Lmx1b controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord

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Summary

The differentiation and migration of superficial dorsal horn neurons and subsequent ingrowth of cutaneous afferents are crucial events in the formation of somatosensory circuitry in the dorsal spinal cord. We report that the differentiation and migration of the superficial dorsal horn neurons are regulated by the LIM homeobox gene *Lmx1b*, and its downstream targets *Rnx* and *Drg11*, two transcription factors implicated in the development of dorsal horn circuitry. An analysis of *Lmx1b* mutants shows that *Lmx1b* normally acts to maintain the expression of the Ebf genes and to repress the Zic genes. *Lmx1b* mutants also exhibit the disruption of the cutaneous afferent ingrowth, suggesting that the dorsal horn cells might provide important cues guiding sensory axons into the dorsal spinal cord. Our results thus indicate that *Lmx1b* has a pivotal role in genetic cascades that control the assembly of circuitry in the superficial dorsal horn.

Supplemental data available online

Key words: *Lmx1b*, Dorsal horn, Migration, Differentiation, Cutaneous afferents, Mouse

Introduction

Dorsal spinal cord formation begins with neuronal and glial progenitors exiting the cell cycle in the ventricular zone (VZ), and is followed by sequential migration and further differentiation of migrating neurons and glia. Once neurons have reached their appropriate region of the dorsal spinal cord, they differentiate further into distinct layers or laminae that carry out distinct physiological function (Christensen and Perl, 1970; Rexed, 1952). For example, lamina I primarily contains relay and local interneurons, whereas lamina II (substantia gelatinosa) consists of few projection neurons but contains numerous small-size interneurons, many of which make synaptic contacts with lamina I neurons (Molander and Grant, 1995; Willis, 1995). The primary afferents of dorsal root ganglia (DRG) neurons project to distinct laminae of the spinal cord and establish precise neuronal connections with their targets. Small-diameter DRG neurons that express TrkA, a high-affinity neurotrophin receptor for nerve growth factor (NGF), project to laminae I-II and relay noxious and thermal sensory information, whereas large-diameter DRG neurons that express the neurotrophin receptor TrkC make connections with motoneurons in the ventral horn and relay muscle proprioceptive information (Christensen and Perl, 1970; Huang and Reichardt, 2001; Lawson and Biscoe, 1979; Scott, 1992; Snider, 1994).

During spinal cord development, the specification of dorsal interneurons is mediated by both extrinsic and intrinsic factors (Caspary and Anderson, 2003; Helms and Johnson, 2003; Lee and Jessell, 1999; Liem et al., 1997). Specifically, the intrinsic factors that control the specification and development of several classes of early-born neurons (dI1-6) have been analyzed in greater detail. In mice lacking transcription factors *Lbx1* (*Lbx1h* – Mouse Genome Informatics) or *Rnx* (*Tlx3* – Mouse Genome Informatics), the specification of dI5 neurons is affected, as indicated by the loss or downregulation of *Lmx1b* (dI5 marker) (Gross et al., 2002; Muller et al., 2002; Qian et al., 2002). These two genes are also involved in the development of late-born neurons that make up the dorsal horn of the spinal cord (Caspary and Anderson, 2003). In addition, the transcription factors *Drg11* (*Prxxl1* – Mouse Genome Informatics), *Ebf1*, *Ebf3* and *Zic1* have also been shown to be important for the early specification of the dorsal spinal cord neurons (Aruga et al., 1998; Aruga et al., 2002b; Chen et al., 2001; Ebert et al., 2003; Garcia-Dominguez et al., 2003; Garel et al., 1997; Wang et al., 1997). Among them, *Zic1* has been shown to be a negative regulator of the differentiation of the dorsal horn neurons in mice (Aruga et al., 2002a; Aruga et al., 1998; Aruga et al., 1996a; Aruga et al., 2002b; Aruga et al., 1994; Aruga et al., 1996b). Despite these studies, our knowledge about the molecular mechanisms that govern the development of early-born neurons and the assembly of the dorsal horn circuits is still rather fragmentary.

