Homeotic gene action in embryonic brain development of *Drosophila*

Frank Hirth, Beate Hartmann and Heinrich Reichert*

Institute of Zoology, University of Basel, Rheinsprung 9, CH-4051 Basel, Switzerland
*Author for correspondence (e-mail: reichert@ubaclu.unibas.ch)

SUMMARY

Studies in vertebrates show that homeotic genes are involved in axial patterning and in specifying segmental identity of the embryonic hindbrain and spinal cord. To gain further insights into homeotic gene action during CNS development, we here characterize the role of the homeotic genes in embryonic brain development of *Drosophila*. We first use neuroanatomical techniques to map the entire anteroposterior order of homeotic gene expression in the *Drosophila* CNS, and demonstrate that this order is virtually identical in the CNS of *Drosophila* and mammals. We then carry out a genetic analysis of the *labial* gene in embryonic brain development. Our analysis shows that loss-of-function mutation and ubiquitous overexpression of *labial* results in ectopic expression of neighboring regulatory genes. Furthermore, this analysis demonstrates that mutational inactivation of *labial* results in regionalized axonal patterning defects which are due to both cell-autonomous and cell-nonautonomous effects. Thus, in the absence of *labial*, mutant cells are generated and positioned correctly in the brain, but these cells do not extend axons. Additionally, extending axons of neighboring wild-type neurons stop at the mutant domains or project ectopically, and defective commissural and longitudinal pathways result. Immunocytochemical analysis demonstrates that cells in the mutant domains do not express neuronal markers, indicating a complete lack of neuronal identity. An alternative glial identity is not adopted by these mutant cells. Comparable effects are seen in *Deformed* mutants but not in other homeotic gene mutants. Our findings demonstrate that the action of the homeotic genes *labial* and *Deformed* are required for neuronal differentiation in the developing brain of *Drosophila*.

Key words: Homeotic gene, Brain development, Neuronal fate, Axogenesis, *labial*, *Deformed*, *Drosophila*

INTRODUCTION

The homeotic genes encode a network of transcription factors that are implicated in the regulation of axial patterning in animals as diverse as insects and mammals. In insects, the homeotic genes of *Drosophila* have been studied in greatest detail (for recent reviews see McGinnis and Krumlauf, 1992; Manak and Scott, 1994; Duboule and Morata, 1994; Carroll, 1995).

In *Drosophila*, there are eight homeotic genes located in two chromosomal complexes, the Antennapedia Complex and the Bithorax Complex, and these genes are expressed along the anteroposterior body axis in a spatial order that is colinear with their arrangement in the chromosomal complexes. Genetic analysis of homeotic gene function in ectodermal and mesodermal tissue established the role of these genes in specifying segmental identity along the anteroposterior axis. The homeotic genes are expressed at the highest levels in the developing CNS and genes of the Bithorax Complex are known to act on neural tissue. However, virtually nothing is known about the role of these genes in embryonic brain development in *Drosophila*.

Most of the recent progress in understanding the role of homeotic genes in brain development comes from studies on vertebrates (reviewed by Krumlauf, 1994; Keynes and Krumlauf, 1994; Lumsden and Krumlauf, 1996; Maconochie et al., 1996). The vertebrate homeotic (Hox) genes are arranged in four paralogous chromosomal clusters, and these genes are colinear in their chromosomal arrangement and their patterns of expression along the anteroposterior embryonic axis. Genetic studies indicate that the *Hox* genes are involved in axial regional specification and patterning and suggest that a major site of their action is in the rhombomeres of the hindbrain and the spinal cord.

Here we analyse the role of homeotic genes in patterning the embryonic brain of *Drosophila*. Using molecular neuroanatomical techniques, we map the expression domains of all of the homeotic genes in the embryonic CNS, and show that the anteroposterior order of homeotic gene expression is virtually identical in the CNS of *Drosophila* and mammals. We then carry out a mutant analysis and demonstrate that two of the homeotic genes, *labial* and *Deformed*, are essential for regionalized axonal patterning in the developing brain. Furthermore we show that regionalized axonal patterning defects in the mutant brains are due to cell-autonomous and cell-nonautonomous effects; mutant cells do not extend axons and axons of wild-type neurons do not enter the mutant domain. Remarkably, cells in the mutant domain do not express

neuronal markers, indicating a complete lack of neuronal identity in the affected cells.

MATERIALS AND METHODS

Fly stocks

The wild type was Oregon-R. Mutant alleles were: lab^{a76}, lab^{vd1} (Merrill et al., 1989); pb^{10}, pb^{22}, pb^{27} (Bloomington stock center); Dfd^{w21}, Dfd^{r11} (Merrill et al., 1987); Scr^4, Scr^{a68}, Scr^{a72} (Pattatucci et al., 1991); $Antp^{73b}$ (Scott et al., 1983). Mutant alleles were balanced over TM3, Ubx-lacZ. Homozygous null mutants were identified by the absence of Ubx-lacZ. Mutant alleles of the Bithorax Complex genes were the deficiency line Df(P109) in which Ubx and abd-A are deleted, the double mutant Ubx^{9-22}, $Abd-B^{M1}$ and $abd-A^{M1}$, which is an amorph abd-A allele (Bloomington stock center); homozygous mutants were identified by the absence of anti-UBX or anti-ABD-A or anti-ABD-B immunoreactivity, respectively. To identify former lab-expressing tritocerebral cells in the labial mutant background, we used line 7.31 lab-lacZ/7.31 lab-lacZ; labvd1/TM3, hb-lacZ (Tremml and Bienz, 1992). Homozygous null mutants were identified by the absence of hb-lacZ. For comparison with the wild-type situation, 7.31 lab-lacZ was crossed back to wild type. 7.31 lab-lacZ shows cytoplasmic distribution of β-gal and reflects endogenous lab expression with additional ectopic expression patterns in the deutocerebral anlage. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

Heat-shock protocol

For ectopic overexpression of lab, we used the line p[w+ hs-lab] with a lab heat-shock construct homozygous on the X chromosome (Heuer and Kaufman, 1992). For ectopic overexpression of Dfd, we used the line p[hs-Dfd] with a Dfd heat-shock construct homozygous on the II chromosome (Kuziora and McGinnis, 1988). Embryos were collected on hard agar plates coated with a dab of yeast for 1 hour at 25°C. A precollection of embryos for 1 hour was obtained to ensure that adult females did not retain fertilized eggs. Embryos were then collected for 1 hour and allowed to age at 25°C until they reached stage 10/11, before administering a 20 minute heat shock at 37°C by immersing the plates in a water bath of the appropriate temperature. After heat shock, embryos were allowed to recover at 25°C for 3-4 hours until they were at about stage 14 of development and then fixed and labeled. Successful heat shock resulting in ubiquitous expression of labial or Deformed was examined by anti-LAB or anti-DFD immunostaining, respectively. Control animals were reared in parallel with the experimental animals under identical heat-shock condition.

