ey^R flies had to be regularly selected for small eyes (also see Halder et al., 1998).

Antibodies

The primary antibodies were against Fas II (1:50 dilution) (Grenningloh et al., 1991), D-MEF2 (1:1000 dilution) (Nguyen et al., 1994), Dac (1:20 dilution) (Hybridoma Center; Mardon et al., 1994), anti-β-gal antibody (1:1000 dilution) (Sigma) and bovine Tau (1:500 dilution) (Sigma). The secondary antibody against anti-Fas II, anti-β-gal and anti-bovine Tau primary antibodies was biotin-conjugated anti-mouse IgG (1:400 dilution) (Vector Laboratories) and against anti-D-MEF2 and anti-Dac was biotin-conjugated anti-rabbit IgG (1:200 dilution) (Vector Laboratories).

Whole-mount immunostaining

The heads of larvae were separated from their body at just below the fourth segmentation groove in PBS, immediately fixed with 4% formaldehyde in PEMS (0.1 M Pipes, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$) for 20 minutes, washed with PBS three times for 30 minutes each, and incubated in 10% normal goat serum (NGS) in PBT (1× PBS containing 0.1% Triton X-100) for 30 minutes. The heads were then incubated with primary antibody in 10% NGS overnight at 4°C and washed in PBT three times for 30 minutes each at room temperature. After preincubation with 10% NGS in PBT, the heads were incubated with biotin-conjugated secondary antibody in 10% NGS for 2 hours at room temperature, washed with PBT as before, and incubated for 2 hours at room temperature in a solution of Avidin DH and biotin-conjugated horseradish peroxidase (Vector Labs). Color was developed using the substrate 3, 3′-diaminobenzidine (DAB) with or without 0.05% NiCl. The heads were then washed in PBT, dried in an ethanol series, placed in acetone and then 1:1 acetone/epon. Subsequently, the brains were dissected out of the heads and mounted on slides in epon.

Whole-mount in situ hybridization

Embryos were collected on apple juice agar plates and dechorionated in 50% bleach. Larval brains were dissected out in PBS. Subsequently, the eggs and the heads were fixed in PBS containing 5% formaldehyde and 50 mM EGTA and stored in ethonol. They were then treated with xylene for 2 hours and fixed for a second time in PBS containing 0.1% Tween-20 and 5% formaldehyde. To synthesize digoxigenin-labeled

riboprobe, pBluescript containing *ey* (Pignoni et al., 1997) was linearized with *Sal*I and transcribed with T3 polymerase according to standard protocol. The digoxigenin-labeled probes were hybridized to the fixed embryos or brains in buffer containing 50% formamide at 55°C. Hybridized probe was detected with anti-digoxigenin antibody (Boehringer Mannheim) according to the manufacturer's instructions, following which the samples were dehydrated in an ethanol series and embedded in epon.

DiI injection in embryonic MB neurons

Embryos at stages 14, 15 and 16 were dissected by cutting them along the dorsal midline with a sharpened Tungsten needle and exposing the brain and ventral nerve cord. These 'filet' preparations were glued to a polylysine-coated slide and covered with a drop of saline. They were observed with a Zeiss 100× water immersion lens. The quartet of MB neuroblasts at the dorsoposterior pole of the brain hemispheres represents a distinctive landmark that was used to direct a DiI-filled micropipette. The pipette was pushed through the center of the MB neuroblast quartet, approximately 10 μm beneath the brain surface. DiI was injected iontophoretically under fluorescence light control. Spreading of DiI in the cell bodies and axons of neurons located right underneath the MB neuroblasts could be observed within minutes after injection. Preparations were kept in PBS for 30 minutes, after which they were fixed. DiI was photoconverted with diamino-benzidine (DAB) under fluorescent light (Bossing et al., 1996).

Kenyon cell counts

Kenyon cells at the surface of OR^R, ey^R and *ey*-overexpressing brains were counted (10 hemispheres each) using a Zeiss Axiophot. For ey^R and *ey*-overexpressing brains, generally two classes of Kenyon cell counts were observed: one class that had a similar cell count to that of the wild-type OR^R, and a second class that had a lower cell count. For ey^R and *ey*-overexpression, it is the second class of brains that are reported here. We assumed that the cell count at the surface of the brain is representative of the total number of Kenyon cells. However, wild-type brains have 3-4 layers of Kenyon cells while the ey^R and *ey*-overexpressing brains only have 1-2 layers. Thus the differences between OR^R and ey^R/*ey*-overexpressing brains is even greater than assumed here.

Heat shock treatments

Embryos resulting from a cross between hs-*gal4* and UAS-*ey* were heat shocked at 37ºC for 2 hours during embryonic stage 16 (15-17 hours after egg laying). hs-Notch(intra) embryos were heat shocked at 37°C for about 2 hours at embryonic stages 9 and 10 (3:40 to 5:20 hours after egg laying) and stage 11 (5:20 to 7:20 hours after egg laying).

Hydroxyurea treatment

Actively proliferative cells can be selectively killed using hydroxyurea (Timson, 1975). To kill MB neuroblasts, hydroxyurea was dissolved in water and added to standard fly food to a final concentration of 30 mg/ml. Upon hatching, first instar larvae were immediately placed on the food containing hydroxyurea and incubated at 25°C. After 4 hours they were switched to food without hydroxyurea and allowed to grow at 25°C for 2 days.

RESULTS

eyeless (*ey*) and *dachshund* (*dac*) are expressed in the embryonic progenitors of the mushroom body

Previous studies have reported that the MB neurons are formed by four neuroblasts (MBNBs) that occupy a characteristic position on the vertex of the late embryonic and larval brain hemispheres (Ito and Hotta, 1992; Truman and Bate, 1988). The origin and early embryonic development of the MBNBs have not been studied before. Using the early embryonic expression of *ey*, *dac* (Fig. 1B,C) and other markers (e.g. *seven-up*) that we had previously identified as being expressed in the larval MB, we can show that MBNBs segregate from the central protocerebral neurectoderm as part of the Pc3 group of neuroblasts (Younossi-Hartenstein, 1996) during early embryogenesis (Fig. 2). *ey* and *dac* are expressed in a cluster of approximately 10-12 cells at stage 9, shortly before delamination of brain neuroblasts commences, which will be called MB neurectoderm below. It is possible that the MB neurectoderm, defined by the early *ey* expression, corresponds to a proneural cluster, i.e. the equivalence group of cells that are competent to become MBNBs. Proneural

