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The *ladybird* homeobox genes are essential for the specification of a subpopulation of neural cells

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Abstract

In *Drosophila*, neurons and glial cells are produced by neural precursor cells called neuroblasts (NBs), which can be individually identified. Each NB generates a characteristic cell lineage specified by a precise spatiotemporal control of gene expression within the NB and its progeny. Here we show that the homeobox genes *ladybird early* and *ladybird late* are expressed in subsets of cells deriving from neuroblasts NB 5-3 and NB 5-6 and are essential for their correct development. Our analysis revealed that *ladybird* in *Drosophila*, like their vertebrate orthologous *Lbx1* genes, play an important role in cell fate specification processes. Among those cells that express *ladybird* are NB 5-6-derived glial cells. In *ladybird* loss-of-function mutants, the NB 5-6-derived exit glial cells are absent while overexpression of these genes leads to supernumerary glial cells of this type. Furthermore, aberrant glial cell positioning and aberrant spacing of axonal fascicles in the nerve roots observed in embryos with altered *ladybird* function suggest that the *ladybird* genes might also control directed cell movements and cell–cell interactions within the developing *Drosophila* ventral nerve cord.

Keywords: *Drosophila*; *Ladybird*; Homeobox; CNS; Identity specification; Glial cells

Introduction

The specification of unique cell fates as a function of their position within a developing tissue is a fundamental process for the development of multicellular organisms. The *Drosophila* central nervous system (CNS) is an ideal system to study the molecular mechanisms underlying cellular identity specification because of its genetic and molecular accessibility. The CNS develops from a ventral neuroectoderm as individual cells enlarge and delaminate into the embryo, where they will form a stereotyped array of 30 neuroblasts (NBs) per hemisegment, each NB having a unique identity depending on its position and the timing of delamination (Campos-Ortega and Hartenstein, 1984; Doe, 1992). Following its formation, each NB divides several times asymmetrically to generate several small-sized cells called ganglion mother cell (GMC). Each GMC will divide to form a pair of postmitotic neurons and/or glia (Hartenstein et al., 1987). Every NB generates a unique and invariant cell lineage producing multiple types of neurons and glia (Bossing et al., 1996; Schmidt et al., 1997; Udolph et al., 1993).

Cellular identity is defined by the position of the cell, its morphology and the molecular markers it expresses. In the *Drosophila* CNS, cellular diversity is generated by different groups of genes. Segment polarity genes, like *wingless* (*wg*) and *gooseberry* (*gsb*), divide the neuroectoderm along to the antero-posterior axis in four rows per hemisegment and confer the NBs a specific identity according to the row they belong to (Bhat, 1999; Chu-LaGraff and Doe, 1993; Skeath, 1999; Skeath et al., 1995). The columnar genes, like *ventral nerve cord defective* (*vnd*), divide the neuroectoderm along to the dorso-ventral axis in three columns and confer the NBs a specific identity depending on the column they are in (Chu et al., 1998; Skeath, 1999). Neuroblast identity genes like *runt*

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and *eagle* are expressed in the entire lineage of a subset of NBs (Dittrich et al., 1997; Duffy et al., 1991). Temporally regulated "sublineage genes" like *hunchback*, *Krüppel*, *pdm1/2*, *ming/castor* and *grainyhead* are sequentially expressed by the NBs, and the cells generated at a given time maintain the NB expression pattern (Brody and Odenwald, 2000; Cui and Doe, 1992; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002). These genes are involved in specifying lineage-specific cell identities according to birth order. For example, *hunchback* is required and sufficient to specify the identity of the postmitotic cells generated by early-born GMCs while *Krüppel* seems to be necessary for the neurons born thereafter. This property has been observed in different NB lineages and is independent of the cell type involved (Isshiki et al., 2001; Novotny et al., 2002).

In vertebrates, neurons and glia arise from dividing progenitor cells lining the lumen of the neural tube. Dorso-ventral specification of neuronal cell populations is mediated by a code of overlapping expression domains of b-HLH and homeodomain transcription factors. Initially, their expression is induced by two different signaling centers. Ventrally the notochord and the floor plate secrete the signaling molecule Shh and, dorsally the epidermal ectoderm and the roof plate express several members of the BMP family. Shh regulates the expression of a set of homeodomain proteins that comprises members of the Pax, Nkx, Dbx and Irx families (Briscoe et al., 2000; Pierani et al., 1999; Qiu et al., 1998). The overlapping sets of transcription factors that are expressed as a result of the induction by the organizing centers at different dorso-ventral positions might directly determine early classes of neurons. Most of the participating genes determine cell fates in dividing cells; some transcription factors however, such as Evx1, are expressed in postmitotic cells and have important roles in specifying neuronal identity (Moran-Rivard et al., 2001). Depending on their relative position in the notochord, interneurons can be divided in two different groups, dorsal or ventral. These groups can be further divided into different classes based on the expression of various transcription factors. Ventral interneurons V0, V1, V2 and V3 express *Evx1/2*, *En1*, *Lim3/Chx10* and *Sim 10* whereas dorsal interneurons D1a, D1b, D2, D3 and D0 are marked by the expression of *Lhx2*, *Lhx9*, *Isl1*, *Lim1*/*Lim2* and *Lbx1*, respectively (Liem et al., 2000; Moran-Rivard et al., 2001; Saueressig et al., 1999). Recent analysis of *Lbx1* mutant mice has revealed the role of *Lbx1* homeobox gene in cell fate specification of a subset of dorsal interneurons (Gross et al., 2002; Müller et al., 2002).

Here we analyze the expression pattern and neural functions of the *Drosophila* orthologs of *Lbx1*, namely *ladybird early* (*lbe*) and *ladybird late* (*lbl*) (Jagla et al., 1993, 1994, 1997b). We show that these two closely related homeobox genes are dynamically expressed in a subset of cells deriving from NB 5-3 and NB 5-6, determining the correct development of a subpopulation of glial and neuronal cells generated by these neuroblasts. The *lb* loss-of-function mutants are embryonic lethal. Their ventral nerve cord shows no gross abnormalities but the glial cell population is modified, notably characterized by the absence of a NB 5-6-derived exit glial cell in many hemineuromeres. Additional glial cells deriving from NB 5-6 are induced by ectopic neural expression of *lb* genes. Altogether, the highly restricted expression pattern and observed phenotypes strongly suggest that the *lb* genes, like their vertebrate homologs, play an important role in cell fate specification processes of a subpopulation of neural cells.