Immunocytochemistry

Embryos were dechorionated, fixed and labeled according to Therianos et al. (1995). Primary antibodies were rabbit anti-HRP (FITC-conjugated) 1:100 (Jan and Jan, 1982) (Jackson Immunoresearch), mouse anti-EN 1:1 (Patel et al., 1989) (Developmental Studies Hybridoma Bank), rat anti-EMS 1:200 (Walldorf and Gehring, 1992), rabbit anti-LAB (Grieder et al., 1997), rabbit anti-PB (Pultz et al., 1988), rabbit anti-DFD (Kuziora and McGinnis, 1988), rabbit anti-SCR (LeMotte et al., 1989), each 1:200; mouse anti-ANTP 1:100 (Condie et al., 1991), mouse anti-UBX 1:5 (White and Wilcox, 1989) and rat anti-ABD-A 1:100 (Macias et al., 1990), mouse anti-ABD-B 1:1 (Celniker et al., 1989), rabbit anti-β-GAL 1:400 (Milan Analytika), mouse anti-β-GAL 1:100 (DSHB), mouse anti-Fasciclin II 1:5 (van Vactor et al., 1993), rat anti-ELAV 1:30 (DSHB), mouse anti-HB 1:5 (DiPietro and Patel, personal communication), mouse anti-PROS 1:4 (Spana and Doe, 1995), rabbit anti-ASE 1:500 (Brand et al., 1993), mouse anti-β-tubulin 1:5 (Chu and Klymkowsky, 1989), mouse anti-BP102 1:5 (Seeger et al., 1993) and rabbit anti-ISL 1:50 (Thor and Thomas, 1997). Secondary antibodies were Cy3-conjugated goat anti-mouse, Cy3-conjugated

goat anti-rabbit, Cy3-conjugated goat anti-rat, FITC-conjugated goat anti-mouse, FITC-conjugated goat anti-rabbit, FITC-conjugated goat anti-rat, DTAF-conjugated goat anti-mouse, DTAF-conjugated goat anti-rabbit and DTAF-conjugated goat anti-rat (Jackson Immunoresearch), all 1:250. Embryos were mounted in Vectashield H-1000 (Vector).

Laser confocal microscopy

For laser confocal microscopy, a Leica TCS 4D was used. Optical sections ranged from 0.8 to 2 μm recorded in line average mode with picture size of 512×512 pixels. Captured images from optical sections were arranged and processed using IMARIS (Bitplane). Figures were arranged and labeled using Adobe Photoshop.

RESULTS

Homeotic gene expression domains in the embryonic brain and ventral nerve cord

The embryonic CNS of Drosophila consists of the developing brain and the segmental neuromeres of the ventral nerve cord (VNC). The embryonic brain is divided into a supraesophageal anlage and a subesophageal anlage. The supraesophageal anlage comprises the protocerebral, deutocerebral and tritocerebral neuromeres; the subesophageal anlage is composed of the mandibular, maxillary and labial neuromeres (Hirth et al., 1995; Younossi-Hartenstein et al., 1996, 1997; Reichert and Boyan, 1997).

For a precise determination of expression patterns of all homeotic genes in the embryonic brain and VNC of Drosophila, we carried out immunocytochemical experiments and high-resolution laser confocal microscopy. In these experiments, double immunolabeling was used to relate homeotic gene expression to neuromeric borders (Hirth et al., 1995). To maximize spatial resolution, we focused on the phylotypic stage (Slack et al., 1993) where the germband is fully segmented and segment boundaries are identifiable morphologicaly and molecularly.

Fig. 1 shows the expression patterns for the homeotic genes labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx), abdominal-A (abd-A) and Abdominal-B (Abd-B) at embryonic stage 14 in relation to the CNS landmarks revealed by neuron-specific anti-HRP labeling (Fig. 1A) and to engrailed (en) expression domains (Fig. 1B), which delimit the posterior neuromere boundaries (Patel et al., 1989; Hirth et al., 1995). The relative position of expression of each homeotic gene was determined independently by double immunolabeling with anti-HRP (Fig. C-J) and by double immunolabeling with anti-engrailed (data not shown). A summary of these expression patterns is shown in Fig. 8A.

All five genes of the Antennapedia Complex are expressed in specific domains of the developing brain. The lab gene has the smallest spatial expression domain; it is only expressed in the posterior part of the tritocerebral anlage (Fig. 1C). This contrasts to previous reports that lab is expressed throughout the tritocerebral (intercalary) neuromere (Diederich et al., 1989; Mahaffey et al., 1989; Diederich et al., 1991). The pb gene has the largest anteroposterior extent of expression, however, in contrast to other homeotic genes, pb is only found in small segmentally repeated groups of 15-20 cells per neuromere. These groups of pb-expressing cells range from the

posterior deutocerebrum towards the end of the VNC (Fig. 1D). Since *pb*-expressing cells are found anterior to the *lab*-expressing cells in the brain, this is an exception to the spatial colinearity rule. (Spatial colinearity is conserved in the epidermis, where *pb* expression is posterior to *lab* expression; Pultz et al., 1988). The *Dfd* gene is expressed in the mandibular neuromere and the anterior half of the maxillary neuromere (Fig. 1E) and the *Scr* gene is expressed in the posterior half of the maxillary neuromere and the anterior half of the labial neuromere (Fig. 1F), confirming previous reports (Mahaffey and Kaufman, 1987; Carroll et al., 1988; Diederich et al., 1989; Mahaffey et al., 1989; LeMotte et al., 1989; Diederich et al., 1991). The *Antp* gene is expressed in a broad domain from the posterior half of the labial neuromere towards the end of the VNC (Fig. 1G). Expression of *Antp* in the labial neuromere of the brain was not reported previously (Beachy et al., 1985; Wirz et al., 1986; Carroll et al., 1986, 1988; Hayward et al., 1995).

The three genes of the Bithorax Complex are expressed in the VNC; our findings for the expression domains of these genes confirm earlier reports (Beachy et al., 1985; White and Wilcox, 1985; Carroll et al., 1988; Celniker et al., 1989; Karch et al., 1990). *Ubx* gene expression extends in a broad domain from the posterior half of the T2 neuromere to the anterior half of the A7 neuromere with highest expression levels in the posterior T3/anterior A1 neuromeres (Fig. 1H). The *abd-A* gene is expressed from the posterior half of the A1 neuromere to the posterior half of the A7 neuromere (Fig. 1I).

For the above mentioned genes, the anterior border of CNS expression remains stable from stage 11/12 until the end of embryogenesis. In contrast, the anterior border of CNS expression for the *Abd-B* gene shifts at stage 14. Before this stage *Abd-B* expression extends from the posterior half of neuromere A7 to the end of the VNC, afterwards it extends from the posterior half of neuromere A5 to the end of the VNC with the most intense expression localized to the terminal neuromeres (Fig. 1J). With the exception of the *Dfd* gene (see also Kaufman et al., 1990), the anterior limit of homeotic gene expression in the CNS (arrowheads in Fig. 1) is always parasegmental.

## Mutations in *lab* and *Dfd* result in axonal patterning defects in the brain

To investigate the functional role of the homeotic genes in embryonic brain development, we studied mutations in the five genes that are expressed in brain neuromeres namely *lab*, *pb*, *Dfd*, *Scr* and *Antp*.

In *lab* null mutants, marked defects in axonal patterning are associated with the tritocerebral neuromere. The longitudinal connectives that normally run through this neuromere (Fig. 2A) are missing or reduced (Fig. 2C, arrow). The tritocerebral commissure, which interconnects

the brain hemispheres at the level of the tritocerebrum (Fig. 2B, arrow), is completely absent (Fig. 2D, arrow). Moreover, the frontal connectives no longer project into the tritocerebral neuromere, rather they grow ectopically into the more anterior brain neuromeres (Fig. 2C, arrowhead).

In *Dfd* null mutants, comparable defects in axonal patterning are seen in association with the mandibular neuromere. The longitudinal pathways that normally project through this neuromere are missing or reduced (Fig. 2E, arrow). The mandibular commissure which interconnects the mandibular hemineuromeres (Fig. 2B, arrowhead) is completely absent (Fig. 2F, arrowhead).

