Homeotic gene action in embryonic brain development of *Drosophila*

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**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*^{ab76}, *lab*^{abd1} (Merrill et al., 1989); *pb*^{b108}, *pb*^{b42}, *pb*^{b27} (Bloomingtom stock center); *Dfd*^{w21}, *Dfd*^{w11} (Merrill et al., 1987); *Scr*^{4}, *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(F1)109* in which *Ubx* and *abd-A* are deleted, the double mutant *Ubx*^{w22}, *Abd-B*^{MI} and *abd-A*^{MI}, 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, hh-lacZ* (Tremml and Bienz, 1992). Homozygous null mutants were identified by the absence of *hh-lacZ*. For comparison with the wild-type situation, 7.31 *lab-lacZ* was cross 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 X 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 Theanis 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, FITC-conjugated goat anti-mouse, FITC-conjugated goat anti-rabbit, FITC-conjugated goat anti-rabbit, DTAF-conjugated goat anti-mouse, DTAF-conjugated goat anti-rabbit and DTAF-conjugated goat anti-rabbit (Jackson ImmunoResearch), all 1:250. Embryos were mounted in Vectashield H-1000 (Vector).

**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 germ band is fully segmented and segment boundaries are identifiable morphologically and molecularly.

Fig. 1 shows the expression patterns for the homeotic genes *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*), *Ultrathorax* (*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-*en* (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...
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).
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);
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. 6A,C, 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, 1984).
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.
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 anterior-posterior 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.)

Although the relative order of homeotic gene expression is the same in the CNS of Drosophila and mouse, the 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	extsuperscript{−/−} 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 neurormere-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 similar 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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