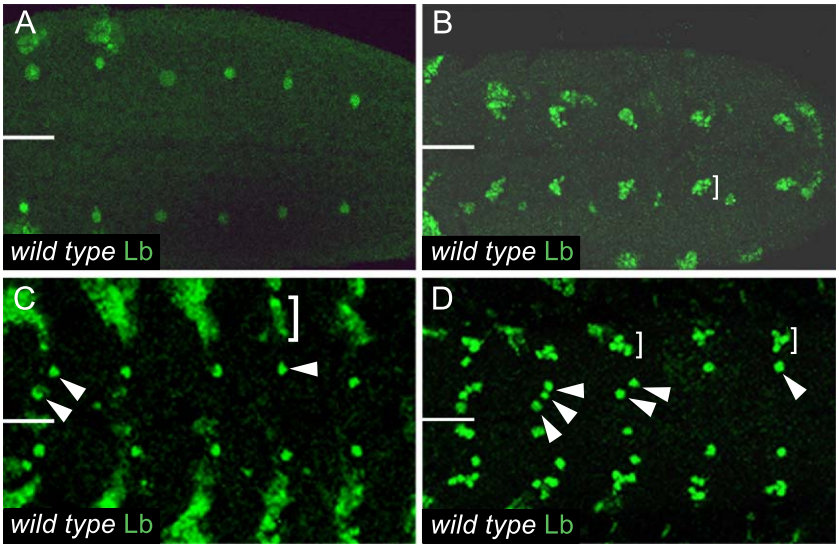
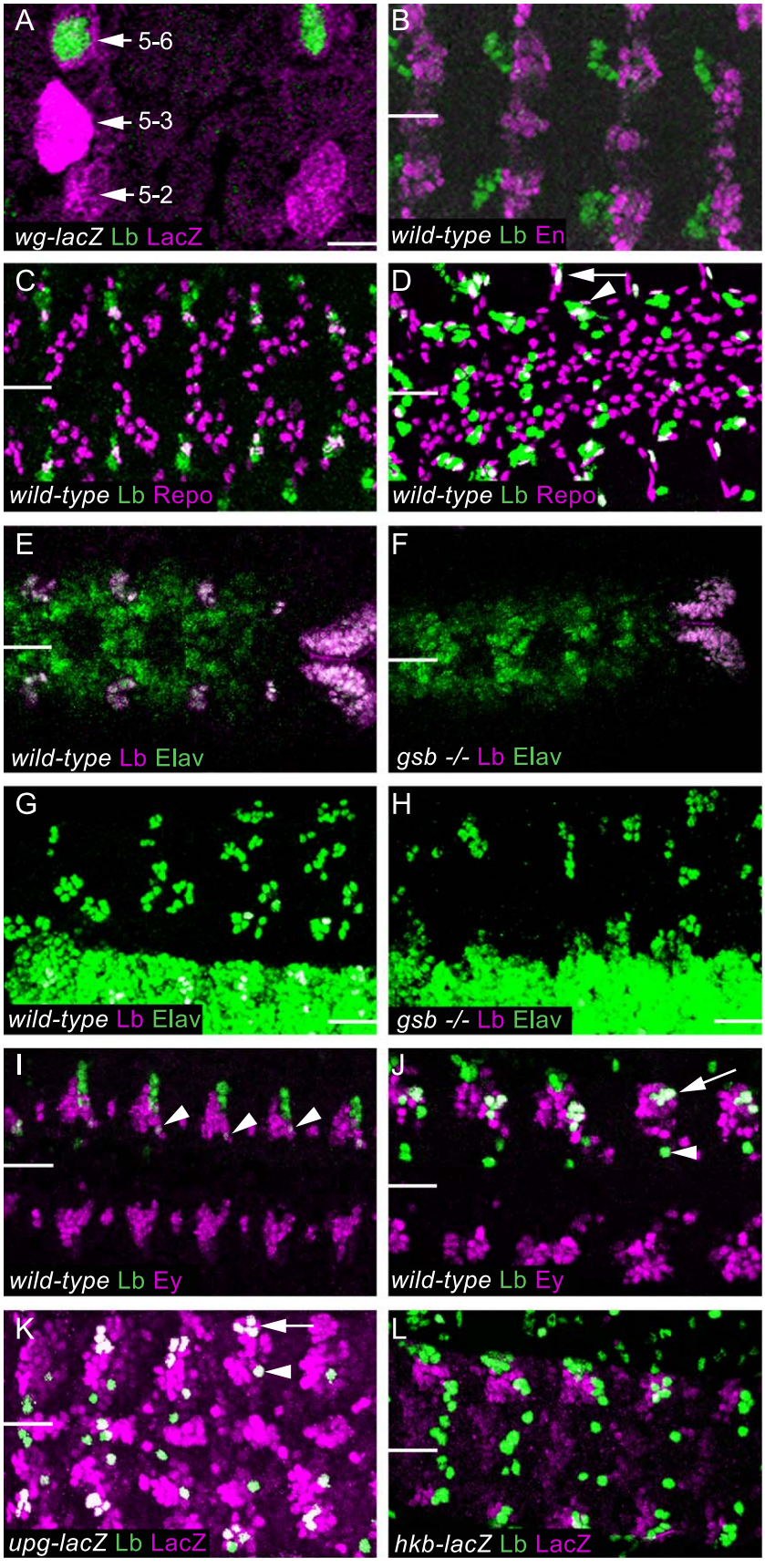


Fig. 1. The expression pattern of the *lb* genes is highly dynamic. (A–D) Ventral views of wild-type embryos stage 9 (A), 11 (B), 13 (C) and 15 (D) analyzed by immunohistochemistry using anti-Lb antibodies. The segments T1 to A2 are shown oriented anterior to the left, white horizontal bars indicate the ventral midline. Note the dynamic and segment-specific pattern of expression of the *lb* genes. Arrowheads point to the medio-dorsal neurons, outer brackets to the latero-dorsal cells.


A
5-6
5-3
5-2
wg-lacZ Lb LacZ
B
wild-type Lb En
C
wild-type Lb Repo
D
wild-type Lb Repo
E
wild-type Lb Elav
F
gsb -/- Lb Elav
G
wild-type Lb Elav
H
gsb -/- Lb Elav
I
wild-type Lb Ey
J
wild-type Lb Ey
K
upg-lacZ Lb LacZ
L
hkb-lacZ Lb LacZ

Materials and methods

Drosophila strains

The following *Drosophila* strains were used: w1118 as wild-type control; 10999 [*Df(gsb)*] from Bloomington Stock Center; *wingless-lacZ*, *unplugged-lacZ* and *huckebein-lacZ* (Doe, 1992); *Df(3L) H99* (White et al., 1994); *5820-GAL4* (*pGawB l(3) 31-1*$^{31\text{-}1}$*/TM6c Sb*^{1} *Tb*) from Bloomington Stock Center; *ladybird* loss-of-function mutants were obtained by crossing the following deficiency strains: *Df(3R) 3/1* obtained by our imperfect excision of the P5545 element inserted between the *C15* and the *S59* genes (Zikova et al., 2003) and *Df(3R) eF1* (Jagla et al., 1997a,b). The expression of the genes from the cluster 93E has been analyzed by immunohistochemistry using antibodies directed against Lbe, Tinman, Bagpipe and S59, and by in situ hybridization for *lbl* and *C15*. These experiments revealed that Df(3R)3/1 inactivates both *lb* genes as well as *C15* and *bagpipe*. To recover the contribution of *C15* to the CNS development, we crossed *Df(3R)3/1 to Df(3R) eF1* that uncovers the genes *tinman*, *bagpipe*, *lbl* and *lbe* but is rescued for *tinman*. The gene *bagpipe* being not expressed in the CNS (Azpiazu and Frasch, 1993), we assume that the observed phenotypes are specific for the loss of both *lb* genes.

Transgenic flies

The *K-lacZ* and *UAS-lb* transgenes are inserted on the second chromosome. Homozygous transgenics were crossed and the resulting females were crossed to a balancer in order to select a recombination event leading to a double-transgenic chromosome over *CyO*. After several genetic crosses, the *K-lacZ* transgene was introduced in the *Df(3L)3/1* background {*+/+;K-lacZ/K-lacZ;Df(3L)3/1/TM6c*} (Jagla et al., 2002).

