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The retinal determination gene, *dachshund*, is required for mushroom body cell differentiation

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SUMMARY

The *dachshund* gene of *Drosophila* encodes a putative transcriptional regulator required for eye and leg development. We show here that *dachshund* is also required for normal brain development. The mushroom bodies of *dachshund* mutants exhibit a marked reduction in the number of α lobe axons, a disorganization of axons extending into horizontal lobes, and aberrant projections into brain areas normally unoccupied by mushroom body processes. The phenotypes become pronounced during pupariation, suggesting that *dachshund* function is required during this period. GAL4-mediated expression of *dachshund* in the mushroom bodies rescues the mushroom body phenotypes. Moreover, *dachshund* mutant mushroom body clones in an otherwise wild-type brain exhibit the phenotypes, indicating an autonomous role for *dachshund*. Although *eyeless*, like *dachshund*, is preferentially expressed in the mushroom body and is genetically upstream of *dachshund* for eye development, no interaction of these genes was detected for mushroom body development. Thus, *dachshund* functions in the developing mushroom body neurons to ensure their proper differentiation.

Key words: *dachshund*, Mushroom body, Neuron, Development, *Drosophila*

INTRODUCTION

Mushroom bodies (MB) are insect brain structures that are required for learning and memory. Flies without MB or with abnormal MB exhibit defects in associative learning paradigms such as olfactory classical conditioning (de Belle and Heisenberg, 1994; Heisenberg et al., 1985). Several proteins required for normal olfactory learning are preferentially expressed in the MB. These include the products of *dunce*, *rutabaga* and *DCO*, genes that encode components of the cAMP/PKA pathway (Han et al., 1992; Nighorn et al., 1991; Qiu and Davis, 1993; Skoulakis et al., 1993). These lines of evidence, along with results from parallel studies in other insects (Hammer and Menzel, 1995; Meller and Davis, 1996), emphasize the importance of MB for learning and memory in *Drosophila*.

The MB have a unique architecture. The cell bodies are located in the dorsal posterior brain above the neuropil areas housing their dendritic processes, which are known as the calyces. These unipolar neurons project anteriorly through the brain, sending dendritic branches into the calyces and continuing anteriorly as a nerve termed the peduncle. The peduncle diverges in the anterior brain into five discrete areas of neuropil known as lobes. The α and α′ lobes are columns of neuropil oriented vertically in the brain and the β, β′ and γ lobes are columns oriented horizontally (Crittenden et al., 1998). This basic structure, in which the peduncle gives rise to vertically and horizontally oriented lobes, first emerges late in embryogenesis (Tettamanti et al., 1997).

The adult MB develop from four neuroblasts per brain hemisphere by the sequential generation of three classes of neurons (Crittenden et al., 1998; Ito and Hotta, 1992; Ito et al., 1997; Lee et al., 1999). The γ neurons, which are unbranched and project their axons into the γ lobe, are born from embryogenesis through the third larval instar (Armstrong et al., 1998; Lee et al., 1999). The α′β′ neurons are branched and project axon collaterals into both the α′ and β′ lobes. These neurons are born from the third larval instar through puparium formation (Lee et al., 1999). The αβ neurons are also branched and project axon collaterals into the α and β lobes. These neurons are born after puparium formation (Lee et al., 1999). The larval MB are simpler in structure than adult MB in having only two lobes: a vertically oriented, α-type lobe and a horizontally oriented, β-type lobe. Although γ neurons in the adult are unbranched and project only into the γ lobe, larval γ neurons send projections into both the α-type and β-type lobes (Lee et al., 1999). The larval γ axons then degenerate early in pupariation and subsequently regrow as unbranched projections to form the adult γ lobe (Lee et al., 1999; Technau and Heisenberg, 1982).

Four genes are known to be responsible for the determination of the *Drosophila* retina. Two of these genes, *eyeless* (*ey*) and *sine-oculis* (*so*), encode homeobox proteins. Two others, *dac* and *eyes absent* (*eya*), encode nuclear proteins

that lack protein motifs suggestive of specific function (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994). Protein interaction studies have shown, however, that SO binds EYA, and EYA binds DAC (Chen et al., 1997; Pignoni et al., 1997). Furthermore, DAC and EYA are capable of activating transcription in yeast if fused to the GAL4 DNA binding domain (Chen et al., 1997). These studies have therefore suggested that EYA and DAC are cofactors for the transcription factor, SO.

A fascinating insight into the function of these genes was obtained from ectopic expression studies. Ectopic expression of EY, EYA or DAC produces ectopic eyes on the fly (Bonini et al., 1997; Chen et al., 1997; Halder et al., 1995; Shen and Mardon, 1997), and this capacity for eye determination is enhanced when most combinations of the four genes are combinatorially expressed (Chen et al., 1997, 1999; Pignoni et al., 1997). Furthermore, ectopic expression of a single retinal determination gene induces the expression of others (Bonini et al., 1997; Chen et al., 1997; Halder et al., 1998; Pignoni et al., 1997; Shen and Mardon, 1997). These previous findings have suggested a model in which EY functions earlier than the three other retinal determination genes, but through multiple feedback loops in which each protein can induce the expression of the others.

We show here that DAC is also required for the normal development of the mushroom bodies. The MB in *dac* mutants are disorganized and their processes demonstrate a failure in normal targeting. Our findings indicate that the retinal determination gene, *dac*, serves the differentiation of neurons in the central brain.

MATERIALS AND METHODS

Fly stocks

The *dac Drosophila* mutants were identified by their yellow body or mouthhook color from *yw*; dac^{null}/CyO^{y+} stocks. Mutants were dissected from their pupal cases at the approximate time of normal eclosion by monitoring for wing darkening and the presence of abdominal bristles. Control animals of the same age were of the genotype *yw*;+/+. The dac^{4} allele is a deletion of most of cytological band 36A and thus removes the entire *dac* locus. The eye and leg phenotypes of dac^{1} and dac^{3} homozygotes are identical to those of dac^{4} homozygotes, indicating that all three alleles are phenotypic nulls (Mardon et al., 1994). In addition, these animals are protein nulls by immunohistochemistry.

Stocks for MARCM experiments were obtained from L. Luo (Stanford University). Animals of the genotype $GAL4^{C155}$, UAS-mCD8-GFP, hs-FLP; dac^{null} FRT^{40A}/GAL80 FRT^{40A} or $GAL4^{C155}$, UAS-mCD8-GFP, hs-FLP; dac^{+} FRT^{40A}/GAL80 FRT^{40A} were collected within 2 hours of larval hatching and heat shocked for 30 minutes at 38°C.

P element lines for the enhancer detector screen were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, USA), Berkeley Drosophila Genome Program (Torok et al., 1993), D. Glover (Deak et al., 1997), N. Perrimon (Harvard University), C. Goodman (University of California, Berkeley) and A. Schneiderman (Cornell University). DNA flanking the PZ element of the dac^{P} line was identified by inverse PCR and plasmid rescue.

First instar larvae collected within 2 hours of hatching were fed on sugar food containing 0.1 mg/ml BrdU for 45 minutes, then immediately dissected.