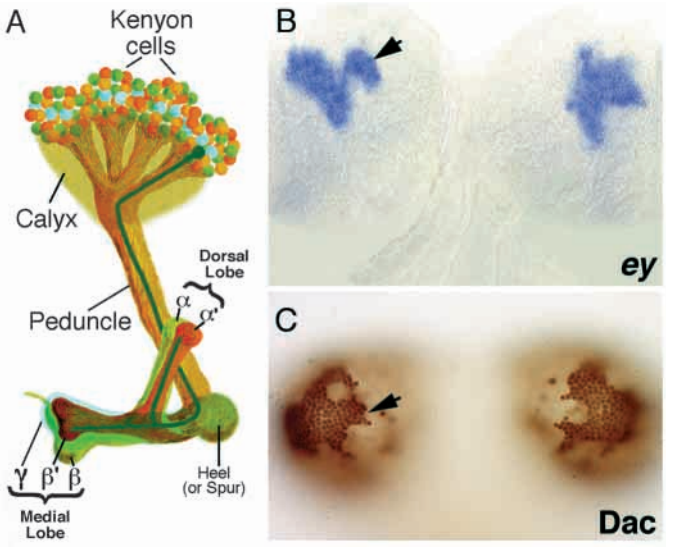

Fig. 1. Diagrammatic view of the adult mushroom body (MB) and larval expression of the regulatory genes *ey* and *dac*. (A) The anterior view of the left MB is shown. In each brain hemisphere, MB Kenyon cells are produced as a result of the continuous proliferation, from the mid-embronic to the late pupal stages, of only four identical neuroblast (Ito and Hotta, 1992; Truman and Bate, 1988). Each single neuroblast is capable of giving rise to a clonal unit of cells (Kenyon and glial cells), which are capable of generating the entire structure of an MB (Ito et al., 1997); however, functional heterogeneities may be present or develop later among the Kenyon cells. Through differential antibody staining, it has been found that MB axons are made up of three different pedunclular tracts (Crittenden et al., 1998), indicating that they are derived from three different subpopulations of Kenyon cells (αβ, α′β′ and γ neurons). The first tract branches into the α and β lobes, the second into the α′ and β′ lobes and the third into the γ lobe and heel (Crittenden et al., 1998; Armstrong et al., 1998; Lee et al., 1999). In adults, the medial lobe is composed of the β, β′ and γ lobes and the dorsal lobe is made up of α and α′ lobes; however, from late embryogenesis to the early third instar larva, the dorsal and medial lobes of MB each have only a single axon tract made up of the γ-type axons, originating from γ-type Kenyon cells (Lee et al., 1999). Typical branching pattern of an individual Kenyon cell axon is shown in dark green. (B) Third larval instar brain, dorsoposterior view, in situ hybridization using *ey* antisense riboprobe showing expression of *ey* in the Kenyon cells. (C) Second instar larval brain, dorsoposterior view, immunostaining with anti-Dac antibody (Mardon et la., 1994) showing Dac in Kenyon cells. Arrowheads point to Kenyon cells.

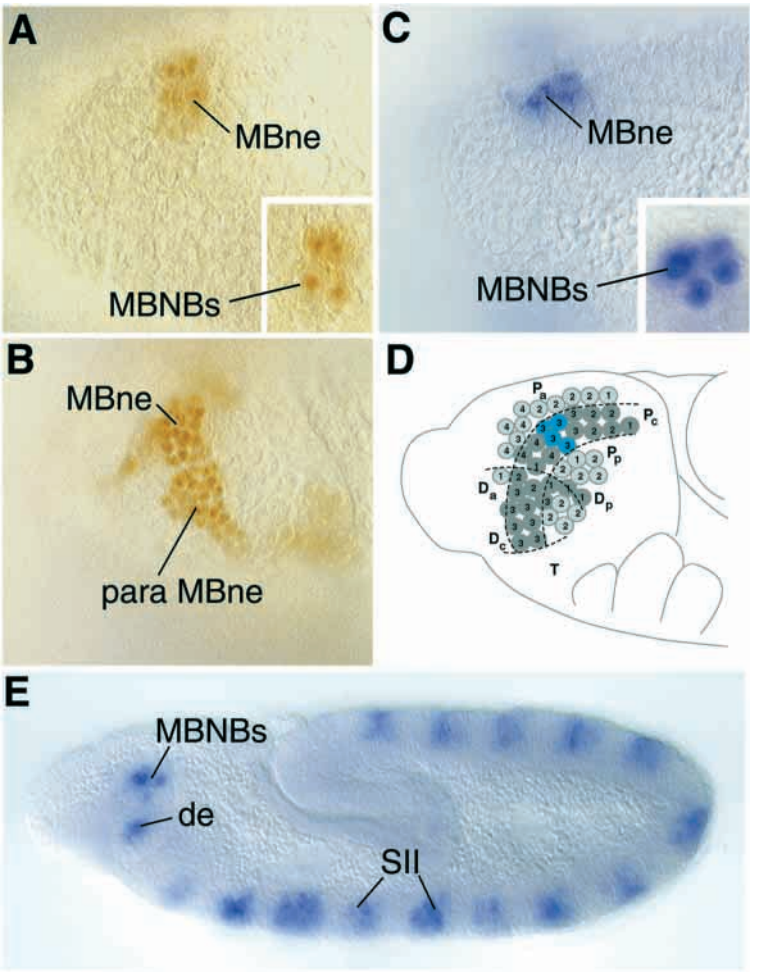

Fig. 2. Expression of *dac* (A,B) and *ey* (C,E) in the procephalic ectoderm of the early embryo that gives rise to the MB. (D) Schematic brain neuroblast map where MBNBs are labeled blue (see below for abbreviations). *ey* expression is visualized by in situ hybridization, *dac* expression by an antibody against the Dac protein. All panels show lateral views; anterior is to the left, dorsal to the top. Both *dac* and *ey* are turned on during stage 9 (A,C) in a cluster of 10-12 cells, the MB neurectoderm (MBne), located in the central protocerebral neurectoderm (Pc in D). Four neuroblasts (MBNBs) delaminate from the MB neurectoderm during embryonic stage 9 (insets in A,C). These neuroblasts belong to the Pc3 group identified previously (blue cells in D; Younossi-Hartenstein et al., 1996). During stage 10, *dac* is also expressed in cells laterally adjacent to the MB neurectoderm in a cluster of cells we call para-MB neurectoderm (para-MBne, B). (E) Beside the MBNBs, segmentally reiterated groups of SII neuroblasts in the head (de, deuterocerebrum) and trunk (SII) express *ey*. In D, the anterior, central and posterior regions from which protocerebral neuroblasts will arise are labeled Pa, Pc and Pp; the anterior, central and posterior regions from which deuterocerebral neruoblasts will arise are labeled Da, Dc and Dp; the region from which the tritocerebrum will arise is labeled T (Younossi-Hartenstein et al., 1996). The number on each neuroblast refers to the nomenclature introduced in Younossi-Hartenstein et al. (1996).

genes of the AS-C, whose expression defines proneural clusters in the ventral neurectoderm (Skeath and Carroll, 1992), are expressed in wider domains in the head and include the MB neurectoderm (Younossi-Hartenstein et al., 1996; see Discussion).

As they delaminate, the four MBNBs keep expressing *ey*, whereas expression in the ectodermal cells that stay at the surface diminishes. *dac*, on the other hand, remains expressed in the MB ectoderm as well as the MBNBs throughout embryogenesis (Fig. 2B). Ectodermal expression of *dac* expands to include a cluster of cells laterally adjacent to the MB neurectoderm, referred to as 'para-MB neurectoderm' below. It should be noted that this designation is not meant to imply any other than a purely topological relationship between MB and para-MB neurectoderm. As discussed below, the para-MB neurectoderm does not contribute to the formation of the MB in any way.

ey and *dac* are also expressed in other embryonic neuroblasts. *ey* is expressed in a small group of neuroblasts in the deuterocerebrum and tritocerebrum and in segmentally reiterated groups of three SII neuroblasts in the ventral nerve cord (Fig. 2E). Expression of *dac* at an early stage (stage 9-10) is restricted to the MB and para-MB neurectoderm and the MBNBs. Later, scattered groups of neurons in both ventral nerve cord and brain, as well as other embryonic tissues, turn on this gene (Fig. 3C).

Once the MBNBs have delaminated, the MB and para-MB neurectoderm does not seem to give rise to any more neuroblasts. However, the fate of this part of the head ectoderm is an unusual one: all cells of the MB and para-MB ectoderm are incorporated at mid-embryogenesis into the cortex of the brain (Fig. 3). Neurons and glial cells in *Drosophila* are typically produced by neuroblasts that delaminate at an early stage (stage 9-11) and proliferate inside the embryo. Cells that remain at the surface after neuroblast delamination typically form the epidermis of the larva. We have previously described portions of the brain that do not stem from neuroblasts, but form small 'placode'-like groups of ectoderm cells that invaginate during stage 13 (Younossi-Hartenstein et al., 1996). The MB and para-MB ectoderm form a subset of these placodes (Fig. 3A). Following their invagination from the surface, these cells are positioned at the surface of the lateral part of the brain hemisphere. Abundant cell death removes part of the cells, particularly in the case of the MB neurectoderm (Fig. 3B). The remaining cells spread out over the lateral aspect of the brain hemisphere.