Lmx1b is an LIM homeobox-containing gene, and was originally isolated as a mouse ortholog of the chicken *Lmx1* (Chen et al., 1998a; Riddle et al., 1995; Vogel et al., 1995). In the chicken, *Lmx1* is involved in the specification of the dorsal cell

fate in the limb, and the differentiation and morphogenesis of the isthmic organizer (Adams et al., 2000; Matsunaga et al., 2002; Riddle et al., 1995; Vogel et al., 1995). Mice lacking *Lmx1b* exhibit abnormal limbs and kidneys (Chen et al., 1998a). Mutation of *Lmx1b* results in nail patella syndrome in humans, an autosomal dominant disease characterized by abnormal skeletal patterning and renal dysplasia (Dreyer et al., 1998). In the central nervous system, *Lmx1b* is involved in multiple developmental processes, including the formation of eye, dopaminergic neurons, serotonergic neurons and the trajectory of the motor axons in the limb (Cheng et al., 2003; Ding et al., 2003; Kania and Jessell, 2003; Kania et al., 2000; Pressman et al., 2000; Smidt et al., 2000). In the nematode *Caenorhabditis elegans*, the *Lmx1b* ortholog, *Lim6*, has been shown to be important for the differentiation of GABAergic neurons (Hobert et al., 1999).

In the developing dorsal spinal cord, *Lmx1b* is expressed in dI5 neurons and late-born neurons destined to populate the superficial layer of the dorsal spinal cord (Chen et al., 2001; Gross et al., 2002; Muller et al., 2002). In this study, we performed detailed studies of the dorsal spinal cord development in *Lmx1b* mutants. Our study reveals that *Lmx1b* is important for the development of dI5 neurons, late-born dorsal horn neurons and the projection of cutaneous afferents in the dorsal spinal cord. Our study uncovers a central role for *Lmx1b* in transcriptional cascades that govern the dorsal horn development.

Materials and methods

Genotyping and maintenance of animals

Lmx1b, *Rnx* and *Drg11* mutant mice were generated and genotyped as previously described (Chen et al., 1998a; Roberts et al., 1994). The age of embryos was determined according to the plug date (the plug date is considered to be E0.5). Because the mating time among the mice may differ, the actual developmental stage of the embryos was further ascertained according to the spinal cord morphology by the use of Nissl staining, as well as the criteria described previously (Kaufman, 1998). The $Lmx1b^{+/-}$, $Rnx^{+/-}$ and $Drg11^{+/-}$ mice were maintained in a mouse facility according to protocols approved by the Division of Comparative Medicine at Washington University School of Medicine.

BrdU labeling and detection by immunocytochemistry

Pregnant female mice derived from timed matings between *Lmx1b* or *Rnx* or *Drg11* heterozygous mice were given a single intraperitoneal injection of BrdU (5 mg/ml solution in PBS and 60 μg/g of body weight) at 11.5 days postcoitum (dpc) and 12.5 dpc. After time periods of 2 hours, 1 day, 2 days or 3 days, embryos were removed, genotyped and sectioned as described (Chen et al., 1998a; Chen et al., 2001; Roberts et al., 1994). The sections were processed and stained sequentially with a mouse anti-BrdU antibody (Dako), biotinylated donkey anti-mouse IgG (Jackson Immunoresearch) and ABC Elite reagents (Vector). For double staining with anti-LMX1B antibody (Kania et al., 2000), the slides were first incubated with a guinea pig anti-LMX1B antibody detected enzymatically through production of a brown precipitate. For anti-BrdU antibody detection, a nickel intensification technique was used, producing a black precipitate. To quantify the distribution of BrdU-labeled neurons, we used the Photoshop (Adobe) program after dividing the dorsal horn into two parts: medial one-third and lateral two-thirds (from the midline to the lateral edge of the dorsal horn, Fig. 2). BrdU-labeled neurons in ten sections each from wild-type (n=6) and mutant (n=6) embryos were counted at the thoracic segmental level, and a comparison was performed using Student's t-test.

Nissl staining, in situ hybridization and immunocytochemistry

Nissl staining, in situ hybridization and immunocytochemistry were performed as described (Chen et al., 1998b; Chen et al., 2001). For double staining of LBX1 with BRN3A, PHOX2A with PAX2, and LMX1B and PHOX2A, Cy3-labeled donkey anti-guinea IgG (Jackson) and FITC-labeled donkey anti-rabbit or anti-mouse IgG (Jackson) were used. Antibodies included: rabbit anti-BRN3A (Fedtsova and Turner, 1995), guinea pig anti-ISL1 (Tanabe et al., 1998), guinea pig anti-LMX1B, guinea pig anti-LBX1 (Muller et al., 2002), mouse anti-MAP2 (Sigma), rabbit anti-PHOX2A antibody (Tiveron et al., 1996), rabbit anti-PAX2 (Zymed Lab), rabbit anti-trkA and rabbit anti-Peripherin 57K (Chemicon). For cell counting, 10-15 consecutive sections each from wild-type (n=6) and homozygous mutant (n=6) embryos at thoracic levels were counted, and statistical analysis was performed by the use of Student's t-test.