Immunohistochemistry

Embryos were stained with the following primary antibodies: monoclonal anti-Lbe (1:20) (Jagla et al., 1997a,b); polyclonal anti-Lb (1:500) (Jagla et al., 1997a,b); monoclonal anti-BP102 (1:50) (DSHB); monoclonal anti-Elav (1:50) (DSHB); monoclonal anti-Cut (1:1000) (DSHB); monoclonal anti-Engrailed (1:20) (DSHB); rabbit anti-Eyeless (1:500) (provided by U. Walldorf); monoclonal anti-β-Galactosidase (1:500) (Sigma); rabbit anti-Repo 1:500 (provided by M. Noll); rabbit anti-β-Galactosidase (1:5000) (Cappel Laboratories); rabbit anti-Even Skipped (1:2000) (provided by M. Frasch); rabbit anti-pMAD (1:150) (provided by C.-H. Heldin); FITC conjugated affinity purified goat anti-horseradish peroxidase (1:200) (Jackson Immunoresearch Laboratories). Labeled cells were detected using the ABC-Elite-peroxidase kit (Vector Laboratories) with diaminobenzidine as substrate, or with secondary antibodies conjugated with FITC, Cy3 or Cy5 (Jackson). Whole-mount embryos were visualized using an axiophot Zeiss microscope under Nomarski optics or an Olympus FV300 confocal microscope.

Results

lb expression in the CNS is highly dynamic

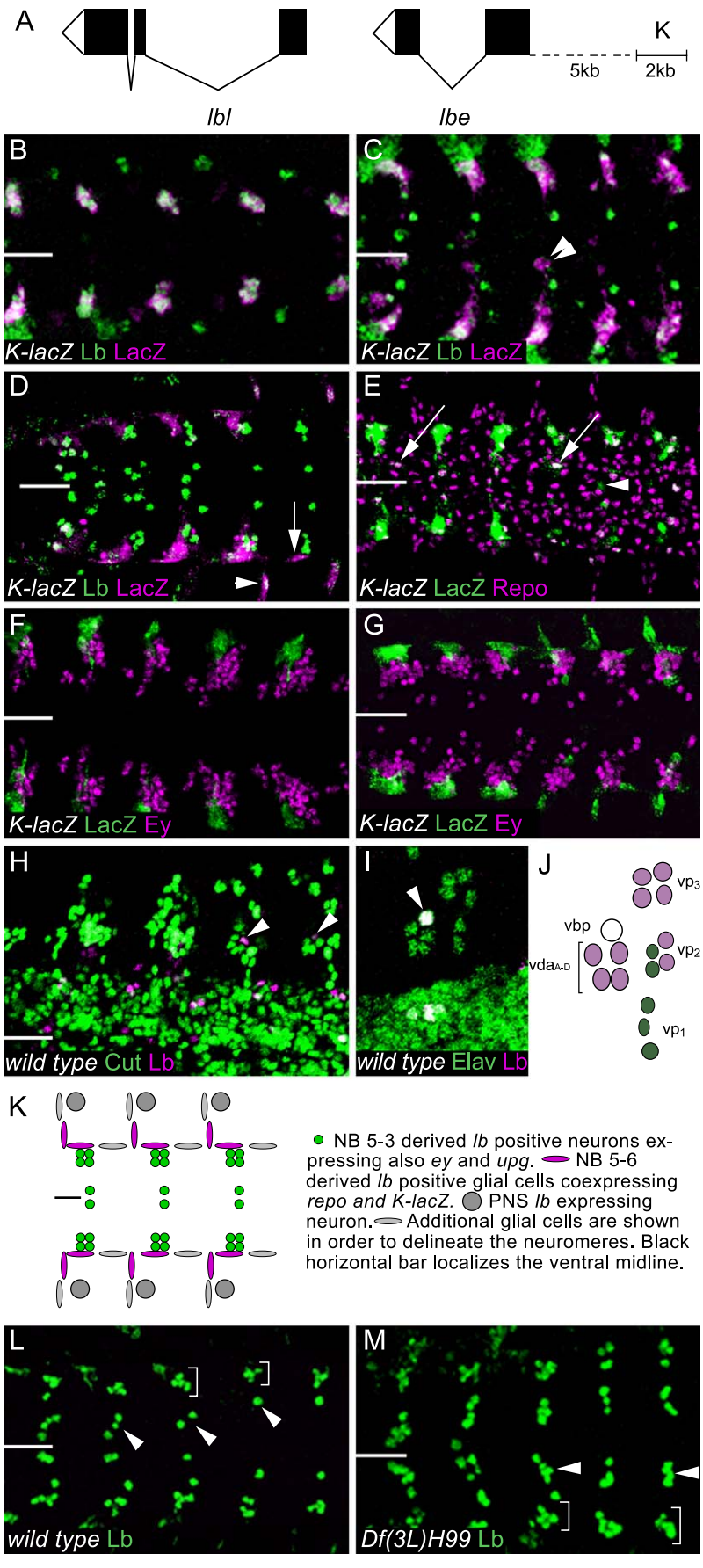
Both *lbe* and *lbl* are expressed during the development of the *Drosophila* embryo nervous system. Similar to their expression in the heart and muscle precursor cells, they are coexpressed in the same cells, *lbl* being transcribed a little later than *lbe* (data not shown). In the following, we will therefore refer generally to *lb* expression.

The first *lb* expression within the developing CNS is detected at stage 8/early 9 (stages according to Campos-Ortega and Hartenstein, 1984), in a single laterally lying neuroblast per hemineuromere that could be identified as NB 5-6 (Figs. 1A and 2A; see below). During stage 10 and 11, the number of *lb*-expressing cells increases rapidly. These cells remain lateral (Fig. 1B, outer bracket). At stage 12–13, additional *lb*-positive cells appear in a mediodorsal position, one in each abdominal hemineuromere, two in the third and three in the first and second thoracic hemineuromeres (Figs. 1C,D arrowheads). After stage 12, the number of the lateral *lb*-positive cells decreases progressively to reach a stable number of four (abdominal) and five (thoracic) at stage 14 (Fig. 1D, outer brackets). Three patterns of *lb*-expressing cells can be identified at this stage. In the thorax, five latero-dorsal cells are associated with three medio-dorsal neurons in T1–T2 vs. two in T3, the abdominal segments show four latero-dorsal and a single medio-dorsal *lb*-positive cell.

lb genes are expressed in cells of the NB 5-3 and 5-6 lineages

The first *lb* expression is detected in NB 5-6, which is identified by the expression of β-Gal in a *wg-lacZ* enhancer

Fig. 2. The *lb* genes are expressed in cells deriving from NB 5-6 and 5-3. Ventral (A–F, I–L) and ventro-lateral (G,H) views of *wingless-lacZ* stage 9 (A), wild type stage 11 (B,E), stage 12 (C), stage 13 (I) and stage 16 (D,G,J); *Df(gooseberry)* stage 11 (F), stage 16 (H); *unplugged-lacZ* stage 16 (K) and *huckebein-lacZ* stage 16 (L) embryos. Segments T1 to A2 are shown except for panels (E,F) that show A6 to A9, and (A) that shows one hemisegment, white horizontal bars indicate the ventral midline. Analysis was done using anti-Lb antibodies (A–L) together with anti-β-Gal (A,K,L), anti-Engrailed (B), anti-Repo (C,D), anti-Elav (E–H), anti-Eyeless (I,J) antibodies. In panels I and J, the upper half shows Lb and Ey double staining, the lower half Ey staining only. At stage 9, *lb* expression is detected in NB 5-6 (A). Its progeny are anterior to the En-positive rows 6 and 7 (B). NB 5-6 produces *lb*-positive glial cells (C,D). From stage 13 onward, *lb* expression is detected in neurons produced by NB 5-3 as indicated by coexpression of *eyeless* (I,J) and *unplugged* (K) in medio-dorsal and latero-dorsal neurons (arrowhead and arrow, respectively).