In *Scr* null mutants, no gross abnormalities are seen in brain patterning (data not shown). Longitudinal and commissural pathways are present and appear normal. However, since the maxillary organs of the peripheral nervous system are duplicated in *Scr* null mutants, abnormal central projections of the axons from these ectopic sensory organs may exist. In *pb*
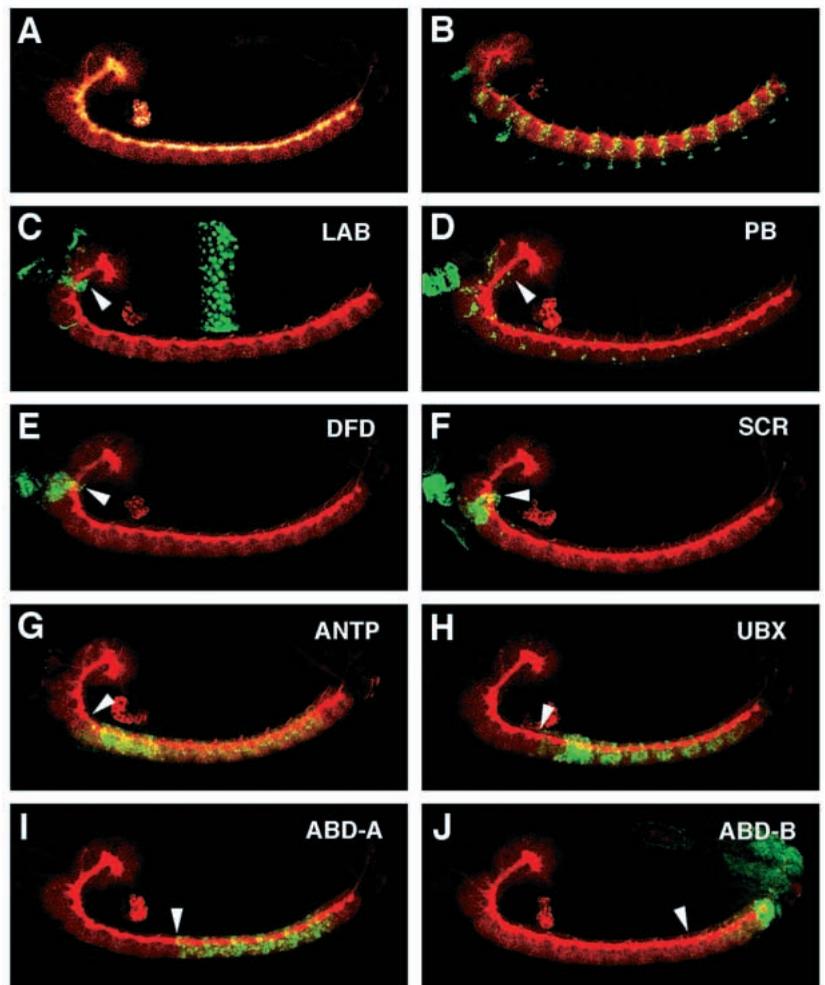
Fig. 1. Homeotic gene expression in the embryonic CNS. Laser confocal microscopy of wild-type stage 14 embryos, lateral views; reconstructions of optical sections. (A) Anti-HRP immunostaining reveals embryonic brain and VNC. (B) Neuromeric borders revealed by *en* expression; anti-EN (yellow/green) and anti-HRP (red). (C-J) Homeotic gene expression. Double labeling with homeotic gene-specific antibodies (yellow/green) and anti-HRP (red). Arrowheads indicate anterior expression borders of homeotic genes along neuraxis. (C) *lab*, (D) *pb*, (E) *Dfd*, (F) *Scr*, (G) *Antp*, (H) *Ubx*, (I) *abd-A*, (J) *Abd-B* .

Fig. 2. Axonal brain patterning defects in homeotic mutants. Laser confocal microscopy of stage 14 embryos, lateral views, embryos labeled with anti-HRP. Orientation as in Fig. 1. (A,C,E) Superpositions of optical sections; (B,D,F) single midline optical sections. (A,B) Wild type. (A) Arrow indicates longitudinal connectives; arrowhead indicates frontal connectives. (B) Arrow indicates tritocerebral commissure; arrowhead indicates mandibular commissure (cross-section). (C,D) *lab* null mutant. Defects are associated with the tritocerebral neuromere; longitudinal connectives are reduced (C, arrow), frontal connectives project ectopically (C, arrowhead) and the tritocerebral commissure is missing (D, arrow) whereas the mandibular commissure is present (D, arrowhead). (E,F) *Dfd* null mutant. Defects are associated with the mandibular neuromere; longitudinal connectives are reduced (E, arrow) and the mandibular commissure is missing (F, arrowhead) whereas the tritocerebral commissure is present (F, arrow) and the frontal connectives project correctly (E, arrowhead).
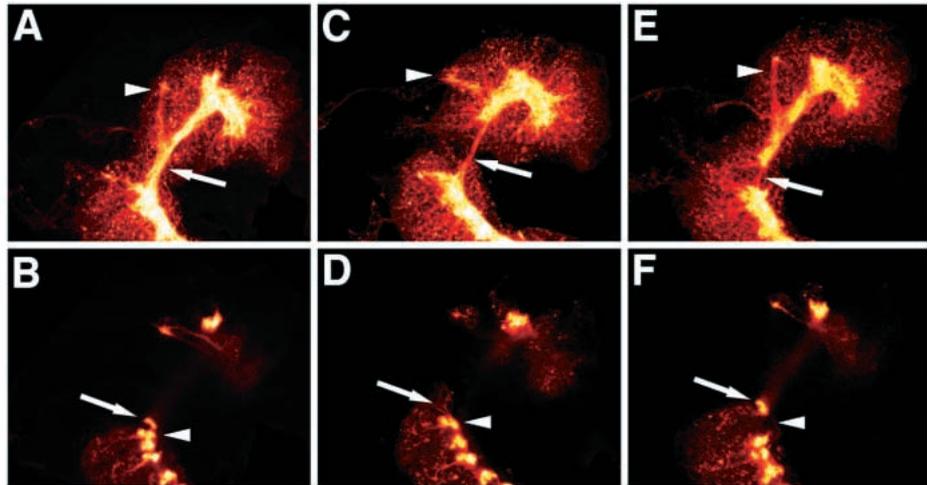
null mutants and in *Antp* null mutants, no gross morphological defects are seen in the embryonic brain (data not shown).

Although they are not expressed in the embryonic brain, we also studied the effects of mutations in the genes of the Bithorax Complex on the development of the embryonic CNS. No gross morphological defects were seen in the embryonic CNS in any of the single or double mutants (see materials and methods) of the *Ubx*, *abd-A* or *Abd-B* genes (data not shown).

Cells in the posterior tritocerebrum are generated in *lab* mutants

For a developmental genetic analysis of the observed brain patterning defects, we first focused on the *lab* gene and characterized the range of patterning defects seen in *lab* null mutants. While the tritocerebral commissure is always missing in *lab* mutants (Fig. 3B, arrowhead; compare to wild type in Fig. 3A), the longitudinal pathways are affected in a more variable manner. In weaker phenotypes, the longitudinal axon pathways are reduced in the tritocerebrum, but not absent (Fig. 3B arrows, compare to wild type in Fig. 3A; Fig. 3D arrow, compare to wild type in Fig. 3C). In contrast, in stronger phenotypes, the longitudinal axon pathways are missing altogether in the tritocerebrum (Fig. 3E, arrow) or reduced to very small ectopically projecting fascicles (Fig. 3F, arrow).