Histology

Frontal cryosections of adult heads were prepared and stained for *lacZ* as described by Han et al. (1992). For immunohistochemistry, flies were fixed in Carnoy's fixative for 4 hours, dehydrated, cleared in methylbenzoate overnight and embedded in paraffin. Paraffin sections were dried overnight at 42°C. Paraffin was removed by immersing the slides in xylenes, followed by rehydration through a graded ethanol series and 10 minutes in PBHT (0.02 M PO_4, 0.5 M NaCl, 0.2% Triton X-100, pH 7.4). Slides were blocked in PBHT+NGS (PBHT + 5% normal goat serum) for 2-5 hours. Primary antibody incubations (overnight) were carried out in PBHT+NGS at the following dilutions: mouse anti-DAC^{MAb1-1} at 1:30 and rabbit anti-LEO (Skoulakis and Davis, 1996) at 1:2500. Horseradish peroxidase-based antibody detection was carried out with the Vectastain Elite ABC kit (Vector labs). For fluorescent detection, primary antibody incubation was in PBHT+NGS at the following dilutions: mouse anti-DAC^{MAb1-1} at 1:10, rabbit anti-LEO at 1:1000 and rabbit anti-DCO at 1:300. Secondary antibodies (FITC-conjugated goat anti-mouse and Texas Red®-conjugated goat anti-rabbit; from Rockland Antibodies) in PBHT+NGS were added at 1:200. Slides were mounted in Vectashield (Vector labs). For whole-mount immunohistochemistry, the CNS of larvae (or brains of adults/pupae) were dissected in PP (1× PBS, 4% formaldehyde, 0.1% Triton X-100) and fixed for the following times: 20 minutes for L1, 30 minutes for L3, and 45 minutes for adult/pupal brains. After fixation, L3 CNS and brains were digested for 5-10 minutes in enzyme (collagenase, dispase and hyaluronidase, each at 1 mg/ml in 1× PBS). The brains were blocked for 30 minutes in PBT (1× PBS, 0.1% Triton X-100, 0.1% BSA)+NGS, followed by primary antibody incubation overnight at 4°C. For fluorescent detection, mouse anti-DAC^{MAb1-1} was used at 1:10, rabbit anti-DCO at 1:500 and rat anti-BrdU at 1:10 (Harlan Sera-Lab). Fluorescent secondary antibodies (above) were used at 1:1000 and Texas Red®-conjugated goat anti-rat (Rockland) was used at 1:300. Staining of embryos was carried out using procedures described by Patel (1994) and alkaline phosphatase detection.

RESULTS

dachshund is preferentially expressed in adult mushroom bodies

Because of the importance of MB for olfactory learning, we screened 1643 enhancer detector lines for preferential expression of the reporter in these cells. The dac^{P} line was one selected in the screen (Fig. 1B). DNA flanking the enhancer detector element was isolated by inverse PCR and plasmid rescue, which revealed that the insertion occurred 11 base pairs downstream of the putative *dac* transcription start site (Fig. 1A). To confirm that the enhancer detector was accurately reflecting DAC expression, we stained adult fly heads with an anti-DAC monoclonal antibody. The antibody revealed robust DAC expression in the nuclei of MB neurons (Fig. 1C) along with scattered neurons throughout the brain. This robust expression in the MB was also observed for *D. virilis* (Fig. 1D), suggestive of a conserved function over 60-80 million years of evolution.

DAC expression in the MB during development

To gain insights into the role of DAC in MB development or function, we stained animals at various stages. DAC expression was evident in the MB nuclei at 48, 72 and 96 hours after puparium formation (Fig. 2A,B). It was also observed in the area of the MB in larvae at all stages (Fig. 2C) and embryos

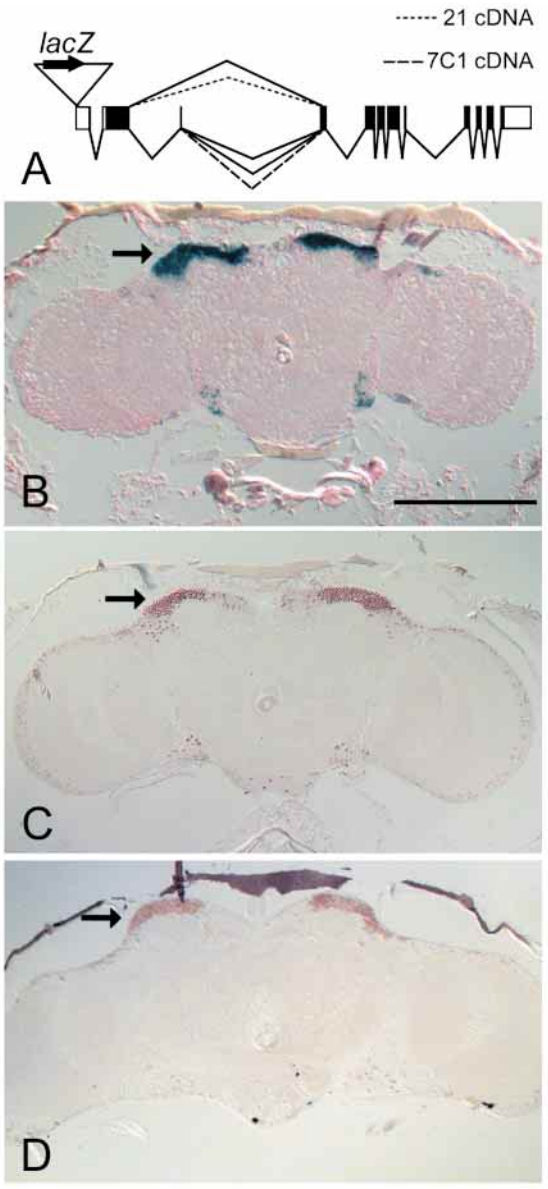

Fig. 1. *dac* expression in adult mushroom bodies. (A) Structure of the *dac* gene. The insertion site of the dac^P enhancer detector element was determined by sequence analysis of plasmid rescue and inverse PCR products. Alternative splicing generates at least five cDNAs of which two, 7C1 and 21, were used here. The translation start site is within the second exon. (B-D) Frontal sections through the mushroom bodies at the level of the calyces. In each panel, the arrows indicate staining of the mushroom body nuclei. (B) Cryosection of a dac^P heterozygote, stained for *lacZ* and counterstained with Nuclear Fast Red. (C) Paraffin section of a Canton-S fly stained with an anti-DAC antibody. (D) Paraffin section of a *Drosophila virilis* fly stained with an anti-DAC antibody. Bar, 200 μm.

as young as stage 9 (Fig. 2D and not shown). The time of expression in embryos coincides with when MB neuroblasts (NB) segregate from the neuroepithelium and begin dividing to produce neurons (Younossi-Hartenstein et al., 1996). To conclusively identify the DAC-positive nuclei as those of MB neurons, we stained third instar larval brains for DAC along with DCO, a cytoplasmic marker for MB (Skoulakis et al., 1993). Optical sections through the brains revealed DCO in the MB soma, axons and dendrites, and DAC in the nuclei of these DCO-labeled cells (Fig. 2E). Thus, DAC expression in the MB begins in embryogenesis at about the time that MB neuroblasts segregate and begin dividing. This preferential expression remains into adulthood.