● NB 5-3 derived *lb* positive neurons expressing also *ey* and *upg*. ⬭ NB 5-6 derived *lb* positive glial cells coexpressing *repo and K-lacZ.* ● PNS *lb* expressing neuron. ⬭ Additional glial cells are shown in order to delineate the neuromeres. Black horizontal bar localizes the ventral midline.

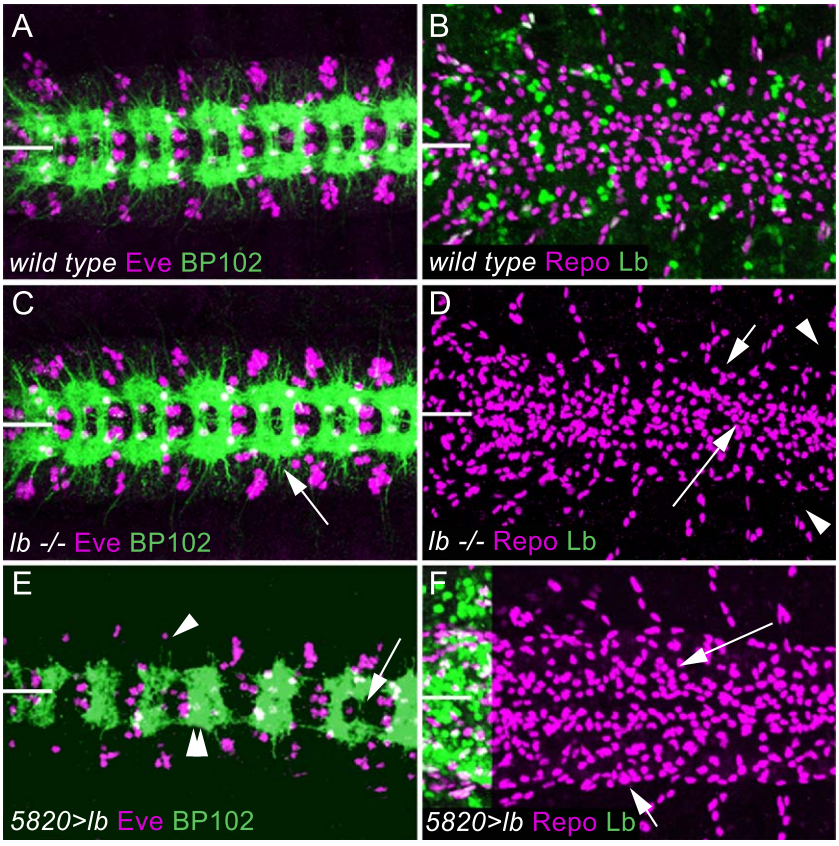


Fig. 4. The *lb* genes affect glial and neuronal development. Ventral views of stage 15 wild-type (A,B), *Df(lb)* (C,D) and *5820 > lb* (E,F) embryos analyzed with anti-Even-skipped and BP102 (A,C,E) or anti-Repo and anti-Lb (B,D,F) antibodies. Segments T1 to A3 are shown, horizontal bars indicate the ventral midline. The ventral nerve cord of the *lb* mutants shows a higher number of BP102-positive lateral projections (C, arrow) and a modified population of glial cells characterized in some segments by a higher (long and short arrows) or lower (arrowhead) number of cells. Note the reduced number of Even-skipped-expressing neurons in the gain-of-function mutants (E, arrowhead), the absence of the posterior commissure (E, arrow), the thickening of the anterior commissure (E, double arrowhead), and a modified population of glial cells characterized in some areas by a higher number of cells (F long and short arrows). In panel F, the left inset shows the extent of *5820-GAL4*-driven expression.

trap line (Fig. 2A). Colabeling experiments using anti-Lb and anti-Engrailed antibodies confirmed that the *lb*-expressing cells are located immediately anterior to the *en*-positive rows 6 and 7 (Fig. 2B).

The NB 5-6 is a lateral S1 neuroblast and its lineage is tagmaspecific containing 10–14 local interneurons projecting across the midline via the anterior and posterior commissures (Schmid et al., 1999; Schmidt et al., 1997). Additionally, a variable number of subperineural and exit glial cells are produced by this NB. Immunohistochemical staining of stage 12 and stage 16 embryos using antibodies against Lb and the glial marker Reversed Polarity (Repo; Halter et al., 1995) indicated that the *lb* gene product is not only expressed within the newly formed NB 5-6 but at later stages persists in the abdomen in a subset of NB 5-6-derived glial cells, namely one subperineural and one exit glial cell (Figs. 2C,D arrowhead and arrow, respectively).

Fig. 3. Identification of a NB 5-6-specific regulatory element and *lb* expression in the PNS. (A) Schematic representation of the genomic locus covering the *lb* genes and the 2 kb enhancer named as K-fragment, located 5 kb upstream of the *lbe* gene transcription start site. (B–G) Ventral views of stage 11 (B), 13 (C,E,F) and 16 (D,G) *K-lacZ* transgenic embryos analyzed with anti-β-Gal together with anti-Lb (B–D), anti-Repo (E) or anti-Ey (F,G) antibodies. Segments T1 to A2 are shown except for panel (I) that focuses on the ventral part of the PNS in one abdominal segment. Horizontal bars indicate the ventral midline. Note the coexpression of the *LacZ* reporter and the *lb* genes in the lineage of NB 5-6 (B–D) and the persistence of the β-Gal protein in those cells in later staged embryos (C,D). Double arrowheads, arrowhead and arrow point to the 5-6 medio-dorsal, the 5-6 exit and subperineural glial cells, respectively. The medially lying β-Gal-positive cell is a glial cell (E, arrow). Note the absence of β-Gal and Ey coexpression confirming specific expression of β-Gal in lineage 5-6 (F,G). (H,I) ventro-lateral views of wild-type stage 16 embryos stained with anti-Lb together with anti-Cut (H) or anti-Elav (I). Note the expression of the *lb* genes in one multidendritic neuron of the PNS (arrowhead). (J) Schematic representation of the vp 1-3 lineages illustrating the *cut* (green), *cut* and *elav* (violet) or *cut*, *elav* and *lb* (white) expressing cells; vp: ventral papilla; vda: ventral multidendritic neurons; vbp: ventral bipolar neuron. (K) Schematic representation of *lb*-expressing cells in three abdominal segments of a stage 16 *Drosophila* embryo nervous system. (L,M) Ventral views of stage 15 wild-type (K) and *Df(3L)H99* (L) embryos stained with anti-Lb antibodies. Note the supernumerary *lb*-positive cells in *Df(3L)H99* embryos (arrowheads and outer brackets).