To determine if the axonal patterning defects in the *lab* mutant brains are due to a loss of cells in the mutant domain, we analysed transgenic flies in which a *lab-lacZ* reporter construct was introduced into a *lab* null mutant background (Tremml and Bienz, 1992). Expression of this reporter construct in a wild-type background reflects endogenous *lab* expression in the tritocerebrum, with an additional small ectopic expression domain in the deutocerebrum (data not shown). A comparison of endogenous *lab* expression in the wild type (Fig. 4A,B) with *lab-lacZ* reporter gene expression in *lab* null mutants (Fig. 4C,D) shows that the tritocerebral domain, which expresses *lab* in the wild type, is not deleted in the *lab* null mutant. In the mutant, the *lab-lacZ*-expressing cells have the same relative position in the brain as do the normal *lab*-expressing cells in the wild type. Despite the severe axonal patterning defects observed in this domain, mutant cells are

generated and appear to be properly positioned in the developing posterior tritocerebrum of the *lab* null mutant. Moreover, mutant cells in this domain remain present
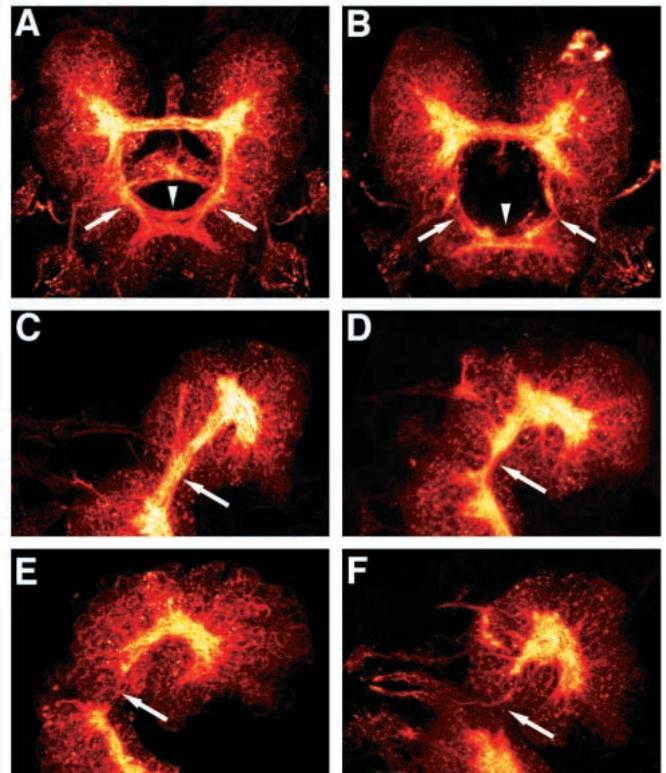
Fig. 3. Different degrees of axonal patterning defects in *lab* null mutants. Laser confocal microscopy of stage 14 embryos, reconstructions of optical sections. (A,B) Frontal views, anterior to top, (C-F) lateral views, orientation as in Fig. 1. (A,C) Wild type; (B,D-F) *lab* null mutant. In all embryos, the brain is labeled with anti-HRP (red/orange). In *lab* null mutants, the tritocerebral commissure is always missing (B, arrowhead; compare to A). Longitudinal axon pathways in the tritocerebrum are reduced (arrows in B and D), absent (arrow in E), or reduced to ectopically projecting fascicles (arrow in F, compare to C).
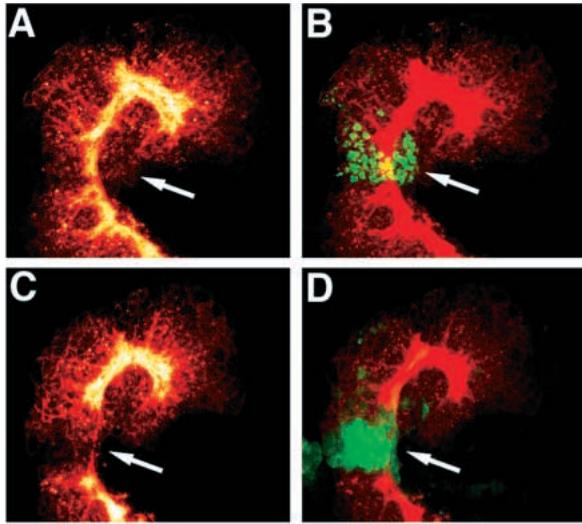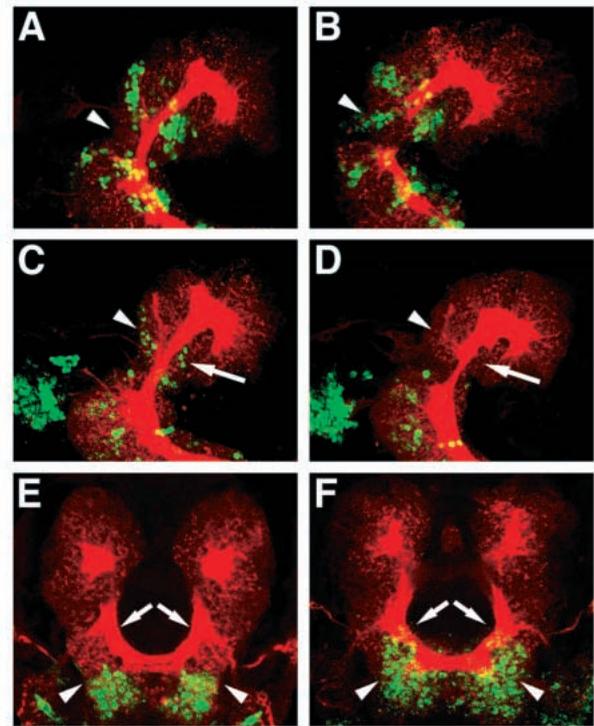
Fig. 4. The tritocerebral domain, which expresses *lab* in the wild type, is not deleted in *lab* mutants. Laser confocal microscopy of stage 14 embryos, reconstructions of optical sections. Lateral views, orientation as in Fig. 1. (A,B) Wild type, same preparation; (C,D) *lab* null mutant carrying a *lab-lacZ* reporter gene construct, same preparation. In all embryos, the brain is labeled with anti-HRP (red, orange). Double labeling (yellow/green) is with anti-LAB (B) or anti-β-GAL (D). Cells in the posterior tritocerebrum (A, arrow), which express *labial* in the wild type (B, arrow), are still present in *lab* mutants (C, arrow), as revealed by anti-β-GAL staining (D, arrow).

Fig. 5. Altered gene expression patterns in *lab* loss-of-function mutants and transgenic *hs-labial* flies. Laser confocal microscopy of stage 14 embryos, reconstructions of optical sections. (A-D) Lateral views as in Fig. 1; (E,F) frontal views with anterior to top. (A,C,E) Wild type; (B,D) *lab* null mutant; (F) transgenic flies carrying the *lab*-coding region under *hsp70* promoter control. In all embryos, the CNS is labeled with anti-HRP (red). Double labeling (yellow/green) is with anti-EMS (A,B), anti-PB (C,D), or anti-DFD (E,F). Cells in the posterior tritocerebrum that do not express *ems* in the wild type (A, arrowhead) show ectopic *ems* expression in *lab* mutants (B, arrowhead). Cells in the deutocerebrum and tritocerebrum that express *pb* in the wild type (C, arrow and arrowhead) lack *pb* expression in *lab* mutants (D, arrow and arrowhead). Cells in the tritocerebral domain that do not express *Dfd* in the wild type (E; delimited by arrows and arrowheads) show ectopic *Dfd* expression in *hs-lab* flies after heat shock (F; delimited by arrows and arrowheads).

throughout remaining embryogenesis. This suggests that the pattern of proliferation in the tritocerebrum is initiated correctly in the absence of the *lab* gene product, but that the cells that normally express *lab* might become incorrectly specified in the *lab* mutant leading to defects in axogenesis. It is noteworthy, that many of the *lacZ*-expressing mutant cells (Fig. 4D) are not labeled with the neuron-specific anti-HRP antibody (Fig. 4C), and that these mutant cells (Fig. 4D) are less compactly aggregated than in the wild-type domain (Fig. 4B). Since *lab-lacZ* reporter gene expression in the tritocerebral domain of *lab* null mutants continues until the end of embryogenesis, autoregulation of *lab* in the embryonic brain does not seem to occur.