DAC expression in the MB lineage

Because DAC plays a role in retinal determination, we questioned whether DAC was expressed in MB neuroblasts, their immediate descendants (ganglion mother cells, GMC), or in MB neurons. The MB NB are the only cells in the dorsal region of the brain that divide during the first 8 hours after larval hatching, so that BrdU is specifically incorporated into the MB NB and their progeny during this time (Ito and Hotta, 1992). We examined DAC expression in MB NB, GMC and neurons after a 45 minute feeding period. This time is sufficient for the MB NB, GMC and a few MB neurons (not shown) to incorporate BrdU (Fig. 2H). The NB were identified by their large and diffuse nuclei; the GMC by their smaller size and location next to an NB; and the MB neurons by their yet smaller size and proximity to an MB NB. None of the BrdU-positive cells, including the NB, GMC or young MB neurons, were labeled strongly with the anti-DAC antibody (Fig. 2F). Only MB neurons born during embryogenesis showed strong DAC expression. Therefore, DAC is strongly expressed in MB neurons that are 8-10 hours old (not shown), but not in NB, GMC or newly born MB neurons. This is consistent with a role for DAC in mature MB neurons, rather than in the developmental events such as cell division that lead to their birth.

dac mushroom bodies

The preferred expression of DAC in the post-mitotic MB neurons during embryonic, larval and pupal stages suggested that DAC might function in their differentiation. To test this idea, we examined the morphology of MB neurons in *dac* null mutants. The structure of the MB neurons in the mutants was grossly altered. The calyx and peduncle are normally well defined (Fig. 3C,H), but in the mutants these neuropil areas exhibited irregular edges due to a failure to contain MB processes to their normal domains (Fig. 3D,I). In addition, aberrant projections were often observed emanating from the calyx in the direction of the tips of the α lobes (Fig. 3D,I). In

Table 1. Penetrance of *dachshund* mushroom body phenotypes in adults

Category	Number of animals showing phenotype		
	dac^1/dac^3	dac^1/dac^4	dac^3/dac^4
Aberrant fibers from calyx	35/36	17/18	16/18
Along peduncle	32/34	12/14	9/9
Toward tip of α lobe	28/36	11/13	10/14
Reduced α lobe	24/24	15/15	24/24
Projection from heel	2/24	0/15	1/24
Disorganized medial lobes	24/24	15/15	24/24
Aberrant γ-lobe projections	13/24	12/14	16/22

Values are the fractions of *dac* null brain hemispheres that exhibited a particular phenotype. The total examined within a genotype differ because only animals in which all relevant sections were present and in good condition were evaluated. No control animals exhibited any of the phenotypes.

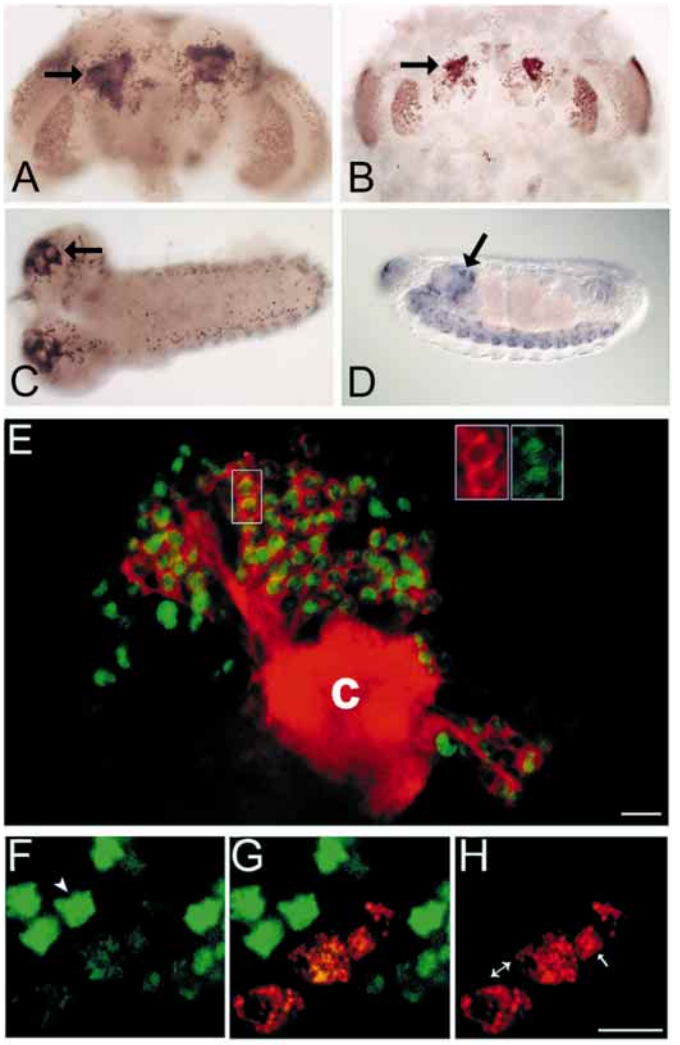

Fig. 2. DAC expression in the mushroom bodies during development. Wholemount preparations stained with an anti-DAC antibody. (A) Dorsal posterior view of a 96-hour pupal brain. A cluster of mushroom body (MB) nuclei are indicated by the arrow. DAC-positive cells are concentrated in the MB. (B) Posterior view of a 48-hour pupal brain. A cluster of MB nuclei are indicated by the arrow. Additional staining was observed in the optic lobes. (C) Dorsal view of the CNS from a first instar larva. The arrow indicates the region where MB nuclei are clustered. Each of the unstained areas within the cluster of MB neurons represents the location of a MB neuroblast. (D) Lateral and slightly oblique view of a stage-15 embryo. The arrow indicates clusters of DAC-positive cells in the MB. DAC-positive cells appear purple (alkaline phosphatase detection). (E) Frontal optical section through one hemisphere of a wandering third instar larva. Anti-DCO antibody (red) labeled the cytoplasm of MB neurons. The MB processes extend from the cell bodies into the calyx (c). Anti-DAC antibody (green) was detected within the nuclei of the MB neurons as well as in other non-MB cells. The insets show two MB neurons with DAC immunoreactivity in their nuclei and DCO immunoreactivity in the cytoplasm. Bar, 10 μm. (F-H) Optical section through the brain of a first instar larva fed with BrdU within 1 hour after hatching. (F) Anti-DAC staining (green). An MB nucleus is indicated by the arrowhead. (H) Anti-BrdU detection (red). The double-headed arrow indicates two MB neuroblasts, identified by their large nuclei. The arrow indicates a ganglion mother cell, identified by its location adjacent to the NB and by its smaller size. The unstained area within the lower left NB nucleus is the nucleolus. (G) Merged images of F and H. Note the near absence of DAC staining in the nuclei of the neuroblasts and ganglion mother cells relative to the MB neurons. Bar, 10 μm.

the wild type, the α lobe is thick and straight and is wrapped by the α′ lobe (Fig. 3J). In *dac* mutants, the dorsal projection was severely reduced in size and exhibited a posterior slant, giving the impression that either α or α′ was missing (Fig. 3K). Of the horizontally oriented lobes, γ is the most dorsal and anterior, β is the most ventral, and β′ is sandwiched between the two (Fig. 6B). In wild-type animals, these lobes are readily distinguishable (Fig. 3A). In *dac* null animals, the horizontal lobes appeared grossly disorganized (Fig. 3B). The β, β′ and γ lobes were not distinguishable as individual lobes in either frontal or sagittal sections. In more anterior sections where only the γ lobe is present, the *dac* mutants exhibited irregularities of the neuropil edges, again suggestive of a disregard to boundaries (3F,G). The disorganization of the horizontal lobes and the reduced vertical lobes are completely penetrant phenotypes, others are partially penetrant (Table 1).