However, judged by their position the three to four dorso-lateral *lb*-positive neurons as well as the dorso-medial neuron within the abdominal neuromeres of stage 16 embryos cannot derive from NB 5-6. Instead, they are more likely generated by NB 5-3, 5-4, 5-5, or 7-4. The latter NB is ruled out since we see no *lb* expression in *gsb*-deficient embryos (Figs. 2F,H compare to E,G) indicating that all the *lb*-positive cells are generated by row 5 NBs (*gsb* mutant embryos show a specific transformation of row 5 into row 3 NB identities; see Skeath et al., 1995). Instead, we favor NB 5-3 as the parental NB for these neurons since they are *eyeless* (*ey*)-positive and NB 5-3 is the only *ey*-positive NB in row 5 (Figs. 2I,J and data not shown). In accordance, we found that the *lb*-expressing neurons are also *unplugged-lacZ*-positive, a marker expressed within the NB 5-3 and 5-5 lineages (Fig. 2K; Doe, 1992) and we never detected coexpression of *lb* and *huckebein (hkb)-lacZ* (Fig. 2L), which is not expressed in the lineage of NB 5-3 but in those of NB 5-4 and 5-5 (McDonald and Doe, 1997).

NB 5-3 is another S1 NB delaminating from the medial column of row 5. It generates 9 to 15 interneurons, one to three cells are lying medially while the others are located laterally (Bossing et al., 1996; Schmid et al., 1999). Most of the interneurons project across the posterior commissure, but according to Bossing et al. (1996), there are also some projections across the anterior commissure. In the thoracic and the first abdominal segments, NB 5-3 gives rise to a motoneuron located laterally that projects to innervate a subset of lateral muscles (Schmid et al., 1999). However, colabeling experiments for Lb and Connectin indicated that *lb* is not expressed in the NB 5-3 motoneuron (data not shown). Identical results were obtained using antibodies directed against Lb and the motoneuronal marker pMAD (Marques et al., 2002; data not shown).

Although NB 5-6 is initially expressing *lb*, in abdominal segments only two of the NB 5-6 derived glial cells seem to maintain *lb* expression at later stages while the *lb*-positive neurons are deriving from another NB, namely NB 5-3. To monitor this, we made use of a transgenic line bearing a *lacZ* reporter construct driven by a 2-kb fragment (called K) located 5-kb upstream of the *lbe* gene transcription start site (Fig. 3A). Double labeling for β-Gal and Lb as well as Ey showed the expression of this transgene specifically in NB 5-6 and its progeny since we never find β-Gal/Ey colocalization (Figs. 3B,C,D,F,G). In contrast to Lb, the β-Gal protein persists in NB 5-6 progeny allowing the tracing of the cells belonging to this lineage, which normally express *lb* transiently (Figs. 3C,D). Indeed, using the *K-lacZ* reporter in stage 11 embryos, we saw that all β-Gal-positive cells are also *lb* positive (Fig. 3B). At stage 13, we found fewer double-stained cells in the lateral position but some solely β-Gal-positive cells in more medial position. This suggests that between stage 11 and 13 initially *lb*-positive NB 5-6 derived cells migrate medially and lose *lb* expression (Fig. 3C, double arrowhead). A double staining for β-Gal and Repo indicated that one of these cells is a glial cell (Fig. 3E, arrows). Occasionally, a medially lying β-Gal-positive neuron can be detected in addition (Fig. 3E, arrowhead). Consistent with the endogenous *lb* expression, *K-lacZ* marks preferentially the *lb*-positive exit and subperineural glia in late-stage embryos (Fig. 3D, arrowhead and arrow, respectively). Thus, the *K-lacZ* transgene can be considered as a marker specific for the *lb*-expressing cells deriving from NB 5-6 and is a convenient tool for monitoring the identity and the location of NB 5-6 progeny in different genetic contexts.

Starting from stage 14, *lb* expression is detected in one additional cell that lies outside of the CNS (Figs. 3H,I, arrowheads). To determine the identity of this cell, we performed double-staining experiments using anti-Lb and anti-Cut antibodies as well as anti-Lb and anti-Elav antibodies (Figs. 3H,I). This enabled us to identify the multidendritic neuron vbp as being *lb* positive (Fig. 3J, white cell; Brewster and Bodmer, 1995). This neuron belongs to the group of anterior ventral multidendritic neurons (Campos-Ortega, 1997). In summary, in a late-stage embryo, the expression of the *lb* genes is detected in eight neuronal cells, a ventral bipolar neuron in the PNS, two glial cells derived from NB 5-6 and five neurons derived from NB 5-3 (Fig. 3K).

NB 5-3/5-6-derived lb-positive cells are eliminated by apoptosis

To check whether any *lb*-expressing cells are eliminated by programmed cell death during embryonic development, we have analyzed homozygous embryos bearing a deficiency (H99) covering the apoptosis genes *reaper*, *grim* and *hid* (Chen et al., 1996a,b; Grether et al., 1995; White et al., 1994, 1996). Indeed, we found a constant number of additional *lb*-positive neurons. In the abdominal neuromeres, *lb* is expressed in five lateral (Fig. 3M, outer brackets) and four medio-dorsal (Fig. 3M, arrowhead) neurons vs. four and one, respectively, in wild-type embryos (Fig. 3L). Within the thoracic segments *lb* is detected in six lateral and six medio-dorsal cells vs. five and two to three, respectively, in wild-type embryos (Figs. 3L,M). Thus, there is a reproducible elimination of *lb*-positive cells in wild-type embryos showing that apoptosis is part of the normal development of the NB 5-3 and/or NB 5-6 lineages.

The lb genes are involved in the development of NB 5-6 derived glial cells

To analyze *lb* mutant embryos, we used two overlapping deficiencies (see Materials and methods). Labeling experiments with antibodies directed against general neuronal markers like BP102 (Figs. 4A,C,E) indicated no dramatic change in the ventral nerve cord morphology of *lb*-deficient embryos (Fig. 4C) (Carney et al., 1997).

However, there seem to be slightly more BP102-stained lateral projections in *lb* − / − embryos compared to wild type (Fig. 4C, arrow), which might result from axonal misrouting of neurons whose identity depends on *lb*. To drive ectopic expression of *lb*, we took advantage of the GAL4/UAS system (Brand and Perrimon, 1993). We crossed *5820-GAL4* driver flies that express the GAL4 transcriptional activator in all NBs, GMCs and neurons (Jacobsen et al., 1998) with flies carrying two copies of the *UAS-lbe* or *UAS-lbl* transgenes. As a result, all embryos that derived from this cross express *lb* in all cells of the developing central nervous system. In contrast to the loss-, the gain of *lb* function leads to much more severe changes in the CNS morphology (Fig. 4E). Stainings with BP102 antibodies indicated a thinning or absence of the posterior commissures in some neuromeres (Fig. 4E, arrow) and a fusion of the commissures in others (Fig. 4E, double arrowheads). These results indicate that the ectopic expression of *lb* affects the proper differentiation of neurons. This can also be demonstrated by anti-Even-skipped staining, since overexpression of *lb* interferes with the development of the EL neurons deriving from NB 3-3 (Bossing et al., 1996), which are reduced in number (Fig. 4E, arrowhead), while all other *even-skipped* (*eve*)-expressing cells could still be detected.