Homeotic gene expression patterns are altered in *lab* mutants

To determine if the defects in *lab* loss-of-function mutants correlate with altered homeotic gene expression patterns in the tritocerebrum, we used molecular markers to study the expression of the homeotic genes *pb*, *Dfd* and *Scr* in *lab* null mutants (summarized in Fig. 8B). We also studied the expression of the *empty spiracles (ems)* gene (Dalton et al., 1989; Walldorf and Gehring, 1992) which in the wild-type brain is expressed in a large domain anterior to *lab* (Hirth et al., 1995).

In *lab* loss-of-function mutants, the *ems* gene is expressed ectopically in the tritocerebral domain in which *lab* is normally expressed (Fig. 5A,B); this ectopic *ems* expression occurs with 100% penetrance and ranges from 5-7 cells per hemisegment. (The tritocerebral location of the ectopic *ems*-expressing cells

was confirmed by co-localization of the *lab-lacZ* reporter construct in a *lab* null background; data not shown). The expression of *pb* disappears in the deutocerebrum and tritocerebrum of *lab* loss-of-function mutants but not in more posterior neuromeres (Fig. 5C,D). In contrast, the expression patterns of *Dfd* and *Scr* remain unaltered (data not shown). Thus, in the tritocerebral domain in which *lab* is normally expressed, two changes in regulatory gene expression occur, namely activation of *ems* and inactivation of *pb*.

To determine if ubiquitous overexpression of *labial* also alters regulatory gene expression patterns in the tritocerebral domain, we used transgenic flies carrying the *lab* gene under control of a heat-inducible promoter (Heuer and Kaufman, 1992). In these mutants, ubiquitous overexpression of *lab* following heat-shock (see Materials and Methods) results in ectopic expression of the posteriorly active *Dfd* gene in the posterior tritocerebrum (Fig. 5E,F; summarized in Fig. 8B);
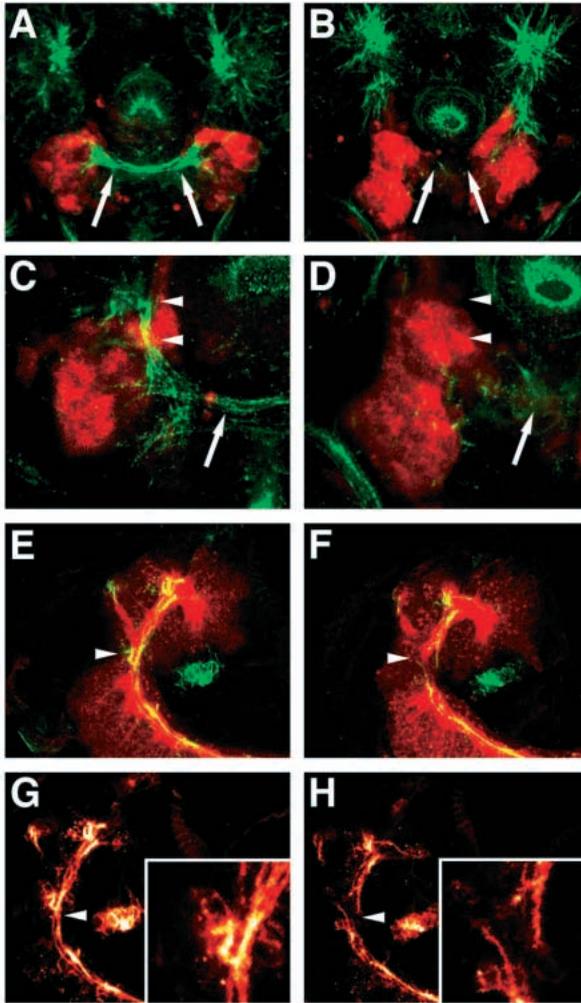
Fig. 6. Cell-autonomous and cell-nonautonomous axogenesis defects in *lab* mutants. Laser confocal microscopy of stage 14 embryos, reconstructions of optical sections. (A,B) Frontal views at the level of the tritocerebrum with anterior to top; (C,D) details of A,B at higher magnification; (E-H) lateral views with orientation as in Fig. 1. (A,C) Wild type with *lab-lacZ* reporter construct; (B,D) *lab* mutant with *lab-lacZ* reporter construct; (E,G) wild type; (F,H) *lab* mutant. (A-D) Double labeling with anti-βGAL (red) and anti-β-tubulin (yellow/green); (E,F) double labeling with anti-HRP (red) and anti-Fasciclin II (yellow/green); (G,H) Same embryos as in E,F, showing only anti-Fasciclin II staining (yellow/red), inset of the *lab* domain at higher magnification. In the wild type, anti-β-tubulin staining reveals commissural axons of *lab-lacZ*-expressing neurons within the tritocerebral commissure (A,C arrows) and longitudinal axons in the tritocerebral domain (C, arrowheads). In *lab* null mutants, the tritocerebral commissure is deleted (B,D, arrows) and no longitudinal axons project through the mutant domain (D, arrowheads). Longitudinal axons from other parts of the brain, which in the wild-type project through the tritocerebral *lab* domain (E,G, arrowhead), stop at the border of the mutant domain or avoid this domain and extend ectopically (F,H, arrowhead).

this occurred with 100% penetrance when the heat shock was given around embryonic stage 10/11. Expression patterns for *ems*, *pb* and *Scr* remained unchanged in these experiments (data not shown).

Cell-autonomous and cell-nonautonomous axogenesis defects in *lab* mutants

As shown above, in *lab* mutants the tritocerebral commissure does not form and longitudinal pathways from adjacent neuromeres are perturbed or absent. This suggests that mutation of the *lab* gene leads to cell-autonomous as well as cell-nonautonomous defects in brain axogenesis.

Evidence for cell-autonomous defects in axogenesis is provided by combining anti-tubulin immunocytochemistry together with *lab-lacZ* reporter gene expression. In the wild type, anti-tubulin immunostaining reveals the axons of the normal tritocerebral commissure that extend from the *lab-lacZ*-expressing neurons of the tritocerebrum (Fig. 6A,C arrows). Anti-tubulin immunostaining also shows longitudinal axon fascicles in the tritocerebral domain (Fig. 6C, arrowheads). In the *lab* null mutant, there are no labeled commissural axons projecting from the *lab-lacZ*-expressing neurons of the tritocerebrum (Fig. 6B,D arrows); the tritocerebral commissure is deleted. Moreover there are no labeled longitudinal axon fascicles in the mutant tritocerebral domain (Fig. 6D, arrowheads). We conclude that the cells in the mutant domain of the tritocerebrum do not generate commissural or longitudinal axons. Although there are no axons associated with the mutant domain, some 'microspike'-like tubulin labeling does appear there.