The MB were not the only brain region disrupted in *dac* mutants; other regions of the brain were affected as well. The antennal lobe and much of the central complex appeared grossly normal in *dac* mutants, but the ellipsoid body (EB) of the central complex was notably disrupted. This structure appears as a circular hub with distinct lateral projections in wild-type animals (Fig. 5D). In *dac* mutants, the hubs were misshapen and seemingly fused with their lateral projections (Fig. 5F). The optic lobes, which consist of the lamina, medulla, lobula and lobula plate, are normally organized into a highly ordered array. In the mutants, the optic lobes were small and disorganized. Some of these effects may be secondary to the failure in retinal development and brain innervation.

Developmental onset of the mushroom body phenotypes

The growth of MB axons occurs in two phases. The first is the initial outgrowth of γ and α′/β′ axons and occurs throughout embryonic and larval development. The second begins early in pupariation when α/β neurons initiate axon outgrowth. Also during this second period, γ neurons retract their axons, which then regrow to help establish the adult MB morphology (Lee et al., 1999). We analyzed the MB of late stage *dac* larvae to determine whether DAC was required during both phases or just one. Only 5-10% of the *dac* larval brain hemispheres showed a marked size reduction of the α-type lobe, and the

Table 2. Developmental penetrance of reduced α-type lobes

	% of animals showing a reduced α-type lobe	
Genotype	3rd instar	Adult
yw; *dac*^{1}/*dac*^{3}	3.2% (8/248)	100% (24/24)
yw; *dac*^{1}/*dac*^{4}	8.9% (16/179)	100% (15/15)
yw; *dac*^{3}/*dac*^{4}	10.5% (8/76)	100% (24/24)
yw;+/+	0% (0/85)	0% (0/30)

The percentage of third instar larval hemispheres that showed a reduced α-type lobe is greatly reduced compared to adults of the same genotype. Values in parentheses are the fraction of animals showing the phenotype.

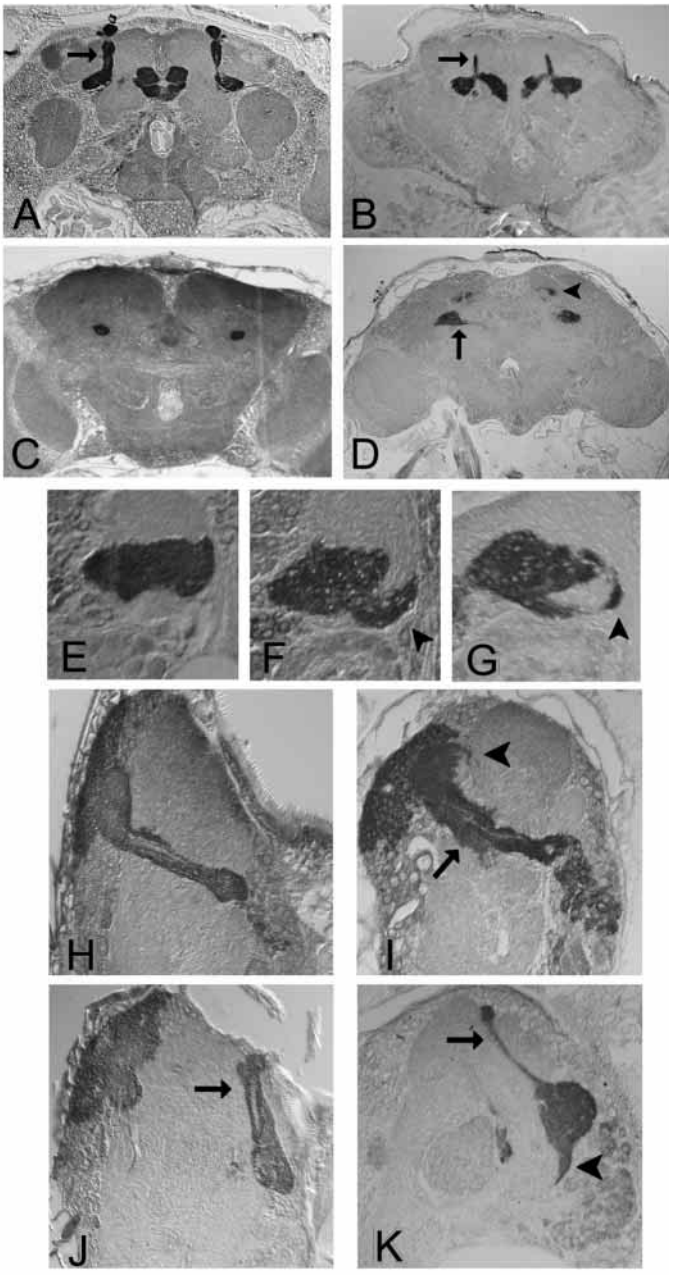

Fig. 3. Mushroom body phenotype of *dac* mutants**.** Paraffin sections of peri-eclosion adults stained with an anti-LEO antibody to reveal the architecture of MB processes. (A,C,E,H,J) Sections from control flies, (B,D,F,G,I,K) *dac* null mutants. (A,B) Frontal sections at the level of the α lobes in (A) Canton-S, (B) *yw; dac*^{1}*/dac*^{3}. Note that the α lobes (arrows) are severely reduced in size in *dac* null animals. (C,D) Frontal sections at the level of the peduncle in (C) Canton-S and (D) *yw; dac*^{1}*/dac*^{3} animals. The arrow in D indicates diffuse staining around the peduncle in the mutant, and the arrowhead to a cross-section of MB fibers dorsal to the peduncle in the mutant. (E-G) Frontal sections through the MB γ lobes in (E) Canton-S and (F,G) *yw; dac*^{1}*/dac*^{3} animals. Arrowheads indicate typical γ lobe aberrations observed in mutants. F shows a thick ventral projection and G a thin ventral projection enclosing non-MB neuropil. (H,I) Sagittal sections showing the calyx and peduncle in (H) *yw; +/+* and (I) *yw; dac*^{1}*/dac*^{4}. The arrow in J indicates fibers from the region of the calyx that grow along the peduncle, and the arrowhead fibers dorsal to the peduncle that emanate from the calyx (see also 3D). These fibers frequently appeared to grow toward the tip of the α lobe. (J,K) Sagittal sections through the α lobes of (J) *yw; +/+* and (K) *yw; dac*^{1}*/dac*^{4}. Arrows indicate α/α′ lobes. Note the greatly reduced size of the α lobe in the *dac* null animal. The arrowhead in K points to an aberrant ventral projection from the γ lobe. The cross section of the horizontal lobes is enlarged compared to wild type.

defect was frequently unilateral (Fig. 4, Table 2). Not only was the phenotype dramatically less penetrant in larvae than in adults, it was also less severe. No aberrant projections were evident from the β-type lobe, the peduncle or the calyx. Thus, the MB phenotype in larvae at late stages is both less penetrant and less severe than in adults of the same genotype. This indicates that *dac* is required during the second phase of axonal growth.