DiI labeling

For study of the projection from the DRG to the spinal cord, a small amount of 1,1″-dioctadecyl-3,3,3″,3″-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) crystals was placed in the DRG unilaterally. The samples were kept in the fixative at 37°C for 2-4 days, and then were sectioned transversely with a vibratome at a thickness of 50-100 μm. Labeling was observed with epifluorescent or laser confocal microscopy.

In utero electroporation

For in utero electroporation, previously detailed procedures were followed (Saito and Nakatsuji, 2001). Pregnant mice at 12 dpc were anesthetized with sodium pentobarbital (40 mg/kg), followed by the exposure of the uterus and cutting of the uterine wall on both horns along the antiplacental side. pCAGGS-*Lmx1b*:EGFP or pCAGGS-EGFP (Niwa et al., 1991) (1-3 μl; 0.5-1.5 μg/μl) was injected into the central canal of the spinal cord using an orally controlled pipette system. After injection, square electrical pulses were delivered by the use of an Electro Square Porator (ECM830) at a rate of one pulse per second (voltage 35V, five pulses, 50 ms) to embryos by holding the utero with forceps-type electrodes. The embryos were repositioned into the abdominal cavity without sewing the uterine wall. The abdomen was filled with warmed saline and the abdominal wall and skin were sutured. Embryos were allowed to survive for 2 days. Genotyping and analysis of gene expression in the spinal cord of electroporated embryos was performed as described above.

Results

Lmx1b is expressed in dI5 and dorsal horn neurons

To further characterize dynamic expression of *Lmx1b* in the developing spinal cord, in situ hybridization and immunocytochemical studies were performed. At E10.5, *Lmx1b* was expressed in dI5 neurons (Fig. 1A). Beginning at E11.5, *Lmx1b* expression was expanded to the entire region lateral to the VZ of the dorsal spinal cord (Fig. 1B). At E17.5 when laminae I-II and laminae III-V are distinguishable, *Lmx1b* expression was prominent in laminae I-II and weak in laminae III-V (Fig. 1C). *Lmx1b* was not expressed in DRG neurons at any of the stages examined (Fig. 1C, data not shown). To determine whether *Lmx1b* is expressed in postmitotic neurons, we performed BrdU/LMX1B double labeling. After 2 hours of BrdU labeling in either E11.5 or E12.5 embryos, very few $BrdU^+$/$LMX1B^+$ double-labeled neurons were detected: $BrdU^+$ neurons were mostly restricted to the VZ of the dorsal spinal cord and did not overlap with LMX1B-expressing neurons in the subventricular zone (Fig.