Evidence for cell-nonautonomous defects in axogenesis comes from the analysis of Fasciclin II-expressing descending and ascending axons from other parts of the brain. In the wild type, these axons project through the *lab* domain of the tritocerebrum in well-formed fascicles (Fig. 6E,G; arrowheads). In *lab* mutants, the fasciclin axons do not project through the mutant domain. They either stop at the border of the mutant domain or they avoid this domain and extend ectopically (Fig. 6F,H; arrowheads).

Loss of neuronal molecular markers in *lab* mutant cells of the tritocerebrum

The fact that the *lab* mutant cells do not extend axons suggests that these cells might not differentiate into neurons. To investigate this, we first determined whether cells with the characteristics of neuroblasts and ganglion mother cells (GMCs) are present in the mutant tritocerebral domain (labeled by *lab-lacZ* reporter gene expression). This is the case. Cells that have the characteristic position and morphology of wild-type *lab*-expressing neuroblasts, and which also express the neuroblast markers *hunchback* (Tautz et al., 1987) and *asense* (Brand et al., 1993), are present in the mutant tritocerebral domain (data not shown). Similarly, cells that have the characteristic position and morphology of wild-type *lab*-expressing GMCs, and which also express the GMC marker *prospero* (Doe et al., 1991; Vaessin et al., 1991), are also present in the mutant tritocerebral domain (data not shown).

We then studied the expression of several neuronal molecular markers in the posterior tritocerebral domain of wild-type and *lab* mutant embryonic brains. In these experiments, the position of the mutant tritocerebral domain was always determined by *lab-lacZ* reporter gene expression (data not shown).

The neuron-specific RNA-binding protein ELAV is expressed exclusively in neurons of the CNS beginning from the time of birth of the first neurons (Robinow and White,
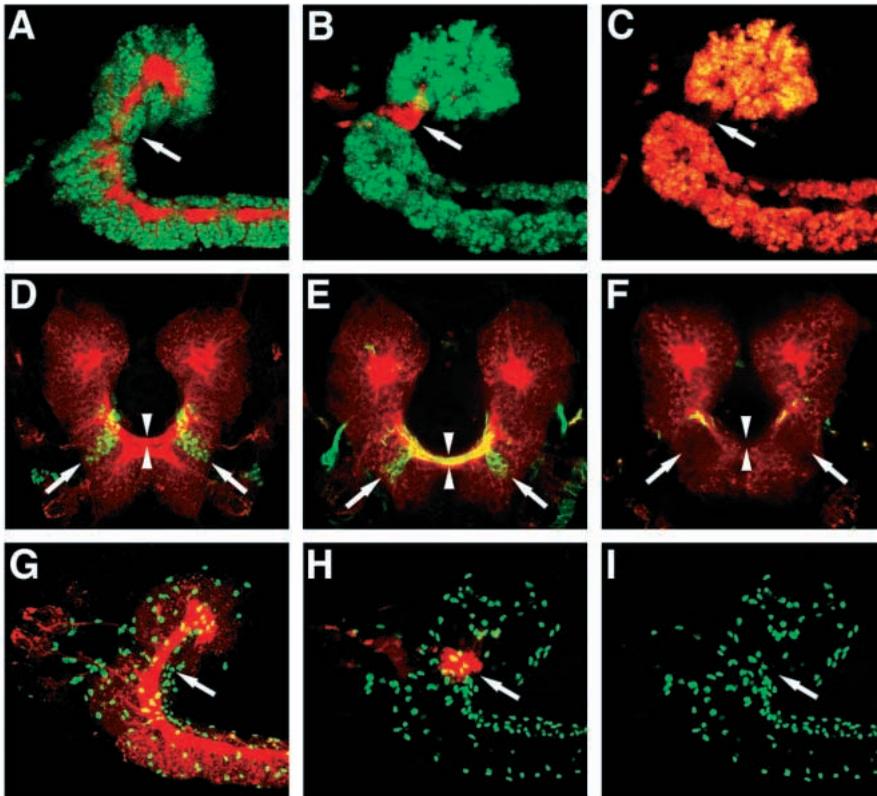
Fig. 7. Expression of ELAV, Fasciclin II and REPO in wild-type and *lab* mutant brains. Laser confocal microscopy of stage 14 embryos, reconstructions of optical sections. (A-C,G-I) Lateral views with orientation as in Fig. 1; (D-F) frontal views with anterior to top. (A,D,E,G) Wild type; (B,C,H,I) *lab* null mutant with *lab-lacZ* reporter construct; (F) *lab* null mutant. (A) Double labeling with anti-HRP (red) and anti-ELAV (green); (B) double labeling with anti-ELAV (green) and anti-β-GAL (red); (C) same embryo as in B, but with only anti-ELAV staining (yellow/red). (D-F) Double labeling with anti-HRP (red) and either anti-LAB (D, yellow/green) or anti-Fasciclin II (E,F, yellow/green). (G) Double labeling with anti-HRP (red) and anti-REPO (yellow/green); (H) double labeling with anti-REPO (yellow/green) and anti-β-GAL (red); (I) same embryo as in H, but with only anti-REPO staining (green). In *lab* mutants, the neuron-specific marker ELAV is missing in the *lab* domain (arrow in B and C) (compare to wild type, arrow in A). Fasciclin II, which in the wild type is expressed in the *lab* domain by a subset of neurons (arrows in E, compare to D) and their axons within the tritocerebral commissure (arrowheads in D,E), is absent in *lab* mutants (F, arrows and arrowheads). In *lab* mutants, the glia-specific marker REPO is present in the *lab* domain (arrow in H and I; compare to wild type, arrow in G).

1991; Koushika et al., 1996). In the wild type, anti-ELAV immunostaining reveals all of the neurons in the embryonic brain including the neurons in the tritocerebral *lab* domain (Fig. 7A). Anti-ELAV immunostaining is found in neuronal cell bodies and, in contrast to anti-HRP immunolabeling, does not stain the neuropil. In *lab* mutants, anti-ELAV immunostaining is no longer seen in any of the mutant cells in the posterior tritocerebral domain, but continues to be expressed in all of the other neuronal cells of the embryonic brain (Fig. 7B,C).

The cell adhesion molecule Fasciclin II (Grenningloh et al., 1991) is expressed in the wild type by a subset of the LAB-positive neurons in the tritocerebrum and by the axons that these neurons project into the tritocerebral commissure (Fig. 7E, compare to 7D). In *lab* mutants, none of the cells in the corresponding posterior tritocerebral domain and none of the axons in that region express Fasciclin II (Fig. 7F).

Immunocytochemical analysis shows that a number of other molecular labels are present in the tritocerebral *lab* domain of the wild type, but are absent in the corresponding tritocerebral domain of *lab* mutants (data not shown). Among these are the LIM-homeodomain gene *islet* (Thor and Thomas, 1997), the neuron-specific NAC epitope recognized by the anti-HRP antibody (Katz et al., 1988; Jan and Jan, 1982), the axon-specific BP102 epitope (Seeger et al., 1993) and the segment polarity gene *engrailed* (Patel et al., 1989).

Taken together, these findings imply that the *lab* mutant cells have not acquired a neuronal identity. This suggests that the *lab* mutant cells either fail to differentiate into neurons or adopt another cell fate such as that of glial cells. To determine if these cells do differentiate into glia, we studied the expression of the glial-specific *repo* gene (Campbell et al., 1994; Xiong et al.,

1994; Halter et al., 1995) in the *lab* mutant tritocerebral domain. Our results indicate that the pattern and number of *repo*-expressing cells in the tritocerebral *labial* domain is similar in the wild type (Fig. 7G) and in the *labial* null mutant (Fig. 7H,I). This indicates that the *lab* mutant cells have not acquired a glial identity. It also suggests that gliogenesis is not affected in the *lab* mutant domain. We therefore postulate that the *lab* gene is necessary for the establishment of correct neuronal cell fate, but not glial cell fate, in the part of the developing brain in which it is normally expressed.