Blocking neuroblast formation in the early embryo reduces the MB size

The time at which the MB neuroblasts can be first distinguished from other neuroblasts, and at which most MB-specific markers start being expressed is in the late embryo

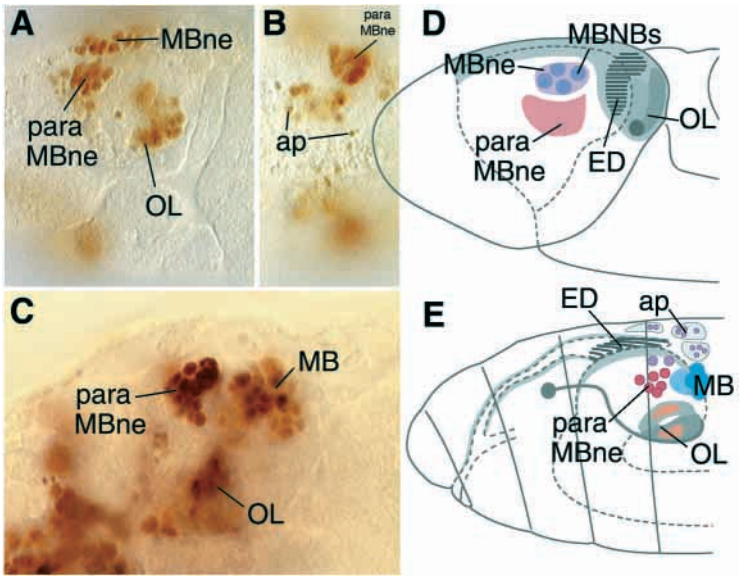

Fig. 3. Fate of the MB neurectoderm (Mbne) and para-MB neurectoderm (paraMBne), labeled by anti-Dac antibody, during later embryogenesis. Dac expression persists in MBne and para-MBne during stages 11 and 12 (A, lateral view). Two additional clusters located in the optic lobe primordium (OL) express Dac. During stage 13 (B, dorsal view) the MBne and paraMBne invaginate from the surface ectoderm and become attached to the surface of the brain hemisphere. Apoptotic cell death (ap; note the small Dac-positive grains that represent cell fragments incorporated in macrophages) removes most cells of the MBne. (C) The brain of a stage-15 embryo (lateral view), revealing the spatial relationship between former paraMBne cells, optic lobe clusters and mushroom body (MB). (D,E) Diagrammatic views of the Dac-expression pattern at extended germ band stage (D, shortly after formation of MB neuroblasts, MBNBs) and at stage 16 when Dac-positive cells have become incorporated into the brain (E). The diagrams also clarify the position of the Dac-expressing neurectoderm relative to the eye disc primordium (ED).

(stage 15). Surprisingly, however, MB neuroblasts are among the earliest neuroblasts delaminating from the head ectoderm. To confirm this observation we determined the phenocritical period at which the activation of the hsp70-Notch(intra) construct is able to affect the MB neuroblasts. It was shown in previous studies that N(intra) activation abolishes neuroblast delamination, resulting in the loss of the structures normally produced by these neuroblasts (Struhl et al., 1993; Hartenstein et al., 1994). Heat pulses applied to embryos during stages 9 and 10 strongly reduced the number of *ey* and *dac* positive MB neurons in the late embryo (Fig. 4A), whereas the later pulse (stage 11) had no such effect (Fig. 4B). This finding supports the notion that the MBNBs are born at an early stage.

Early and late proliferation of MBNBs

Similar to most other neuroblasts of the brain and ventral nerve cord, the MBNBs start to proliferate as soon as they have delaminated, each producing lineages of 15-20 neurons, starting at embryonic stage 9 (Fig. 5A). Neurons keep expressing both *ey* and *dac*, although the level of expression of both genes declines towards mid-embryogenesis (stage 13). MBNBs and their early embryonic progeny form a coherent wedge-shaped cluster that we call the early embryonic MB primordium (*eMBp*; shaded blue in Fig. 5E). During this early phase of MBNB proliferation (between embryonic stage 9-14), about 60-80 Kenyon cells are produced per hemisphere.

Around stage 14, when all the other neuroblasts of the brain and ventral nerve cord have ended their proliferation, the MBNBs keep proliferating. This later phase of proliferation continues uninterruptedly into the larval period and gives rise to the large, circular plate of MB neurons characteristic of the larval brain (late embryonic/larval MB primordium, *lMBp*; shaded orange in Fig. 5F-H). The total number of the Kenyon cells at the end of embryogenesis has been estimated to be between 100 to 300 (Technau and Heisenberg, 1982; Ito and Hotta, 1992; Armstrong et al., 1998).

We believe that early neurons (born between stages 9 and 14) and late neurons (after stage 14) form two different populations. Thus, numerous molecular markers of the larval MB, among them protein kinase A (Skoulakis et al., 1993) and Leonardo (Skoulakis and Davis, 1996) are first produced at embryonic stage 14 in a small number of cells attached to the MB neuroblasts (Fig. 5I). The large population of earlier produced neurons (eMBp) does not show expression of these markers. Beside expressing different genes, early embryonic and larval MB primordium are strikingly different in their growth pattern. The early formed MB neurons form columns of cells that grow from the neuroblast towards the center of the brain, similar to the typical insect neuroblast lineage

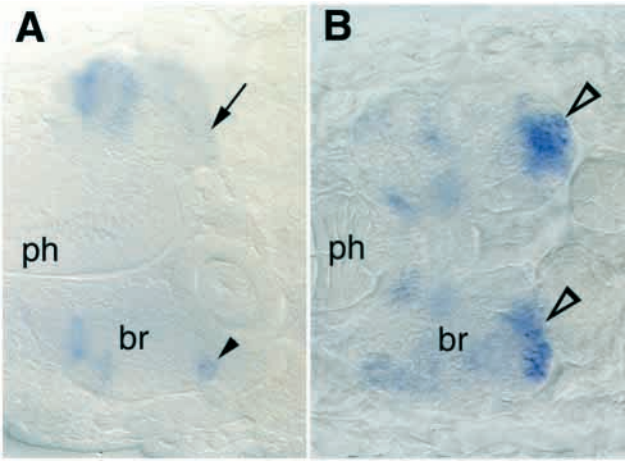

Fig. 4. Reduction of MB in late embryonic brain following heat shock. (A,B) The brain of a stage-16 embryo in dorsal view. MB is visualized by in situ hybridization with an *ey* probe. (A) hs-Notch (intra) embryos were heat shocked for about 2 hours during the embryonic stages 9 and 10 (3:40 to 5:20 hours after egg laying). (B) Control hs-Notch (intra) embryos were heat shocked for 2 hours during the embryonic stage 11 (5:20 to 7:20 hours after egg laying). Note in A the absence on one side (arrow) and strong reduction on the other side (arrowhead) of MB cells as compared to intact MB cells in B, open arrowheads. br, brain; ph, pharynx.