Since a subset of the *lb*-expressing cells in the embryo are NB 5-6 derived glial cells, we further analyzed the effect of *lb* loss-of-function mutation using the glia-specific anti-Repo antibody in stage 16 embryos (Figs. 4B,D,F) (Halter et al., 1995). This staining indicates that in the absence of Lb function, the glial cell population is affected in its positioning and number (Fig. 4D). We observed an increased number of medially located glial cells (long arrow) as well as partial loss (arrowhead) or abnormal positioning (short arrow) of lateral exit and subperineural glial cells. On average (n = 38 hemineuromeres), we counted 2.3 additional glial cells per hemineuromere (VNC and PNS associated glia) in *lb* loss-of-function vs. wild-type situation (n = 30). To determine whether this phenotype is at least partially due to fate switches of cells within NB 5-6 lineage, we analyzed the expression of the *K-lacZ* transgene in a *lb*-deficient background (Figs. 5C,D). These embryos are characterized by the absence of the β-Gal-positive exit glia in 33% of the hemineuromeres (n = 48) (Fig. 5D, asterisks). Interestingly, none of the additional medially lying repo-positive cells are β-Gal-positive, suggesting that they are not misrouted exit glia cells. Instead, they seem to be produced by a neuroblast other than NB 5-6. An opposed phenotype can be seen in the *lb* overexpression experiment since there were supernumerary β-Gal-expressing subperineural and exit glial cells in 45% of the hemineuromeres (n = 56) (Fig. 5F; arrowheads and arrows, respectively). In addition, some medially located β-Gal-positive glial cells could be detected (Fig. 5E, short broken arrow). These correspond most likely to the NB 5-6 derived Lb/β-Gal-positive glial cells that we detected at stage 13 in a wild-type background (Fig. 3E, arrows). This suggests that the *K-lacZ* enhancer is under Lb-positive control and β-Gal expression is sustained due to *lb* overexpression. The same hypothesis explains the presence of additional β-Gal-positive/Repo-negative cells (Fig. 5E, long broken arrows). These observations indicate that the *lb* genes are required for a subpopulation of 5-6 derived cells to acquire a specific glial identity.

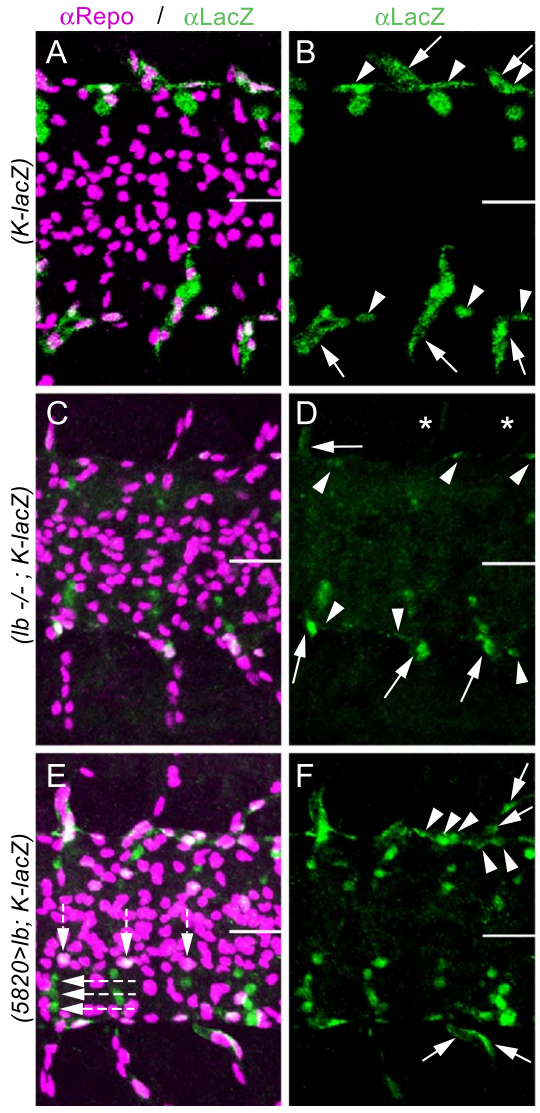


Fig. 5. The *lb* genes are essential for 5-6 glial cell specification. Ventral views of three abdominal segments of stage 16 [*K-lacZ*] transgenics (A,B); [*Df(lb)*; *K-lacZ*] (C,D) and [*5820 > lb*; *K-lacZ*] (E,F) embryos analyzed with anti-Repo and anti-β-Gal antibodies. Horizontal bars indicate the ventral midline. Panels on the right show the β-Gal staining only. β-Gal-positive exit and subperineural glial cells are indicated by an arrow and arrowhead, respectively. [*Df(lb)*; *K-lacZ*] embryos are characterized by the absence of some β-Gal-positive exit glial cells (asterisks). In [*5820 > lbe*; *K-lacZ*] embryos, white arrowheads, white arrows, short and long broken arrows point to additional *K-lacZ*-positive subperineural, exit and medially lying glial cells and neurons, respectively.

The lb-dependent glial defects cause structural aberrations within the segmental nerves

Glial cells are intimately associated with neurons and are essential components of the nervous system. Their roles include structural support, wrapping and insulating neurons and regulating their function with cytokines and growth factors. Developing glia undergo extensive migrations and cell shape changes and provide cues and substrata for neuronal migrations and axon path finding (Bradley, 2001 and references therein). To better visualize the consequences of altered *lb* gene function on the glial cell population in association with the nervous system development, we analyzed wild-type and mutated embryos with anti-Repo and anti-HRP antibodies (Fig. 6). Antiserum against horseradish peroxidase (HRP) labels the surfaces of neurons revealing all axonal pathways (Snow et al., 1987).

The segmental nerves consist of two fascicles, one anterior, the intersegmental nerve (ISN), and one posterior, the segmental nerve (SN) (Fig. 6A). The SN and ISN of each abdominal segment leave the cortex closely attached to each other. On encountering the ventral oblique musculature, the SN and ISN nerves lose their common glial sheath and diverge. A glial sheath formed by two serially arranged cells, called exit glial cells, surrounds both nerves (Klämbt and Goodman, 1991).

Within the abdominal segments, the exit glia posterior to the SN expresses the *lb* genes (Figs. 6A,B, arrows). In the thorax, two to three *lb*-positive exit glia cells are located posterior to the SNa or between the ISN and the SN (data not shown). These *lb*-positive exit glial cells are missing in some segments of *lb* loss-of-function mutants (Fig. 6C, arrow) and can be multiplied in some segments of *lb* gain-of-function mutants (Fig. 6D, arrows). A second obvious phenotype is the variable distance separating the ISN and SN bundles as indicated by the horizontal bracket. This distance is increased in *lb* loss-of-function mutants ($n = 15$) (Fig. 6C) compared to wild type (Fig. 6A). The opposite phenotype can be seen in *lb* gain-of-function mutants ($n = 20$) (Fig. 6D). This strong phenotype cannot be due only to the absence or addition of a few NB 5-6 derived exit glial cells in some segments since we see this in nearly every segment. Instead, the most likely explanation is that this phenotype is caused by an opposite alteration in the properties of the glial cells in the loss- and gain-of-function situations.