Brain patterning defects in *Dfd* and *lab* mutants are comparable

The same type of genetic analysis that was perfomed on embryonic brain development in *lab* mutants was also carried out on *Dfd* mutants (data not shown). In all cases, comparable effects were seen in the *Dfd* mutants, the only major difference being that these effects were observed in the mandibular (and anterior maxillary) neuromere (summarized in Fig. 8B). The cells in the mandibular neuromere are generated in *Dfd* mutants. Homeotic gene expression patterns are altered in *Dfd* mutants; in the null mutant, *lab* is ectopically expressed in the mandibular segment in which *Dfd* is normally expressed. Ubiquitous overexpression of *Dfd* results in ectopic expression of the posteriorly active *Scr* gene in the *Dfd* domain. Cell-autonomous and cell-nonautonomous axogenesis defects occur in the *Dfd* mutants; mutant cells do not project axons, and descending and ascending axons from other parts of the brain do not project through the mutant domain. Finally, the *Dfd* mutant cells of the mandibular neuromere show a loss of neuronal markers such as ELAV, whereas the pattern and number of *repo*-expressing glial cells in the mutant domain is unaffected.

Fig. 8. (A) Schematic comparison of homeotic gene expression patterns in the developing CNS of *Drosophila* and mouse; expression domains are colour-coded. (Top) Homeotic gene expression in embryonic stage 14 *Drosophila* CNS. Borders of the mandibular (S1), maxillary (S2), labial (S3) and VNC neuromeres are indicated by dotted lines; only part of the anterior brain is shown. (Bottom) *HoxB* gene expression in embryonic day 9.5-12.5 mouse CNS based on Graham et al., 1989, Wilkinson et al., 1989 and Hunt et al., 1991. Borders of the rhombomeres are indicated by dotted lines. Note the conserved anteroposterior order of homeotic gene expression. (B) *Drosophila* brain phenotypes in *lab* and *Dfd* loss-of-function mutants and transgenic *hs-lab* and *hs-Dfd* flies; simplified scheme of the deutocerebral (b2), tritocerebral (b3) and mandibular (S1) neuromeres. *en*-expressing cells separating the neuromeres are represented by dots; longitudinal and commissural pathways by bars and arrows. In the wild type *(wt)*, cells in the deutocerebrum and anterior tritocerebrum express *ems* (yellow), cells in the posterior tritocerebrum express *lab* (dark blue) and cells in the mandibular neuromere express *Dfd* (red). In *lab* loss-of-function mutants *(lab⁻/⁻)*, the tritocerebral commissure is missing, tritocerebral longitudinal pathways are

missing or reduced and mutant cells in the tritocerebrum ectopically express *ems* (yellow). In transgenic *hs-lab* flies, cells in the posterior tritocerebrum ectopically express *Dfd* (red). In *Dfd* loss-of-function mutants *(Dfd⁻/⁻)*, the mandibular commissure is missing, longitudinal pathways are missing or reduced and mutant cells in the mandibular neuromere ectopically express *lab* (dark blue). In transgenic *hs-Dfd* flies, cells in the mandibular neuromere ectopically express *Scr* (light blue).
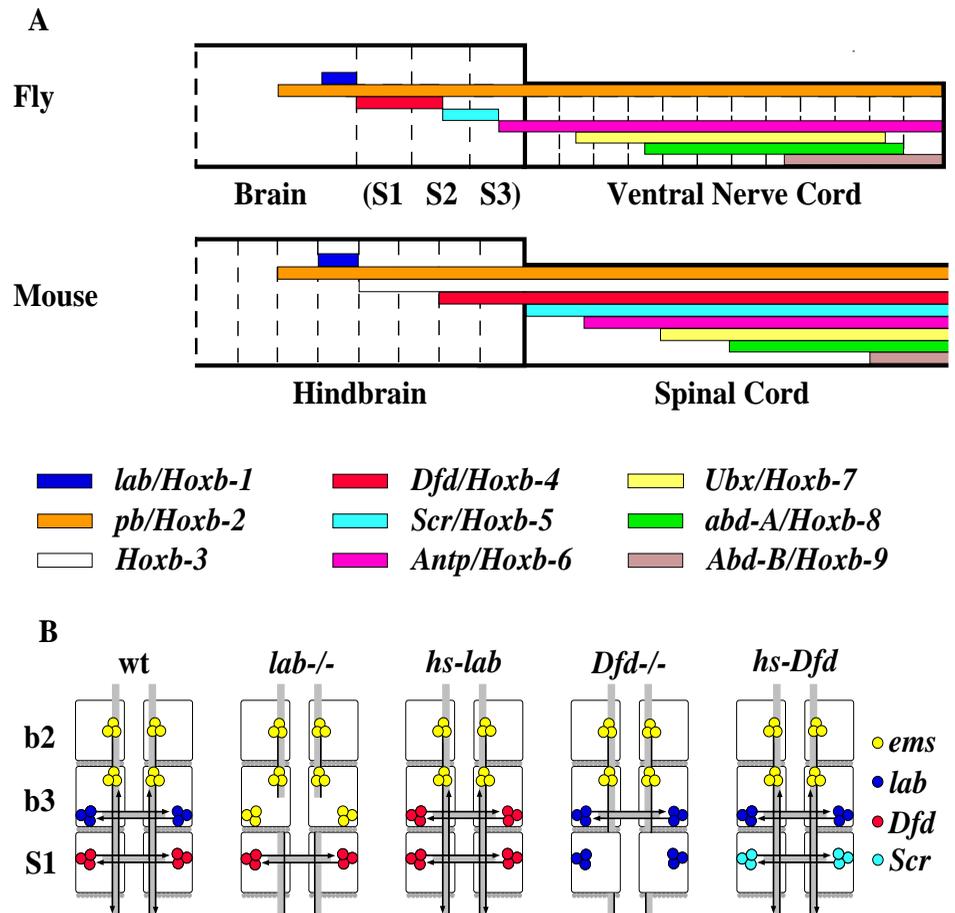
DISCUSSION

Evolutionary conservation of homeotic gene expression patterns in embryonic brain development

In this report, we use molecular neuroanatomical techniques to map the expression domains of all of the homeotic genes in the embryonic CNS of *Drosophila*. In Fig. 8A, we compare homeotic gene expression in the *Drosophila* CNS with homologous homeotic gene expression in the mouse CNS. As only the vertebrate *HoxB* gene complex contains homologs of all of the *Drosophila* homeotic genes, our data from embryonic stage 14 *Drosophila* are compared with those determined for the *HoxB* gene complex in embryonic day 9-12.5 mouse (Graham et al., 1989; Wilkinson et al., 1989; Hunt et al., 1991). This comparison shows that the relative anteroposterior order of homeotic gene expression is virtually identical in both cases. (The only exception is the *Hoxb-3* gene, which has no homolog in *Drosophila*.) The same degree of conservation also holds for a comparison of homeotic gene expression patterns in the embryonic brain of *Drosophila* and man (Vieille-Grosjean et al., 1997).

Although the relative order of homeotic gene expression is the same in the CNS of *Drosophila* and mouse, the relative

extent of homeotic gene expression is in many cases different. With the exception of *Hoxb-1*, most of the homeotic genes in the mouse CNS are expressed in very broad overlapping domains that extend posteriorly. In the fly CNS, this is the case for *pb*, *Antp*, *Ubx*, *abd-A* and *Abd-B*. However, it is not true for *lab*, *Dfd* and *Scr*, which all have discrete, non-overlapping expression domains.