DAC is required in the mushroom bodies for their proper development

The presence of brain phenotypes outside the MB in *dac* mutants prompted us to ask whether DAC is required in the MB for their development or whether the MB phenotypes could be secondary to other defects. We tested this in two ways. First, we used the GAL4-UAS system to drive DAC expression in the MB of *dac* null mutants (Brand and Dormand, 1995). If the MB defects were secondary to other brain defects, then expression of DAC in the MB would not be expected to provide rescue. Conversely, rescue of the MB phenotype would suggest that *dac* function in the MB is required for their proper development. GAL4^{OK107} is an enhancer detector line that exhibits highly preferred expression in larval and pupal MB as well as in the median bundle (Fig. 5G). When the enhancer detector was used to drive the expression in a *dac* mutant background of either of two different *dac* cDNAs, UAS-*dac*-7C1 or UAS-*dac*-21 (Fig. 1A), the MB exhibited morphology very close to wild type (Fig. 5). This rescue was dependent upon temperature, reflecting the temperature-sensitivity of GAL4 activity (Table 3). Nevertheless, the rescue was not complete: the anterior part of the horizontal lobes was slightly disorganized. The incomplete rescue could be due to an improper level of DAC expression, inappropriate developmental onset of GAL4^{OK107}, or a requirement for more than one splice variant of *dac*. The *dac* mutant animals with

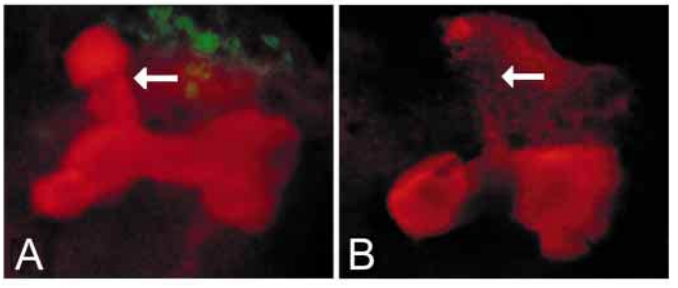

Fig. 4. Mushroom body phenotype of *dac* null larvae**.** Optical sections through the α-type lobe of (A) *yw*;+/+ and (B) *yw; dac*^{1}*/dac*^{3} third instar larvae. Mushroom body architecture was revealed by anti-DCO staining (red) and genotype was confirmed by anti-DAC staining (green). Each panel contains five stacked optical sections that span the entire α-type lobe. Note the reduction in size of the α-type lobe (arrows) in *dac* null larvae.

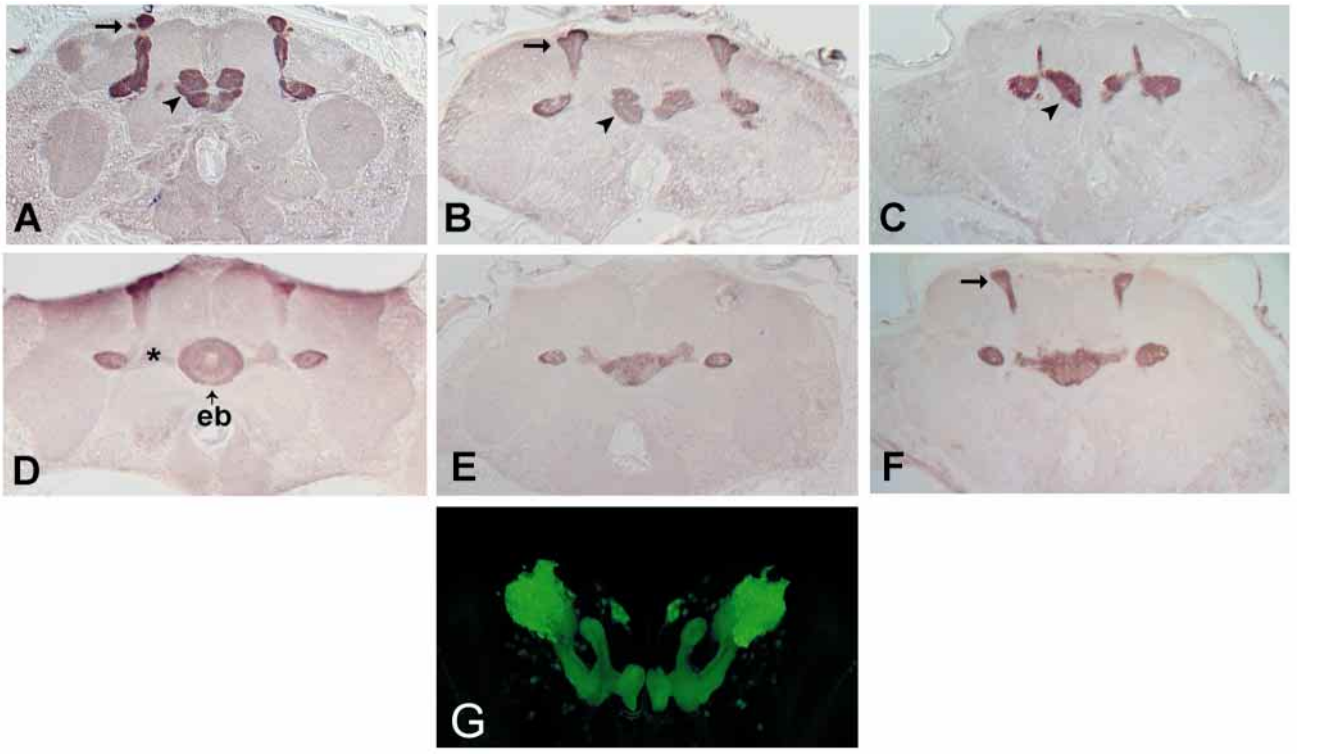

Fig. 5. Gal4 rescue of *dac* mushroom body phenotypes**.** All panels are frontal paraffin sections stained with an anti-LEO antibody to reveal MB and ellipsoid body (EB) structure. (A-C) show the α lobes and the distinct β and γ lobes; (D-F) are at the level of the EB. (A) Canton-S animal. The arrow indicates the tips of the α and α′ lobes, and the arrowhead the β and γ lobes. All lobes are readily discernable. (B) Animal of the genotype *w*; dac^1/dac^3 UAS-*dac*-7C1; $GAL4^{OK107}$. Note that the α and α′ lobes are clearly present and distinguishable (arrow). Note also the separation of the β and γ lobes into separate neuropil areas (arrowhead). (C) *yw;* dac^1/dac^3 animal. Note that the medial lobes are not distinct (arrowhead). Since the dorsal projections are directed posteriorly in *dac* mutants (Fig. 3K), the tips of these lobes appear in F (arrow). (D) Canton-S animal. The central portion of the EB (eb) is normally circular. The asterisk marks the left projection from the EB. (E) Animal of the genotype *w*; dac^1/dac^4 UAS-*dac*-21; $GAL4^{OK107}$. Note that the central portion of the EB appears disorganized, similar to the EB of the *dac* mutant (F). (F) *yw;* dac^1/dac^3 animal. Note that the EB is severely disorganized and there is no α lobe. (G) Confocal image of the expression of GFP in a UAS-GFP; $GAL4^{OK107}$ third instar larval brain. Note the highly preferential expression of GFP in the MB.