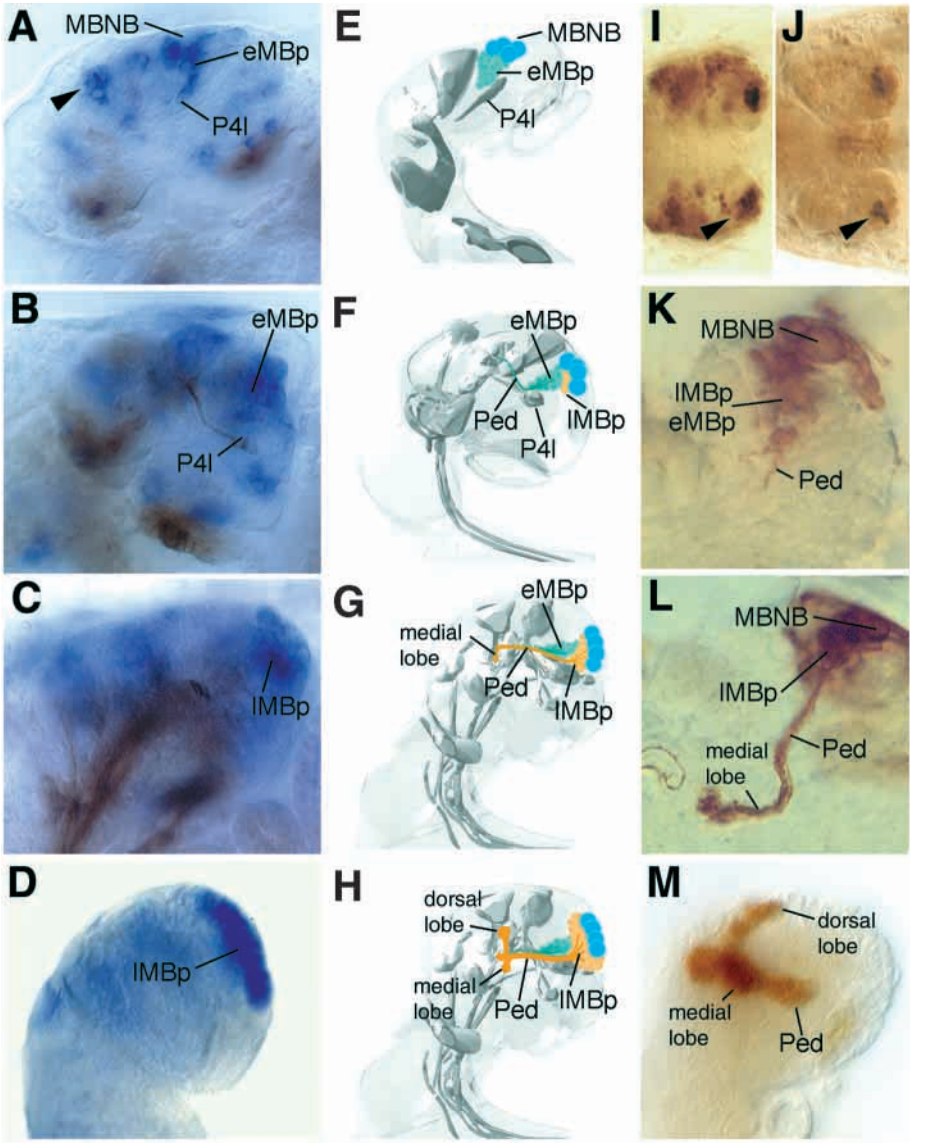

Fig. 5. Morphogenesis of the MB during mid- and late embryonic stages. (A-D) Whole-mount in situ hybridizations with an *ey* antisense RNA probe to detect the expression of *ey* at various embryonic stages (A, late stage 12; B, stage 14; C, stage 16; D, shortly after hatching). (E-H) Schematic views of the MB at stages corresponding to those shown in A-D. Mushroom body neuroblasts (MBNBs) keep expressing *ey* throughout embryogenesis, although the level of expression diminishes. *ey* is also expressed by the neuroblasts' progeny, that is the prospective MB neurons. We distinguish an early group (green in E-H) that forms the early embryonic MB primordium (eMBp), from a late group (orange in F-H), that form the larval MB primordium (lMBp). This distinction is made on the basis of numerous molecular markers that are expressed in the late embryonic and larval MB neurons, but not the embryonic MB neurons (I,J), showing expression of protein kinase A (I) and leonardo (J) in the lMBp of stage 16 brain. (K-L) MB axons labeled by DiI injection. At stage 14 (K), only short straight axons that constitute the forerunner of the peduncle (Ped) have formed. These axons grow along the brain neuropile founder cluster P4l (labeled with anti-FasII in B). At stage 16 (L), distal tips of MB axons show a sharp medial turn that constitutes the medial lobe. The dorsal lobe can only be detected in late stage-17 embryos (M).

(Yaounossi-Hartenstein et al., 1996). In contrast, the later born neurons expand tangentially over the brain surface and form the typical appearance of the Kenyon cells, resembling the head of a mushroom. It appears from our observations that the neurons born early from the MB neuroblasts do not contribute to the Kenyon cells, since they are located centrally, close to the neuropile, as opposed to superficially where one can find the Kenyon cell bodies.

Axonogenesis in the embryonic MB primordium shows separate phases of peduncle, medial lobe and dorsal lobe formation

Axons of MB neurons can be detected with antibody against Fasciclin II (FasII) (Grenningloh et al., 1991) from late stage 17 onward. At this stage, the typical, orthogonally arranged peduncle, dorsal lobe and medial lobe can already be recognized (Fig. 5M). During earlier stages, MB axons are FasII negative. We labeled these axons by applying DiI through a micropipette to the MB neurons located right underneath the easily recognizable quartet of MB neuroblasts. MB axons extend as a short bundle, the forerunner of the peduncle, during stage 14 (Fig. 5K). They grow along one of the brain neuropile founder cells, P4l (Fig. 5B,F). During stage 16, a conspicuous 90° turn can be seen at the tip of the peduncle; this gives rise to the medial lobe (Fig. 5L). The dorsal lobe is formed last by collaterals of the MB axons (Fig. 5M; also see Tettamanti et al., 1997). The above indicate that the medial and dorsal lobes are formed at different times.

ey hypomorph MBs have medial lobes that are reduced or fused

Both hypomorphic alleles of *eyeless*, ey^{R} and ey^{2}, are produced as a result of insertion of transposable elements in a regulatory element in the intron at the 5′ terminus of *ey* (Quiring et al., 1994). This results in reduced expression of *ey* transcripts in the embryonic and larval primordia of the eye imaginal discs. To find a function for *ey* in MB development, we first tested these hypomorphic alleles of *ey*, since at the present time a null allele of *ey* is not available. Neither of the two hypomorphic alleles revealed abnormalities in the embryonic pattern of

MBNBs or the eMBp (data not shown). We then tested these *ey* alleles for defects in the larval MB morphology. Using anti-FasII antibody, 38% of ey^R flies had defects in MBs that could easily be detected. The affected areas were the medial lobes, which were either fused with each other, drastically reduced in the diameter, or completely absent in one or both lobes (Fig. 6B-D). In ey^2 flies, we found fusion of the medial lobes in about 35% of flies, very similar to the medial lobe fusion in ey^R shown in Fig. 6B; however, the other abnormalities were not detected.