The lb genes are essential for the correct development of NB 5-3 derived neurons

A NB5-3 specific neural marker is not available, rendering difficult the analysis of the role of *lb* genes in the specification of cells derived from this lineage. To test whether *lb* is implicated in setting up properties of NB 5-3 progeny, we took advantage of the fact that the NB 5-3 derived neurons are Ey-positive, and examined the *ey* expression pattern in embryos deficient for *lb* or those overexpressing the gene (Kammermeier et al., 2001). The comparison between the wild-type (Fig. 7A) and mutant

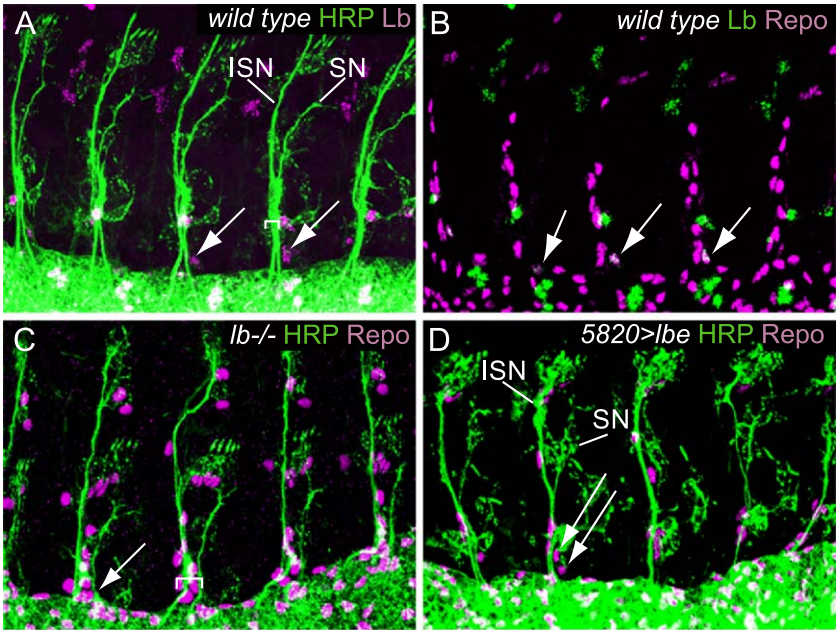


Fig. 6. The *lb* genes interfere with nerve root morphology. Ventro-lateral views of four abdominal segments of stage 15 wild-type (A,B), *Df(lb)* (C) and *5820 > lbe* (D) embryos stained with anti-Lb (A,B), anti-HRP (A,C,D) and anti-Repo (B-D) antibodies. Arrows point to the *lb*-positive glial cells (A,B) that can be missing in *Df(lb)* embryos (C), and can be found in higher numbers in *5820 > lb* embryos (D). Horizontal bracket measures the distance separating the ISN from the SN motoneuron bundle; note that it increases and decreases in *Df(lb)* and *5820 > lb*, respectively. Embryos are oriented anterior to the left.

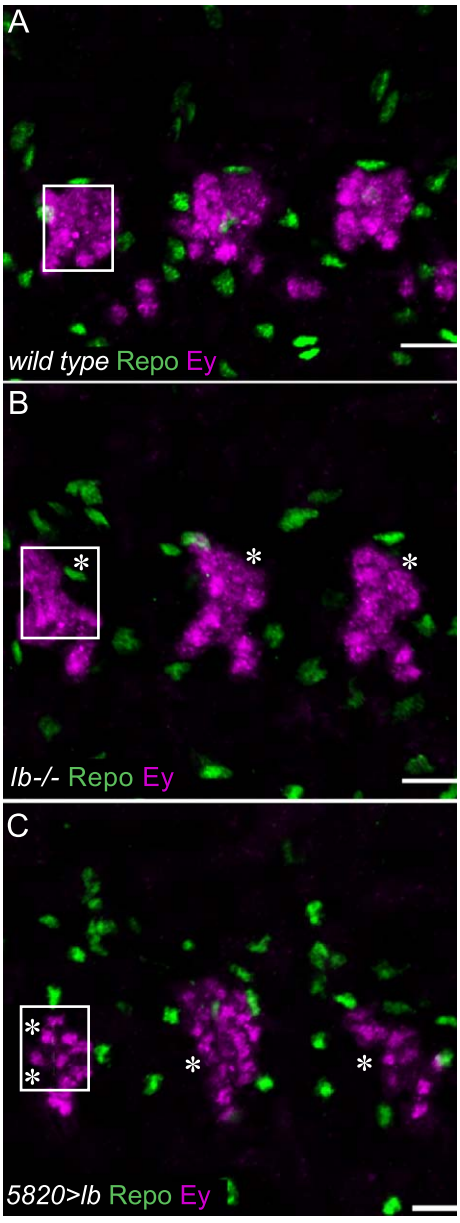


Fig. 7. *lb* genes are required for the specification of a subset of *ey*-positive cells. Ventro-lateral views of three abdominal hemisegments of late stage 14 wild-type (A), *Df(lb)* (B) and *5820 > lbe* embryos stained for Ey and Repo proteins. Embryos are oriented anterior to the left with horizontal bars indicating the ventral midline. Marked area corresponds to rows 4 and 5 *ey*-expressing neural cells in one hemineuromere. Note the reduction of the number of Ey-positive cells in both *Df(lb)* (B, asterisks) and in *5820 > lbe* (C, asterisks) embryos.

phenotype (Fig. 7B) indicates a reduction of the most latero-posterior subset of *ey*-expressing cells in every hemisegment of all mutant embryos observed ($n = 10$) (Fig. 7B, asterisks). Since *lb* is coexpressed with *ey* in laterally located row 5 cells (see Fig. 2J), we conclude that *lb* expression is required for these cells to maintain *ey* activity. The influence of *lb* on the *ey*-expressing population of neural cells is confirmed by the analysis of gain-of-function phenotype (Fig. 7C). In embryos overexpressing *lb* in all neural cells ($n = 15$), we observe a global reduction of *ey*-expressing cells in 50% of the hemisegments. From these, the most affected are the anteriorly located cells most probably belonging to row 4 (Fig. 7C, asterisks). This suggests that *lb* genes provide information required to specify a subset of laterally located Ey-positive cells mostly belonging to row 5, and when misexpressed, they are negatively influencing row 4 *ey*-expressing cells.

Discussion

The animal nervous system captures environmental cues through external sensory organs, integrates them through afferent neurons and commands the body through efferent neurons linked to the musculature. Additionally, glial cells provide support for the correct development and function of this network. The establishment of such a network requires precise cellular interactions based on cellular identity specification and recognition.