Interestingly, in the CNS of both fly and mouse, the criterium of spatial colinearity of homeotic gene expression in the embryo and of gene arrangement on the chromosome is not strictly fulfilled. This is because the anterior expression boundary of *lab/Hoxb-1* is posterior to that of *pb/Hoxb-2*. In contrast, in embryonic epidermal structures of *Drosophila*, spatial colinearity of homeotic gene expression is perfectly preserved (Kaufman et al., 1990). This means that the relative order of homeotic gene expression is more similar in the CNS of the fly and the mouse, than in the CNS and the epidermis of the fly.

Mutations in two homeotic genes cause regionalized axonal patterning defects in embryonic brain development

Previous genetic studies on general development in *Drosophila* have demonstrated cross-regulatory interactions among those

homeotic genes that have overlapping expression domains. For example, for *Antp* and *Ubx*, loss-of-function mutations result in homeotic transformations of epidermal structures towards an anterior identity. In contrast, for the genes *lab* and *Dfd*, loss-of-function mutations in embryos are accompanied by structural deficiencies but show no detectable transformation towards other segmental identities. The epidermal defects seen in *lab* and *Dfd* mutants are thought to be related to the absence of overlapping expression domains with other homeotic genes, so that there is no genetic 'backup' (reviewed by Kaufman, 1990; McGinnis and Krumlauf, 1992; Morata, 1993).

Given the non-overlapping expression patterns of the *lab* and *Dfd* genes in the embryonic brain, this may also apply to the brain-specific defects seen in *lab* and *Dfd* mutants. Loss-of-function mutations in *lab* and *Dfd* both cause similar regionalized axonal patterning defects in the embryonic brain; commissures in the affected neuromere do not form and longitudinal pathways are absent or reduced. None of these effects are observed in loss-of-function mutations in the genes *pb*, *Scr* and *Antp*, showing a functional heterogeneity among the homeotic genes in embryonic brain development of *Drosophila*.

Currently, it is difficult to assess the degree of functional heterogeneity that characterizes the actions of homologous vertebrate *Hox* genes in brain development. Indeed, even members of the same paralogous group of vertebrate *Hox* genes may have somewhat different roles. For example, in the mouse, loss of function of the *lab* homolog *Hoxa-1* results in the deletion of specific rhombomeres suggesting a role in hindbrain segmentation (Mark et al., 1993; Carpenter et al., 1993; Dolle et al., 1993). In contrast, loss of function of *Hoxb-1*, a paralog of *Hoxa-1*, indicates that this gene is involved in determining rhombomeric identity, because specific rhombomeric patterning is initiated properly but not maintained (Goddard et al., 1996; Studer et al., 1996).

Evidence for cross-regulatory interactions of homeotic genes in brain development

The cross-regulatory interactions observed in loss-of-function mutations of homeotic genes in *Drosophila* have also led to the identification of a functional hierarchy among the homeotic gene members, in that the more posterior acting genes impose their developmental specificity upon anterior acting genes. This has led to the concept of phenotypic suppression (reviewed by Duboule and Morata, 1994) and has also been demonstrated for the epidermal action of the *lab* gene on the *ems* gene (Macias and Morata, 1996).

Our finding that mutational inactivation of *lab* results in ectopic expression of the *ems* gene in the *lab* mutant domain of the tritocerebrum, implies that *lab* normally represses *ems* expression in this domain of the wild type. This indicates that phenotypic suppression of homeotic genes may also operate during brain development in *Drosophila*. Interestingly, comparable molecular evidence for cross-regulatory interactions has been reported for the *Hoxb-1*^{-/-} mutant phenotype in the mouse hindbrain, where rhombomere 2 molecular markers become ectopically expressed in rhombomere 4 (Studer et al., 1996).

Cross-regulatory interactions between homeotic genes have also been revealed using transgenic flies carrying a heat-shock construct where ectopic overexpression generally causes

posteriorly directed transformations (reviewed by Duboule and Morata, 1994). Our finding that ectopic overexpression of *lab* leads to changes in the expression pattern of *Dfd* suggests that similar cross-regulatory interactions may also occur in the developing brain. The fact that *lab* overexpression causes the more posteriorly active *Dfd* gene to become expressed in a more anterior domain suggests a posteriorizing transformation of the posterior tritocerebral neuromere identity. This is similar to results obtained in the developing chick hindbrain where *Hoxb-1* overexpression experiments also appear to cause a partial transformation of rhombomeric identity (A. Lumsden, personal communication).

The *lab* gene controls neuronal identity in its tritocerebral expression domain

Recent studies have shown that the *otd (orthodenticle)* and *ems* homeobox genes are essential for patterning anterior parts of the *Drosophila* brain (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Mutation of these genes results in dramatic brain patterning defects; neurons in the mutant domains are not generated causing 'gap-like' brain deletions. The analysis of the *lab* gene reported here shows that this homeotic gene acts differently. In the *lab* null mutant, the posterior tritocerebral cells are generated and positioned correctly in the developing brain and, in consequence, deletions are not observed in the *lab* mutant domain. This suggests that metameric patterning and cellular proliferation in the tritocerebrum are initiated correctly in the absence of the *lab* gene product.

Although loss of *lab* function does not result in neuromere-specific deletions, severe patterning defects in the posterior tritocerebrum do result, such as the absence of the tritocerebral commissure, defective longitudinal connectives and ectopic expression of *ems* (Fig. 8B). This appears to be comparable to what is seen in loss of *Hoxb-1* function in rhombomere 4 of the mouse; mutation does not result in rhombomere deletions but in incorrect axonal projections and ectopic expression of anterior molecular markers (Studer et al., 1996). At this level of analysis, the function of the *lab/Hoxb-1* genes in embryonic brain development is simlilar in both animal groups.

However, in addition to these similarities, there is also a striking difference in the roles of *lab* and *Hoxb-1* in embryonic brain development. In *Hoxb-1* mutants of the mouse, specific motor neurons are generated and differentiate in rhombomere 4. These neurons are, however, incorrectly specified and fail to migrate into their proper position resulting in an atypical motor nucleus and loss of the facial nerve. In contrast, in *lab* mutants of *Drosophila*, the cells that are generated in the posterior tritocerebral domain do not appear to differentiate into neurons. Our findings indicate that the neural progenitor cells, which give rise to these cells, are present and correctly located in the mutant tritocerebral domain. Furthermore, our results indicate that the postmitotic progeny of these progenitors, cells that are fated to become neurons in the wild type, are also generated and correctly located in the mutant tritocerebral domain. Indeed, they remain present and correctly located in the mutant embryonic brain throughout subsequent embryogenesis and do not appear to be eliminated by cell death. However, these cells do not extend axons and are not contacted by axons from other parts of the brain. Furthermore, they do not express any of the neuronal molecular markers that positionally equivalent neuronal cells express in the wild type. This indicates that the

lab mutant cells in the brain do not acquire a neuronal identity, nor do they appear to adopt an alternative glial identity. In contrast to the absence of neuronal cell fate in the *lab* mutant domain, the generation of glial cells within this mutant domain appears to be unaffected. This indicates that the expression of the homeotic *lab* gene is necessary for neurons, but not glia, to adopt their proper differentiated cell fate in the developing tritocerebral domain of the embryonic brain. We conclude that appropriate homeotic gene action is essential for the establishment of neuronal identity in this part the *Drosophila* brain.

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