only the UAS-*dac* transgene showed an MB phenotype equal in character and severity to that of *dac* null mutants (Table 3). Interestingly, the MB defects were corrected by GAL4-driven *dac* expression in the MB, other brain defects remained. The optic lobes were small and disorganized and the ellipsoid body was misshapen as in *dac* mutants (Fig. 5D-F). Thus, *dac* expression in the MB rescues the MB defects, but not other structural defects in the brains of *dac* mutants.

In a second approach, we removed *dac* expression from the MB in an otherwise wild-type brain by creating mosaic animals. The mutant cells were positively labeled using the MARCM system (Lee and Luo, 1999; Lee et al., 1999). For this analysis, a P element in which the tubulin promotor drives GAL80 (tubP-GAL80) was placed in *trans* to either a *dac* null allele or a wild-type chromosome (Fig. 6A). GAL80 represses the transcriptional activation of the UAS-mCD8-GFP marker

Table 3. GAL4 rescue of *dac* mushroom body phenotypes

Genotype	Rescue of MB phenotype	
	25°C	18°C
yw; dac^1/dac^4, UAS*dac*-21; $GAL4^{OK107}$	7 full, 6 partial	1 full, 8 partial, 2 no rescue
yw; dac^3/dac^4, UAS*dac*-7C1; $GAL4^{OK107}$	1 full	None examined
yw; dac^1/dac^3, UASdac-7C1; $GAL4^{OK107}$	2 full	6 full, 5 partial, 2 no rescue
yw; dac^1/dac^4, UAS*dac*-7C1; $GAL4^{OK107}$	2 full, 1 partial	1 partial
yw; dac^1/dac^4, UAS*dac*-21	14 no rescue	None examined
yw; dac^1/dac^3, UAS*dac*-7C1	30 no rescue	None examined
yw; dac^1/dac^4, UAS*dac*-7C1	19 no rescue	None examined

The *dac* mutants that contain both *UAS-dac* and $GAL4^{OK107}$ show rescue of the MB phenotype. The rescue was observed with all *dac* alleles and both splice variants of the *dac* mRNA. Rescue was increased at 25°C due to *dac* expression driven by increased GAL4 activity. Control animals containing only the UAS*dac* element failed to exhibit rescue. Full rescue is defined as having normal MB morphology (presence of α and α′ lobes and organization of horizontal lobes into separate neuropil areas), except for the minor disorganization of the γ lobe described in the text.

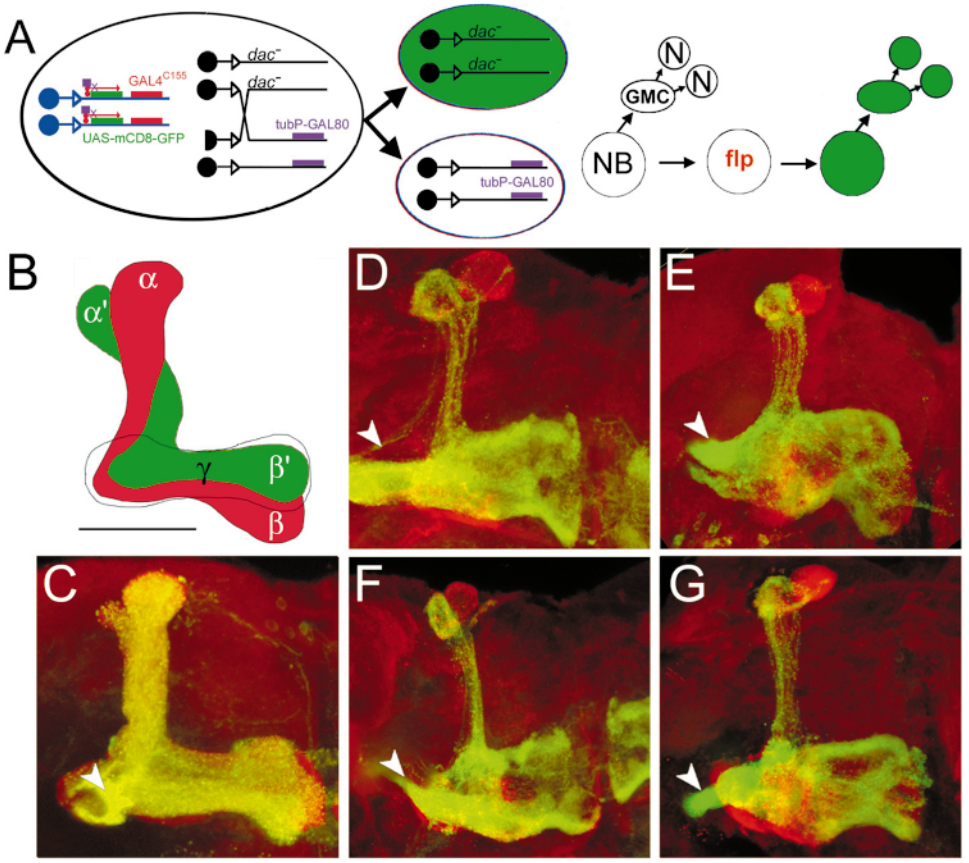

Fig. 6. Mosaic analysis of *dac* mushroom body phenotype. (A) The MARCM system and the generation of a neuroblast clone, (adapted from Lee et al., 1999). Heat-shock induced flippase (flp) activity produces somatic recombination at FRT sites (arrowheads), generating daughter cells without the GAL4-suppressor, GAL80. The neuron-specific GAL4 element, $GAL4^{C155}$, can then drive mCD8-GFP in neurons without GAL80. If this occurs in a neuroblast (NB), all descendants of the NB will express mCD8. GMC, ganglion mother cell; N, neuron. (B) Diagram of the αβ (red), α′β′ (green) and γ (outline) lobes showing that the α′ lobe wraps around the α lobe. Bar, 50 μm. (C-G) Maximum projection images reconstructed from confocal scans through the lobes of a control (C) and four dac^3 (D-G) neuroblast clones. The axons generated by the MB clones were identified by anti-mCD8 antibody staining (green). The MB structure was revealed by anti-DCO antibody staining (red). Note that dac^3 axons failed to contribute to α lobe neuropil and were disorganized in the horizontal lobes (compare D-G against C). The arrowheads at the tip of each heel in the panels show the junction to the partially visible peduncle.

that would normally be driven in all neurons by the neuron-specific driver, $GAL4^{C155}$. Upon induction of a heat-inducible *flp* recombinase, mitotic recombination can result in the loss of GAL80 from one of the daughter cells, allowing the expression of the UAS-mCD8-GFP marker specifically in the cells of the clone (Fig. 6A). An MB neuroblast clone induced in early first instar larvae will produce all three types of MB neurons and represent approximately one quarter of the MB, since each MB develops from four NB and each contributes MB neurons that become part of all lobes (Ito et al., 1997; Lee et al., 1999).