Overexpression of *ey* in MBs results in reduced dorsal lobes and fused medial lobes

Since it appeared that deficient expression of *ey* is associated with developmental defects in MBs, we determined whether overexpression of *ey* also causes abnormalities in MB development. We crossed the driver line 238Y (Yang et al., 1995), which expresses Gal4 exclusively in the MB neuroblasts and Kenyon cells from embryonic stage 13 onward (Tettamanti et al., 1997), with UAS-*ey*, a line which expresses *ey* upon binding of Gal4 to UAS. It should be noted that we checked the reported expression pattern of the 238Y line with UAS-lacZ and UAS-tau, and the expression in the brain of embryo and larva is indeed restricted to the MB (Fig. 6E,F). Among the offspring overexpressing *ey*, 72% had MB defects, consisting of either a unilateral or bilateral reduction or absence of dorsal lobes (Fig. 6G). A fraction of MBs also had fusion of the medial lobes. The result from the 238Y driver line indicates that the observed dorsal lobe defects are caused in an MB-cell-autonomous manner. As controls for the above, we tested lines 238Y and UAS-*ey* individually (without crossing them to each other) and also overexpressed β-galactosidase and bovine Tau proteins in MBs (by crossing the line 238Y to both UAS-lacZ and UAS-tau). Using anti-FasII and anti-bovine Tau immunostaining to detect any abnormality, MBs in the control lines appeared normal and similar in morphology to wild-type flies (not shown). In order to see if the above MB defects are caused by overexpression of *ey* at a specific stage of embryonic development, we induced overexpression of *ey* by heat shocks at various stages of embryonic development using a cross between hs-*gal4* and UAS-*ey* lines. At early embryonic stages, *ey*-overexpressing embryos did not survive, as previously reported (Halder et al., 1995). However, overexpression of *ey* at later embryonic stages (stage 16, 15±2 hours) resulted in the same dorsal lobe defect described above for the 238Y-Gal4-driven *ey* expression (Fig. 6H). This result is consistent with

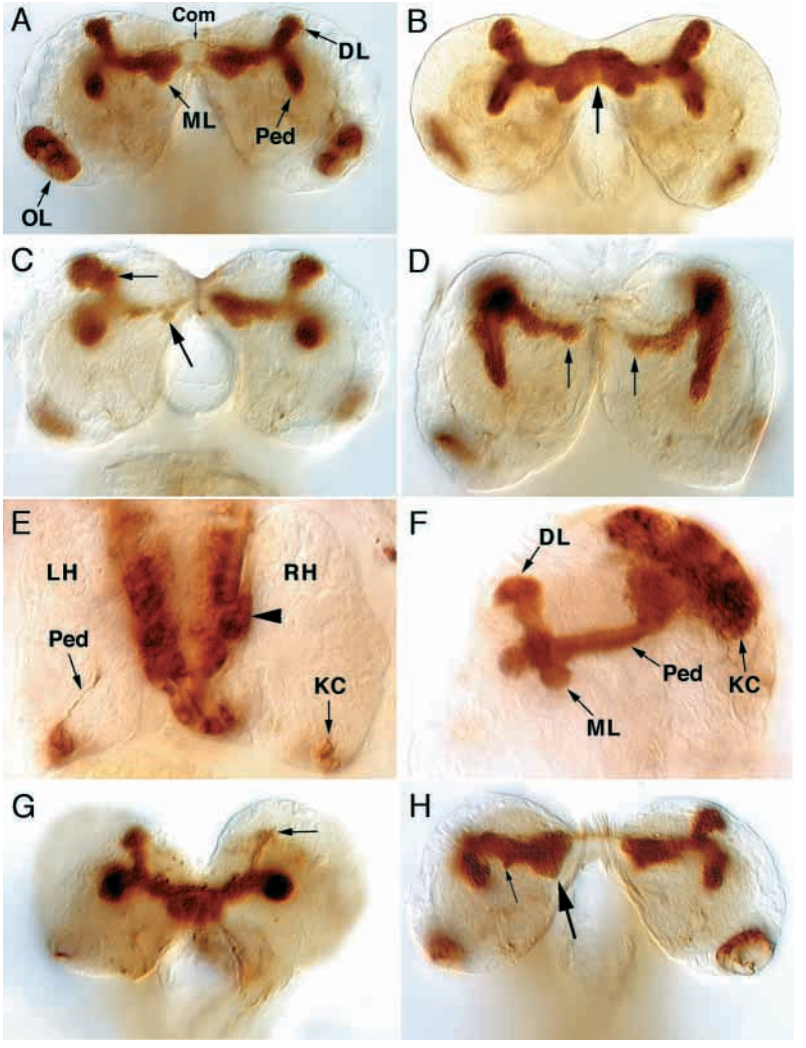

Fig. 6. MB lobe defects in ey^R and *ey*-overexpressing flies, anterior views. (A-D) Whole-mount immunostaining of second instar larval brains labeled with anti-FasII antibody (Grenningloh et al., 1991), which recognizes the MB axons (distal part of the peduncle, Ped; DL, dorsal lobe; ML, medial lobe). (A) Wild-type (OR^R) brain as control. Beside the MB, staining can also be seen in optic lobe (OL). Note the commissural fibers (Com) between the two medial lobes. (B-D) Among the brains of ey^R larvae, 38% had MB defects. Of these, 68% had medial lobes, which were fused (arrow in B); 32% had unilateral reduction of the medial lobe (large arrow in C), often accompanied by an increase in diameter of the dorsal lobe (small arrow in C), or bilateral narrowing of the tips of both medial lobes (arrows in D). (E,F) Stage-15 embryonic (E) and third instar larval (F) brains expressing bovine Tau protein under control of the MB specific driver line, 238Y (Yang et al., 1995). The embryonic brain in E is shown in dorsal view (LH, left hemisphere; RH, right hemisphere). Tau expression in the brain is restricted to a subset of Kenyon cells (KC) and their axons that pioneer the peduncle (Ped). Staining in the middle (arrowhead) represents pharyngeal muscle cells. The larval brain in F is shown in lateral view. Tau is expressed in most of the Kenyon cells (KC) and their axons, but not any other parts of the brain. (G,H) Whole-mount immunostaining of late first (G) and second (H) instar larval brains labeled with anti-FasII antibody. (G) MB defects resulting from 238Y-Gal4 driven overexpression of *ey* in the MB using UAS-*ey* (Halder et al., 1995). Among the flies overexpressing *ey*, 72% had MB defects, consisting of a reduced dorsal lobe; of these, 87% were unilateral (shown in G) and 13% bilateral (not shown). (H) Overexpression of *ey* using hs-gal4 and UAS-*ey*. Note that the absence of the dorsal lobe on the left side is accompanied by a thickening and irregular shape of the medial lobe (arrows).

the finding that dorsal lobe outgrowth occurs during late embryogenesis (Tettamanti et al., 1997).

dac loss of function results in defects of the dorsal lobe of the MB

We used *dac*4, a null allele of *dac* (Mardon et al., 1994) to see if we could detect any defects in the larval MB structure. Staining the larval brains with anti-FasII antibody, we found that about 10% of the larvae have dorsal lobes with extremely reduced diameter (Fig. 7A). The defect was only unilateral, as is usually observed with ey^R and *ey*-overexpression. Although the dorsal lobe defect was most noticeable, there were still other defects such as slanting of the dorsal lobe and widening of various regions of MB neuropile. Unlike with *ey*, since the *dac* null allele is available, mosaic analysis could be performed to see if the function of dac in MBs is autonomous. Indeed, mosaic analysis by Martini et al. (2000) has indicated that the role of *dac* in MB is cell-autonomous. We also used the driver line 238Y-gal4 to specifically overexpress *dac* in MBs, but except for minor defects, we did not observe a major phenotype (Fig. 7B). We examined the expression of *ey* in dac^4 flies and *dac* in ey^R flies. The expression of neither appeared to be decreased or increased in the background of a mutation in the other (not shown). This indicates that these genes may not regulate the expression of each other in MBs, unlike what has been seen for the eye disc. Complementation studies where various *ey* and *dac* alleles were crossed to each other indicated the same result (Martini et al., 2000).