Lineage-specific regulation of neural lb expression

The *lb* genes display an extremely reproducible and highly restricted expression pattern suggesting a complex regulation of their transcription during embryonic CNS development. In the lineages of NB 5-6 and 5-3, the pattern of expression of the *lb* genes differs considerably. In stage 10–11 embryos, we counted 14 to 16 *lb*-positive cells, suggesting that the *lb* genes are expressed in the entire lineage generated from S1 NB 5-6 (Schmid et al., 1999; Schmidt et al., 1997), whereas in NB 5-3 progeny, their expression is restricted to only a few post-mitotic neurons. The timing of *lb* gene expression in these two neural lineages is also different. The *lb* genes start their expression in NB 5-6 just after its delamination at early stage 9. At stage 12 of development, *lb* expression decreases in lineage 5-6 and persists only in the subperineural and exit glial cells. At the same time, *lb* appears in the postmitotic neurons of the neighboring lineage 5-3 as well as in the vp3 multidendritic neuron of the PNS. The pattern of *lb* expression suggests an influence of both segment polarity and columnar genes. The absence of *lb*-positive cells in *gsb*-deficient embryos indicates a genetic control by *gsb*. This was confirmed by an ectopic expression of *lb* in *gsb* gain-of-function embryos (data not shown). Down-regulation of *lb* in NB 5-6 and their absence in NB 5-3 until stage 12 indicate transcriptional control by repressors. One good candidate in the development of NB 5-3 is Runt. This transcription factor is expressed in NB 5-3 from the time of its delamination until stage 11–12, time at which *lb* starts to be expressed (Dormand and Brand, 1998). Interestingly, the enhancer K shows three putative DNA binding sites (DBS) for Runt, one of which is conserved in *Drosophila pseudoobscura*. Their functionality remains to be tested.

The lb genes are involved in specifying certain neural cell identities and properties

Highly restricted and lineage-specific expression of *lb* genes have been previously described in several differentiating embryonic tissues. For example, in the *Drosophila* mesoderm, both *lbe* and *lbl* are expressed in a subpopulation of heart precursors (Jagla et al., 1997a) and in muscle progenitors giving rise to the SBM and LaP (Lateral adult Precursor) (Jagla et al., 1998). Altered *lb* expression leads to transformations of heart cell and myoblast identities, indicating that *lb* functions to determine cell fate decisions during heart and muscle formation (Jagla et al., 1997a, 1998). Here we analyzed the contribution of the *lb* genes to the *Drosophila* CNS development.

Our experiments show that panneural overexpression of *lb* leads to a significantly higher number of exit and subperineural glial cells, while the number of these cells in *lb* loss-of-function mutant embryos is reduced. Both cell types are normally generated by NB 5-6, suggesting that Lb acts in a cell autonomous way with respect to the specification and/or differentiation of these cells. Indeed, the additional cells obtained in the overexpression experiment express the NB 5-6 lineage marker *K-lacZ*, which supports this hypothesis. This could be explained in two ways: either ectopic Lb leads to a transformation of NB 5-6-derived neuronal cells into glia or there is additional proliferation of glial precursors within this lineage. Currently, we cannot distinguish between these possibilities.

Additionally, *lb* seems to be important for later specification and/or differentiation events of a subset of the NB 5-3 progeny. This is based on our finding that in *lb*-deficient embryos the presumptive *ey*-positive NB 5-3 neurons lose their *ey* expression. Due to the lack of appropriate markers, we currently do not know what the fate of these cells is. One possibility is that a part of these cells are transformed into glia since we see additional glial cells in *lb*-deficient embryos in the medio-ventral area of the ventral nerve cord, which do not express the NB 5-6 marker *K-lacZ*.

Particularly interesting in *lb*-deficient embryos is the increased distance separating the ISN and SN axonal bundles and the opposite phenotype in gain-of-function mutants. Since this is the position of the NB 5-6 derived exit glial cell in wild-type embryos, we speculate that this phenotype is due to a defect in glial function. The fact that the opposite phenotypes in loss- and gain-of-function situations are seen in all hemineuromeres, while the number of the exit glial cells can resemble the one in wild type, strongly suggests that the *lb* genes also control the expression of cellular surface proteins governing cellular interactions. This hypothesis is strongly supported by the mislocalization of the PNS-associated glial cells and the highly disturbed axonal network in the *lb* gain-of-function mutants. Another important function of the glial cells is the establishment of the blood–nerve barrier. The isolation of the nerves is achieved, thanks to the pleated septate junction formed at the junctions between subperineural glia that form a continuous sheath around the VNC and in peripheral and exit glia that ensheath peripheral nerves. Mutants for components of these pleated septate junctions such as neurexin IV (Baumgartner et al., 1996) or gliotactin (Auld et al., 1995) show a disrupted blood–nerve barrier; consequently, their axons are exposed to the high K^+ environment of the surrounding hemolymph resulting in the failure of action potentials and paralysis. Misspecification, mislocalization or absence of glial cells as seen in *lb* mutants could also interfere with the establishment of a correct blood–nerve barrier. Only the identification of *lb* target genes will help us in the understanding of their mode of action.

Thus, all these above-discussed discrete alterations observed in the embryonic CNS with affected *lb* gene function suggest a role for the *lb* genes in diversification of neural cell fates as well as in regulating genes necessary for cell–cell interactions.

lb and Lbx1 contribution to the nervous system development reveal conserved functions and evolutionary changes

The *Drosophila lb* genes are expressed in two NB lineages belonging to the dorsal half of the neurectoderm. Likewise, the mouse *lb* ortholog, *Lbx1*, is expressed in the dorsal part of the spinal cord. The expression of *Lbx1* subdivides the differentiating neurons into two classes, the somatosensory relay interneurons and dorsal association interneurons. The latter express *Lbx1* and are dependent on this factor for being correctly specified. While *lb* genes are expressed in a NB, GMCs and postmitotic neurons and glial cells, *Lbx1* is exclusively expressed in postmitotic neurons. However, timewise, both *lb* and *Lbx1* are expressed in two waves giving rise to an early (NB 5-6 lineage in *Drosophila*/dl4-6 in mouse) and a late (NB 5-3 interneurons and PNS bipolar neuron in *Drosophila*/dIL A–B interneurons in mouse) population of *lb/Lbx1* neurons. Both *lb*- and *Lbx1*-expressing cells appear in *Pax* progenitor domains, *gsb* in *Drosophila*, *Pax7/Pax3* in the mouse suggesting conserved regulatory pathways.

Mice lacking *Lbx1* die at birth from a breathing defect, their dorsal spinal cord is severely hypocellular due to the loss of these dorsal association interneuron cell types. These were transformed into relay interneurons and subsequently undergo apoptosis because they lack the right survival signals. Ad contrario, when *Lbx1* is misexpressed in the developing neural tube, it suppresses the differentiation of the relay interneurons that subsequently become dorsal association interneurons and undergo cell death. In *Drosophila*, the lack of adequate molecular markers disabled us to clearly establish a particular differentiation program depending on *lb* but the analysis of the glial and neuronal cell populations in *lb* mutants supports the idea that the *lb* genes control cell identity specification programs.

Like gain-of-function in mice, *lb* gain-of-function in *Drosophila* clearly showed their potential to interfere with

NB differentiation programs of dorsal relay interneurons in mouse and *eve*-positive EL neurons in *Drosophila* for example (Gross et al., 2002; Müller et al., 2002 and this work).

If we now compare and speculate about the contribution of *Lbx1*- and *lb*-expressing neurons to their respective nervous systems, there seems to be a simplification of the *Lbx1* expression pattern vs. *lb*. In *Drosophila*, the *lb* genes are expressed in a higher diversity of neuronal cell types compared to *Lbx1* that is expressed only in dorsal interneurons. This apparent simplification is of course due to the higher complexity of the vertebrate nervous system. Nonetheless, evolutionary conservation of both function and genetic regulatory pathways present *Drosophila* as a good model to study the role of Lb in CNS development. The establishment of new molecular tools and identification of direct targets is required to understand how *lb/Lbx1* genes exert their cell identity functions.

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