We generated NB clones in the MB of *dac* heterozygotes by heat shock just after hatching, as depicted in Fig. 6A. The brains of the resulting adult animals were then stained with anti-mCD8 to reveal the *dac* mutant axons and anti-DCO to visualize the overall MB neuropil, but primarily the tips of the α/α′ lobes where DCO staining is highest. We generated clones in control animals in a similar way, substituting the *dac* chromosome with a normal second chromosome. In each of the 11 control MB NB clones that we examined, the clonal axons were found distributed among all five lobes of the MB (Fig. 6C). Thus, the descendents of a normal MB NB invade and fill all neuropil compartments of the MB rather homogeneously. In contrast, *dac* mutant axons failed to fill the α lobes in 28 *dac* clones. This defect was not due to death of the α/β neurons, since control and mutant neuroblast clones contained the same number of neurons as determined by cell counts (control=313±100, *n*=5; *dac* mutant=354±43, *n*=9). The four examples shown (Fig. 6D-G) illustrate that despite the α lobe defect, the axons do contribute to the α′ lobe. In addition, in contrast to the homogeneous filling of the horizontal lobes by normal axons (Fig. 6C), the *dac* axons fill these neuropils in a disorganized way with substantial variability between clones (Fig. 6D-G). Therefore, *dac* mutant axons fail to become properly organized in the horizontal lobes and to fill the α lobe properly.

Genetic interaction of *dac* and *ey*

Because *ey* is upstream of *dac* in eye development and is also preferentially expressed in the MB (P. Callaerts and V. Hartenstein, personal communication), we hypothesized that *dac* and *ey* might function in the same pathway for the development of MB. If so, a synergistic effect on the phenotype might be detected when both genes are mutant.

The dac^P allele exhibits a mild MB phenotype in homozygous adults and the ey^2 allele produces no apparent MB phenotype in homozygous adults. No difference between the double homozygotes of genotype $dac^P/dac^P; ey^2/ey^2$ and dac^P homozygotes was apparent (Fig. 7A-I). Nevertheless, the double mutants did show a genetic interaction in the eye. The eyes of ey^2 animals are variably reduced whereas dac^P animals have a consistent but less severe reduction in eye size. The eyes of the double mutants were consistently very small or absent (data not shown). We also examined dac^P homozygotes in combination with the more severe and lethal *ey* alleles, ey^{D1Da} and ey^{JD}. The MB of the $dac^P/dac^P; ey^{D1Da}/+$ animals and $dac^P/dac^P; ey^{JD}/+$ animals exhibited an MB phenotype similar in severity to dac^P homozygotes. In addition, we examined double heterozygotes of the severe *ey* alleles ey^{D1Da} and ey^{JD} in combination with each of the three *dac* null alleles. The animals of each genotype had normal MB and eyes. Thus, we

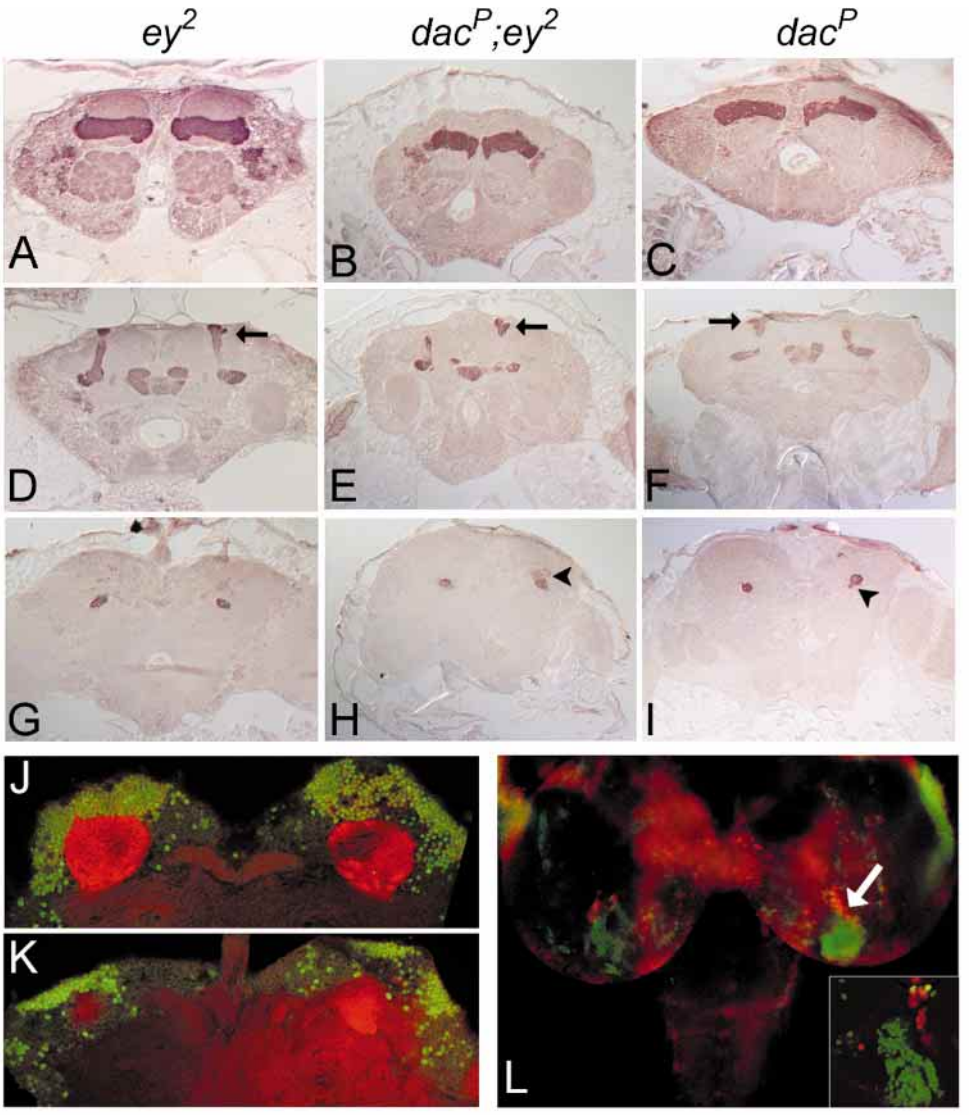

Fig. 7. No evidence of genetic interaction between *dac*^{P} and *ey*^{2}. (A-I) Frontal paraffin sections stained with an anti-LEO antibody. (A,D,G) *ey*^{2} homozygotes; (B,E,H) *dac*^{P}; *ey*^{2} double homozygotes; and (C,F,I) *dac*^{P} homozygotes. (A-C) Sections at the level of the anterior γ lobes. Note that both the *dac*^{P}; *ey*^{2} double homozygote and the *dac*^{P} homozygote have disorganized γ lobes. (D-F) Sections at the level of the α lobes. Note that both α and α′ lobes (arrows) are present in the *ey*^{2}, *dac*^{P}; *ey*^{2} and *dac*^{P} animals and these lobes appear normal in size. (G-I) Sections at the level of the posterior peduncle. Diffuse fibers surround the peduncle (arrowheads) in both the *dac*^{P}; *ey*^{2} and *dac*^{P} animals, but none are present in the *ey*^{2} animals. (J-K) Expression of DAC (green) and DCO (red) in (J) Canton-S and (K) *ey*^{JD} adults. DAC staining is apparent in *ey*^{JD}, although the number of MB neurons and the size of the MB neuropil is reduced. (L) Expression of DAC (green) and β-gal (red) in the CNS of a *so*^{7} third instar larva. Anterior is up, with both brain hemispheres and the ventral nerve cord visible. The region of *so* reporter expression is just anterior to the MB. Inset: Higher magnification showing *so* reporter expression anterior to that of DAC.