As both *ey* and *dac* are expressed in the MB and eye primordia, we tested to see if other genes that interact with these factors in the eye are also expressed in the MBs. The expression of *eyes absent (eya)* and *sine oculis (so)* was tested using in situ hybridization, and lacz enhancer trap lines were used to test those of *decapentaplegic* (*dpp*), *hedgehog* (*hh*) and *wingless* (*Wg*). Except for *hh*, which was expressed in Kenyon cells, no expression was observed for the other genes (data not shown).

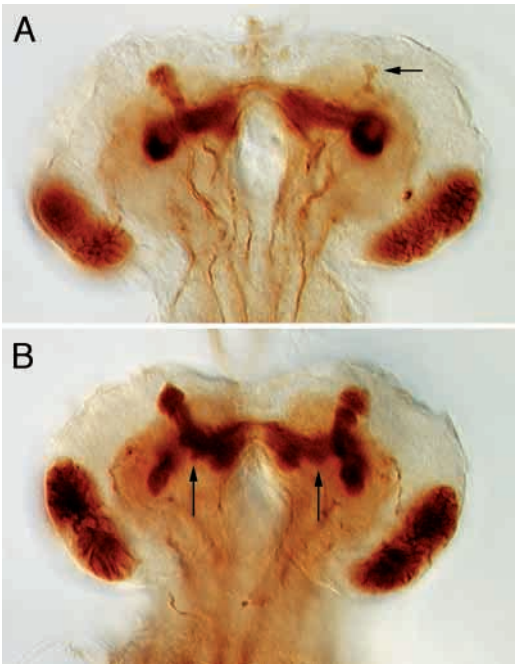

Fig. 7. MB lobe defects in *dac* null allele and *dac*-overexpression, anterior views. Whole-mount immunostaining of late second instar larval brain using anti-FasII antibody. (A) dac^4 MB. dac^4 is a null allele of *dac* (Mardon et al., 1994). Note the extreme narrowing of the left MB dorsal lobe (arrow). (B) Overexpression of *dac* under the control of 238Y-gal4 driver line (Yang et al., 1995). Note abnormal bulging (arrows) in other wise normal-appearing MB.

The number of MB Kenyon cells is reduced in both *ey* hypomorph and *ey* overexpression

Beside defects in axonal patterning, MBs of larvae lacking or overexpressing *ey* also show abnormalities in neuron number. Staining with D-MEF2, which in the brain of *Drosophila* larvae exclusively labels the MB Kenyon cells (Schulz et al., 1996), indicated that the number of Kenyon cells is reduced in both ey^R and *ey*-overproducing flies (Fig. 8B,C). A reduction in the number of Kenyon cells in ey^R adult brains has been already reported by Hinke (1961).

We tested the level of *ey* transcripts in the Kenyon cells of ey^R flies to see if the observed defects are due to reduction or absence of *ey* mRNA. Although the number of cells appeared reduced, as mentioned above, the level of *ey* expression in each Kenyon cell appeared normal (not shown).

Since our observation indicates that the number of Kenyon cells is substantially reduced in ey^R and *ey*-overexpressing flies, why is the diameter of the MB peduncle, which should reflect the number of MB axons, not reduced? One possibility is that the bulk of peduncular axons that we observe during the early and mid-larval stages is formed by a small number of MB neurons. To test this hypothesis we ablated the MB neuroblasts by feeding hydroxyurea for 4 hours immediately after hatching, following the protocol of de Belle and Heisenberg (1994). We let the larvae develop for 2 days and then stained brains with anti-D-MEF2 and anti-FasII antibodies (Fig. 8E-H). The results show that only a small number of Kenyon cells remain; however, there still remain a substantial peduncle, dorsal and medial lobe. Since the MBs were killed at the early first instar larval stage, the only remaining Kenyon cells should be those that were already generated during the embryonic stages.

DISCUSSION

Embryonic origin of the MB and related structures

The *Drosophila* MB has been the object of extensive searches for genes controlling brain structure (Heisenberg, 1980; Heisenberg et al., 1985; de Belle and Heisenberg, 1996), as well as learning and memory (Davis, 1996). Two genes involved in the development of MBs have so far been identified. One is *mushroom body tiny* (*mbt*), which encodes a p21-activated kinase (PAK) (Melzig et al., 1998). Mutation in *mbt* results in a reduced number of Kenyon cells. The other is *linotte/derailed* (*lio/drl*), which encodes a receptor tyrosine kinase (RTK), and its mutation results in missing dorsal lobes (Moreau-Fauvarque et al., 1998). Many additional mutations causing structural defects in MBs have been identified (de Belle and Heisenberg, 1996; Prokop and Technau, 1994; Lee and Luo, 1999), but the identities of the corresponding genes are not yet known. Additionally, the developmental abnormalities resulting in the MB defects remain unresolved,