found no evidence for genetic interaction of *dac* and *ey* in MB development.

Since *ey* is genetically upstream of *dac* for eye development, we hypothesized that *ey* might be required for *dac* expression in the MB. To test this hypothesis, we stained sections of *ey*^{JD} and *ey*^{D1Da} homozygotes with anti-DAC and anti-DCO antibodies. Although the number of MB neurons was reduced in *ey*^{JD} and *ey*^{D1Da} homozygotes, the level of DAC immunofluorescence in each remaining MB neuron appeared equivalent to that of Canton-S animals (Fig. 7J,K).

Because *ey* directs *so* transcription in the *ey* disc and *so* is required for *dac* expression (Chen et al., 1997; Halder et al., 1998), an absence of *so* from the MB might explain the apparent inability of *ey* to direct *dac* expression. We examined the expression of a *so* reporter, *so*^{7}, in the central brain, using anti-β-gal immunofluorescence. The *so*^{7} reporter was expressed in cells just anterior to, but not in, the MB (Fig. 7L). The staining of *so*^{7} adult brains revealed the same pattern of β-gal expression, in neurons immediately anterior to the MB (data not shown).

Collectively, these results point to the likelihood that *dac* and *ey* participate in different aspects of MB development, unlike their unified role in retinal determination in the eye. This occurs in spite of the fact that *ey*, like *dac*, is preferentially expressed in MB and mutation of either results in abnormal MB development.

DISCUSSION

Our results describe a novel function for the retinal determination gene, *dachshund*, in mushroom body cell differentiation. Our data demonstrate that DAC is required autonomously within the MB for three major aspects of MB cell differentiation. First, DAC is required for MB neurites to respect their normal neuropil borders. Second, it is required for axons to arrange themselves properly within the horizontal lobes. Third, and most dramatically, DAC is required for MB axons to be able to fill the α lobe neuropil properly.

The mechanisms for *dac* involvement in these three aspects of MB cell differentiation are unknown, but we offer several hypotheses to account for the observations. DAC likely

functions in the MB to direct the transcription of genes involved in these processes. This idea is consistent with our data showing that DAC is expressed abundantly in the nuclei of post-mitotic MB neurons, but not in the MB NB nor the GMC. In addition, the role ascribed to DAC in retinal determination is that of a cofactor for transcriptional activation. These observations support a conserved biochemical role for DAC as a transcriptional modulator in MB neurons.

But how do the cellular phenotypes arise? The failure of *dac* MB neurites to remain confined to their normal territory suggests that DAC may regulate the expression of cell surface molecules involved in territory recognition. These could be cell adhesion molecules that sequester the processes and prohibit excessive growth. Or, they could be cell surface receptors involved in the recognition of repulsive signals from boundary neuropil or glia. Similar ideas underlie the possibilities for the disorganization of axons within the medial lobes. The normal organization presumably involves attractive and repulsive interactions among the individual axons and their terminals and the postsynaptic elements. An imbalance in these forces could account for the failure of *dac* MB axons to fill the horizontal lobes homogeneously. The loss of axons from the α lobe in *dac* mutants suggests more specific ideas. The neurons that send projections to the α lobe may never extend neurites, or may retract them in the absence of DAC function. Or, the cause may be within the realm of appropriate targeting and pathfinding. The neurites destined to fill the α lobe may take the wrong turn without appropriate guidance cues and invade the medial lobes. Irrespective of which mechanisms ultimately prove to be true, the identification of *dac* as an important regulator of MB cell differentiation offers a foothold through which to probe the molecular events underlying MB development.

When is DAC required for normal mushroom body cell differentiation? Although *dac* null adults had severe MB phenotypes, most mutant larvae had no discernable phenotype. The 6% of mutant larvae that showed a phenotype had a reduced α-type lobe but no other defects. From these data we conclude that the phenotypes in *dac* larvae are both less severe and less penetrant than in adults. Furthermore, because the larvae were dissected within 5 hours of puparium formation, the majority of the MB structural defects must occur during pupariation. At puparium formation, the MB consists of both γ and α′β′ neurons; the α/β neurons are added after pupariation (Lee et al., 1999). The lack of severity and penetrance of the larval MB phenotype along with the inability of *dac* axons to contribute to the α lobe lead to the attractive proposition that the αβ neurons are particularly sensitive to the loss of DAC function. This sensitivity explains the onset of the phenotype as well as the failure to observe α lobe projections in the mutants. Nevertheless, *dac* mutant larvae do exhibit an MB phenotype with low penetrance, so the gene must have some role, albeit minor, in the development of γ and α′/β′ neurons.

It is intriguing that a second retinal determination gene, *ey*, is also expressed preferentially in the MB and is required for their normal development. Although these genes operate in the same genetic pathway in the retina, we found no evidence for genetic interaction in mushroom body development. Indeed, their respective phenotypes are quite different. The *ey* mutations produce a dramatic reduction in the number of MB cells, potentially indicating a role for EY in MB cell survival, division or fate. In contrast, *dac* mutations have no discernable effect upon the number of MB cells produced but affect their post-mitotic differentiation. These observations suggest that *dac* and *ey* function independently in MB development. This conclusion is tempered by the fact that the *ey* alleles available are hypomorphs. However, consistent with independent roles, the two other retinal determination genes (*eya* and *so*) in the *ey/dac* pathway appear not to be expressed in the MB (Fig. 7L and Bonini et al., 1998).

Only a few genes have been identified so far that affect the overall development of mushroom bodies. The gene products of the mutants *mushroom body defect*, *mushroom bodies deranged*, *mushroom bodies miniature* and *short stop*, remain unknown (Lee and Luo, 1999; Heisenberg, 1985). The *linotte* gene has been shown to encode a receptor tyrosine kinase and *mushroom bodies tiny*, a protein related to P-21 activated kinase (Melzig et al., 1998; Moreau-Fauvarque et al., 1998). How these genes fit within the genetic cascade underlying MB development remains unknown. The identification of transcription factors and their accessory proteins, such as DAC and EY, that function early in the developmental hierarchy underlying MB development provides a starting point to dissecting the development of these important neural centers.

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