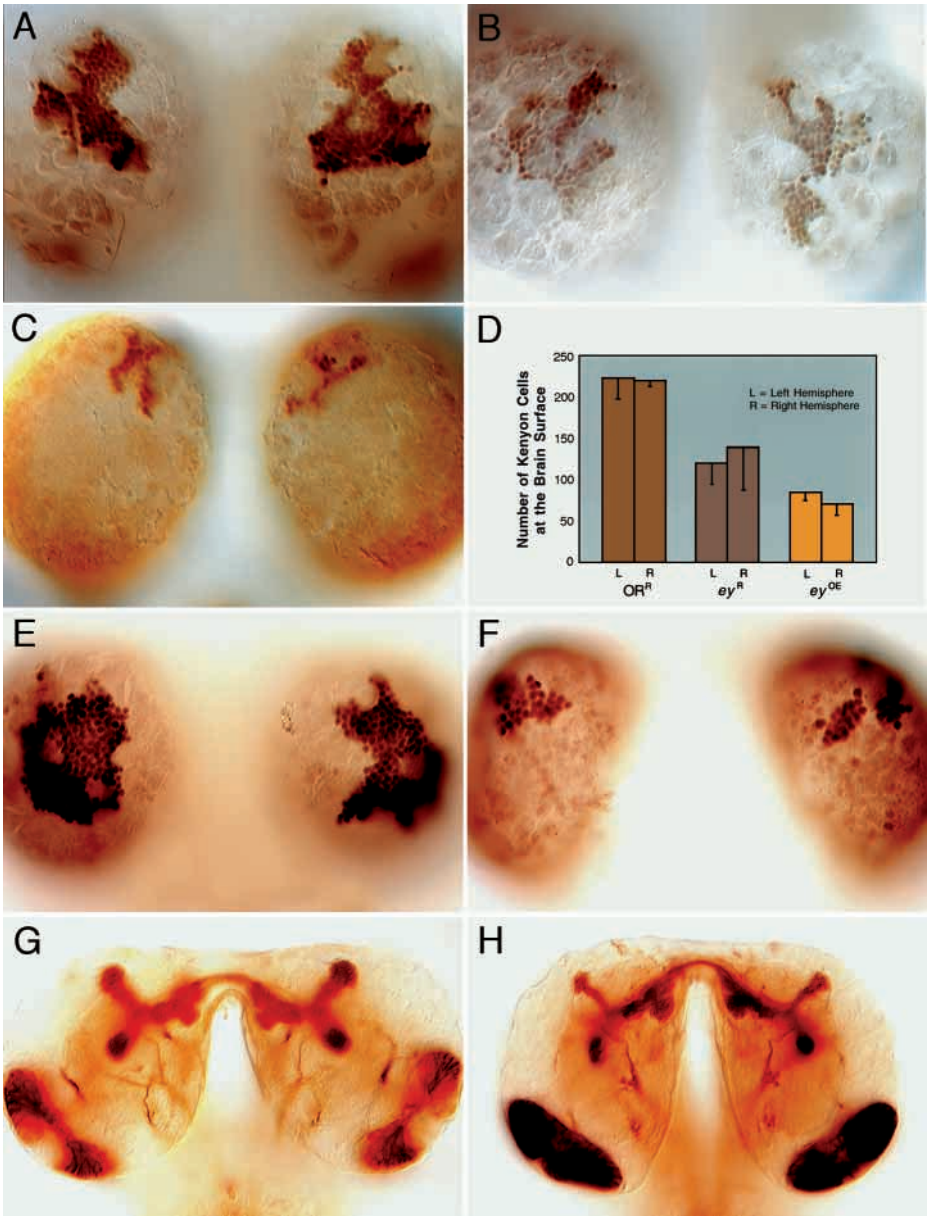

Fig. 8. Reduction in the number of MB Kenyon cell in ey^R, *ey*-overexpressing and hydroxyurea-treated flies, posterior views. (A-C) Whole-mount immunostaining of third instar larval brains using anti-D-MEF2 antibody (Nguyen et al., 1994), which labels nuclei of Kenyon cells. (A) Wild-type control. (B) In ey^R MBs the number of Kenyon cells is reduced in both brain hemispheres. (C) Overexpression of *ey*. Note the drastic reduction in the number of the Kenyon cells. In control experiments, reduction in cell number was not observed in individual lines 238Y and UAS-*ey*, or in a cross of line 238Y to UAS-lacz or UAS-bovine tau (not shown). (D) Graphic presentation of Kenyon cell count in OR^R, ey^R and *ey*-overexpression (ey^{OE}). The results show a significant difference in the number of Kenyon cells (*t*-test between OR^R and ey^R: $t(18)=6.8$, $p<0.0001$; and between OR^R and ey^{OE}: $t(18)=20.7$, $P<0.0001$; ANOVA: $f(2, 27)=79.83$, $P<0.01$). (E-H) The effect of larval MB neuroblasts ablation on Kenyon cell number and MB neuropile. (E,G) Wild-type early third instar brains labeled with anti-D-MEF2 (E) and anti-Fas II (G) as control. (F,H) The corresponding stainings of larval brains treated with hydroxyurea for 4 hours after hatching. The number of Kenyon cells is dramatically reduced (F), but the diameter of peduncle and lobes (H) is affected less strongly.

partially due to the lack of information about the early development of the MBs. In this report we have studied the embryonic origin and morphogenesis of the MB. We have further analyzed the function of *eyeless*/Pax6 and *dachshund*, genes expressed in the MB throughout embryogenesis.

The *Drosophila* brain is formed by a set of approximately 85 neuroblasts that delaminate from the procephalic ectoderm of the embryonic head (Younossi-Hartenstein et al., 1996). These neuroblasts divide in a stem cell mode and produce lineages of 10-20 neurons each that make up the larval brain. Most neuroblasts cease to divide during mid-embryonic stages. After a variable time period of mitotic quiescence, neuroblasts become active again in the larva and produce a second pool of neurons. Both neurons formed during and after embryogenesis contribute to the adult brain (Prokop and Technau, 1991).

Several years ago, Ito and Hotta (1992) identified four neuroblasts that give rise to the MB. These neuroblasts can already be detected in late embryos and early larvae. Since a number of molecular markers, including *ey*, *dac* and *sevenup* (*svp*), are expressed in these neuroblasts, we were able to reconstruct their origin in the early embryo. The four MB neuroblasts are among the first wave of neuroblasts that delaminate from the procephalic ectoderm. They belong to a group of six *svp*-positive neuroblasts called Pc3 (*Pc* for central protocerebral neurectoderm) that appear during late stage 9 (Younossi-Hartenstein et al., 1996). The early origin of the MB neuroblasts came somewhat as a surprise, given that a variety of different molecular markers expressed in MB neurons come on quite late in embryogenesis, around stage 15 (this report). However, supressing neuroblast formation by heat-shock-driven activation of the hsp70-Notch(intra) construct confirms the early origin of MB neuroblasts. Only early heat shocks were able to affect MB formation.

MB neuroblasts begin proliferating immediately after

delamination at embryonic stage 9. Like most other neuroblasts, they 'bud off' chains of ganglion mother cells and neurons in early and mid-embryonic stages. The fate of this early progeny of the MB neuroblasts is enigmatic. Thus, although *seven-up* (*svp*) and *ey* are initially expressed in these cells, expression is downregulated later on. This and the fact that several other molecules expressed in the larval and adult MB are not expressed in the early MB neurons prompted us to consider these cells as a distinct cell population, the early embryonic MB primordium. It is quite possible that the early embryonic MB primordium will not become part of the MB at all. If this is indeed the case, it would be interesting to establish what brain compartment the early embryonic MB neurons contribute to, and whether this compartment is functionally connected to the MB. Lineage studies, injecting the tracer molecule DiI into MB neuroblasts (Bossing et al., 1996), are in preparation to address this issue.

The fact that *dac* remains expressed in the patch of ectoderm cells from which the MB neuroblasts delaminate (MB neurectoderm) allowed us to trace these cells during later stages. Most cells that stay at the surface of the head, when neuroblast delamination is over, form a thin epithelium that folds inside during head involution and becomes the so-called dorsal pouch (Juergens and Hartenstein, 1993). Only small groups of the head ectoderm cells, notably along the dorsal midline, but also scattered at a few locations more laterally, invaginate and become part of the brain. The *dac*-positive MB neurectoderm, as well as *dac*-positive para-MB neurectoderm, belongs to these invaginating placodes. Many *dac*-positive cells undergo apoptosis; the remaining ones spread out on the surface of the brain hemisphere. It will be interesting to establish whether these cells, that are topologically closely related to the MB progenitors in the early embryo, remain functionally connected with the MB.

Another structure whose origin may be close to the MB primordium is the eye imaginal disc. The earliest marker of the eye disc is *eyeless*, which comes on during stage 12 in a strip of cells anteriorly adjacent to the optic lobe (Daniel et al., 1999). During head involution, the eye disc primordium becomes folded in as part of the dorsal pouch. Around the time of hatching the disc primordium invaginates from the pouch as a proper 'disc' (Younossi-Hartenstein et al., 1993). Given the fact that *ey* and *dac* are expressed in both MB and (larval) eye disc, it is tempting to speculate that this could reflect a common origin of both structures in the embryo. It seems quite clear, however, that the MB neurectoderm and eye disc primordium are not overlapping. Thus, the *dac* positive cells that are descended from the MB neurectoderm are located further anterior than the eye disc primordium (see diagram in Fig. 3).

ey and *dac* may have a role in axonal pathway selection

We have shown that in the *ey* hypomorphs, the medial lobes are reduced or not formed, and in *ey* overexpression and a *dac* null allele, dorsal lobes are reduced or not formed. Additionally, in both *ey* hypomorph and *ey*-overexpression, fusion of medial lobes is frequently observed. Thus our results indicate that *ey* and *dac* may have a role in specifying the structural fate of Kenyon cell axons. Interestingly, experiments in a *Pax6* mutant mouse indicate that *Pax6*, similar to *ey*, may have a role in selection of the axonal pathways (Mastick et al., 1997; Engelkamp et al., 1999; Kawano et al., 1999). Due to the simple structure of MB neuropile, the study of the function of ey in MB may be helpful in understanding the role of *Pax6* in the vertebrate nervous system.

The fact that *ey* overexpression by a MB-specific driver line causes a defect similar to the one seen by a global, hs-gal4-induced, expression suggests that *ey* acts autonomously in the MB neurons. Since it is still possible that the MB-specific driver line is expressed at levels below detection with the lacZ or bovine tau reporters, however, we cannot formally exclude the possibility that *ey* expression outside the MB contributes to the reported phenotype. Recently Kurusu et al. (2000), using a null allele of *dac* and a new null allele of *ey*, have shown that MB neuropile is drastically abolished in the pupa. Furthermore, P. Callaerts et al. (personal communication) have found additional *ey* hypomorphs that display axonal defects in adult MB. And Martini et al. (2000) have detected dorsal lobe defects in adults MB that carry a *dac* null alleles and have shown with mosaic analysis that *dac* acts cell-autonomously. The above further support an important function of these genes in MB axon formation. It should be also mentioned that analysis of chimeric mouse indicates that Pax6 acts cell-autonomously during eye and nasal development (Quinn et al., 1996; Collinson et al., 2000).

ey may have a role in neuroblast proliferation

We also found that both loss and overexpression of *ey* result in a significant reduction in the number of MB neurons. A reduction in the number of MB neurons in ey^R flies has already been reported in a previous paper (Hinke, 1961). Since the number of the MB neuroblasts and neurons is normal in the late embryos of ey^R and *ey*-overexpression, the defect in larval MB cell number is probably the result of a failure of MB neuroblasts to proliferate normally. Abnormalities in proliferation have also been observed in mouse *Pax6* mutants (Warren and Price, 1997; Warren et al., 1999).

A model of embryonic MB development

The abnormalities caused by the hypomorphic *ey* allele and *ey* overexpression has to be interpreted in light of the temporal sequence in which medial and dorsal lobes are formed in the embryo. Thus the medial lobe is formed first, when the growth cones of the extending MB axons make a sharp medial turn. The dorsal lobe originates as a collateral at a later stage. Taken together, these findings lead to the following model (Fig. 9). Signals localized near the dorsal midline of the brain act upon the outgrowing MB axons to form the medial lobe (signal 'm' in Fig. 9), followed by a dorsal signal ('d' in Fig. 9) that induces the outgrowth of the dorsal lobe collaterals. Ey may be specifically involved in balancing the reception of the d- and the m-signals, such that it renders growth cones more sensitive to the m-signal and less sensitive to the d-signal. According to this hypothesis, reception of the m-signal is reduced, and reception of the d-signal increased in *ey* hypomorphs. As a result growth cones fail to grow medially and extend dorsally instead, resulting in the absence of medial lobes and normal or thickened dorsal lobes. By overexpressing *ey*, the MB axons get more sensitive to the m-signal, so that the collaterals that should grow dorsally are diverted medially. A thickening of the medial lobe is often observed in brains where the dorsal lobe

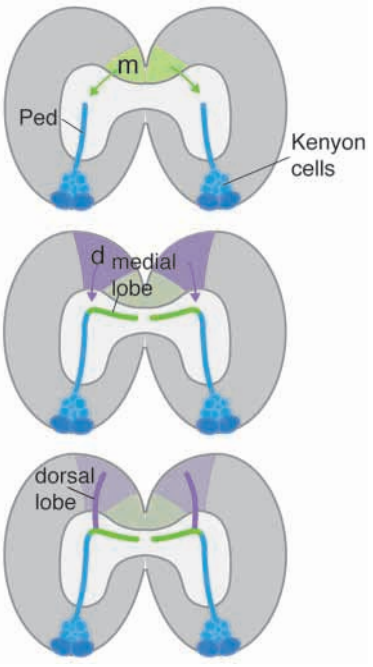

Fig. 9. Model of embryonic MB development. Kenyon cell axons grow out in three phases, corresponding to the formation of the peduncle (Ped; top panel), the medial lobe and dorsal lobe. Local signals (m and d) associated with neurons, glial cells or axons, may be involved in directing the growth of the lobes.

is absent. In contrast to *ey*, *dac* may function only in the regulation of the d-signal, since its null allele results in the absence of the dorsal lobe while its overexpression has little effect.

ey could regulate the m and d signals by regulating the expression of various genes. In the vertebrate CNS, cell adhesion molecules, N-CAM and L1 appear to be under direct control of *ey/Pax6* (Holst et al., 1997; Meech et al., 1999). Studies in chimeric mice made from aggregation of wild-type and $Pax6^{-/-}$ embryos show that the wild-type cells have adhesive properties different from those that have mutant *Pax6* (Quinn et al., 1996; Collinson et al., 2000). *Drosophila* FasciclinII, a homologue of vertebrate N-CAM, may also be regulated by *ey*, since in the *ey*-null mutants it appears to be downregulated (Kurusu et al., 2000). Additionally, in *Drosophila*, the tyrosine kinase receptor, derailed/linotte (*drl/lio*) (Callahan et al., 1995; Bonkowsky et al., 1999), may be the receptor for the d-signal, since its absence causes loss of the dorsal lobes (Moreau-Fovarque et al., 1998). *ey* could act directly or indirectly to downregulate *Drl/lin* expression, which would explain why *ey* overexpression causes the absence of the dorsal lobes. In vertebrates, both the loss of function (Hill et al., 1991) and overexpression (Schedl et al., 1996) of *Pax6* lead to defects in the eye and nervous system.

The identity of the embryonic Kenyon cells

There is some evidence indicating that the embryonic Kenyon cells are made up of γ-type neurons. This is based on hydroxyurea ablation of MB neuroblast in the newly hatched 1st instar larvae. Using various gal4 driver lines, Armstrong et al. (1998) demonstrated that when the early 1st instar larvae are fed hydroxyurea, all the MB lobes are absent in the adult except the γ lobe. Since no MB Kenyon cells are generated after the hydroxyurea treatment, the Kenyon cells that remain are those that were generated during the embryonic period. In the adult, the γ lobe is generated by the γ-type neurons (Lee et al., 1999). Thus the above implies that the MB neurons generated during the embryonic period are the γ neurons; however, after embryogenesis, additional γ neurons are added to the pool of previously existing γ neurons (Armstrong et al., 1998). In the present experiments we observed the formation of both the dorsal and medial lobes after hydroxyurea treatment of the early 1st instar larvae. This is due to the fact that, during the 1st and 2nd larval stages, when we usually observed the larvae (Fig. 8E,F), the axons of the γ neurons are branched and give rise to both the medial and dorsal lobes (Lee et al., 1999). It is interesting to note that when *ey* or *dac* are lacking, the most severe defects are found during the pupal stage (Kurusu et al., 2000), when the axons of the γ neurons are degenerated and reformed along with the formation of the α/β axons (Lee et al., 1999).

Is the *ey* gene network conserved in the MB?

During evolution of a species, a gene network controlling the development of an organ may gain a new function and participate in the development of another organ (Noveen et al., 1998). In order to see if the *ey* gene network is conserved in the mushroom bodies, we tested the embryonic and larval expression of two members of the *ey* network, *eya* and *so*. No expression of *so* or *eya* mRNA was detected using in situ hybridization. This indicates that the *ey* gene network may not be conserved in MBs. Furthermore, there does not appear to be a direct interaction between *ey* and *dac* as tested with their overexpression (this report) and complementation experiments (Martinin et al., 2000), emphasizing that the interaction between *ey* and *dac* is not conserved in MB. Three other genes that are involved in *Drosophila* eye development are *decapentaplegic* (*dpp*), *hedgehog* (*hh*) and *wingless* (*wg*). *dpp* has been shown to induce the expression of *eya*, *so* and *dac* (Chen et al., 1999; Curtiss and Mlodzik, 2000) and ectopic eyes (Pignoni and Zipursky, 1997; Chen et al., 1999). Additionally, *eya* can regulate the expression of *dpp* and *wg* (Hazelett et al., 1998). Thus there appears to be crosstalk between the *ey* and *dpp* gene networks in eye development. Only *hh* is expressed in the larval MB, however, and *dpp* and *wg* are absent. These results indicate that gene networks in eyes and MBs are at best only partially conserved during evolution.

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