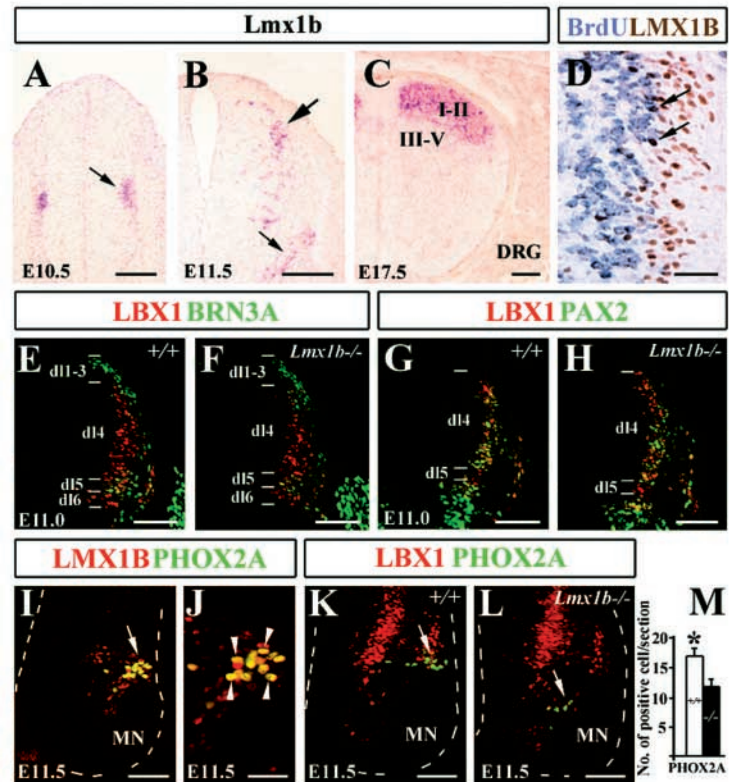

Fig. 1. Expression of *Lmx1b* and specification of early-born neurons in the spinal cord of *Lmx1b*$^{-/-}$ mutants. (A-C) Expression of *Lmx1b* in the dorsal horn detected by in situ hybridization. (A) *Lmx1b* is expressed in dI5 interneurons at E10.5 (arrow). (B) At E11.5, in addition to dI5 neurons (small arrow), *Lmx1b* is expressed in neurons emerging from the dorsal VZ (large arrow). (C) *Lmx1b* expression is concentrated in laminae I-II neurons at E17.5. (D) Detection of BrdU$^+$ (blue)/LMX1B$^+$ (brown) in the spinal cord of wild-type E12.5 embryos. Arrows in D show nascent neurons co-labeled with anti-LMX1B and anti-BrdU antibodies. (E,F) Detection of LBX1 (dI4-6 marker, red) and BRN3A (dI1-3 and dI5 marker, green) in wild-type (E) and *Lmx1b* mutant (F) embryos at E11. (G,H) Detection of LBX1 (red) and PAX2 (dI4 and dI6 marker, green) in wild-type (G) and *Lmx1b* mutant (H) spinal cord at E11. (I,J) Detection of LMX1B (red) and PHOX2A (green) in dI5 neurons at E11.5; arrow in I and arrowheads in J (higher magnification of I) indicate double-stained cells. (K,L) Detection of LBX1 (red) and PHOX2A (green) in wild-type (K) and *Lmx1b* mutant embryos (L) at E11.5. Arrows indicate PHOX2A$^+$ cells. (M) Statistical comparison of the number of PHOX2A$^+$ cells in wild-type (white bar) and *Lmx1b* mutant (black bar) embryos. Asterisks indicate significant difference using Student's *t*-test $P<0.001$. Scale bars: 100 µm in A-C,E-I,K,L; 50 µm in D; 25 µm in J. MN, motoneurons.

1D). Thus, *Lmx1b* expression is largely confined to post-mitotic neurons.

Phox2a marks a subset of dI5 neurons and is partially lost in *Lmx1b* mutants

Previous studies have shown that *Lmx1b* expression is either absent or downregulated in dI5 neurons of *Lbx1* or *Rnx* mutant mice (Gross et al., 2002; Muller et al., 2002; Qian et al., 2002). These observations raise the possibility that *Lmx1b* plays a role in the development of dI5 neurons, and that *Lbx1* and/or *Rnx* may mediate the specification of dI5 neurons via *Lmx1b*. To characterize the development of dI5 neurons in the absence of *Lmx1b*, we examined the expression of *Lbx1* (dI4-6), *Brn3a* (*Pou4f1* – Mouse Genome Informatics) (dI1-3, dI5) and *Pax2* (dI4, dI6), all markers of distinct dorsal neuronal types in *Lmx1b* mutants (Gross et al., 2002; Muller et al., 2002). Immunocytochemical staining revealed a similar staining pattern of LBX1, BRN3A and PAX2 between *Lmx1b* mutants and wild-type control at E11.0 (Fig. 1E-H). Our study, however, was complicated by the observations that *Lbx1* and *Brn3a* are also expressed in neurons located adjacent to dI5 neurons. To identify a more dI5-specific marker, we examined the co-expression of LMX1B and PHOX2A at E10.5 and E11.5 spinal cord (Tiveron et al., 1996). We found that PHOX2A expression overlaps with LMX1B in a subset of dI5 neurons, and no neurons expressing PHOX2A alone were found in the same region (Fig. 1I,J). Thus, *Phox2a* is a dI5 neuron-specific marker (Fig. 1I,J). In *Lmx1b* mutants, *Phox2a* expression was either lost or decreased in dI5 neurons in the mutants compared with that in wild-type control (Fig. 1K,L; data not shown). Moreover, the location of PHOX2A$^+$ neurons was shifted more ventrally in the spinal cord of *Lmx1b* mutants, indicating that dI5 neurons migrated aberrantly in *Lmx1b* mutants (Fig. 1K,L). Cell counting confirmed that the number of PHOX2A$^+$ neurons was indeed reduced in the mutants (Fig. 1M). Together our data suggests that the development of dI5 neurons is impaired in the absence of *Lmx1b*.

A migratory defect in the dorsal spinal cord of *Lmx1b* mutants

To examine the effect of *Lmx1b* mutation on the dorsal horns of *Lmx1b*$^{-/-}$ embryos, we performed Nissl staining. At E12.5, the dorsal horns of *Lmx1b*$^{-/-}$ embryos and wild-type controls were indistinguishable (Fig. 2A,B). At E15.5, in wild-type embryos, numerous laminae I-II neurons, distinguishable from laminae III-IV neurons by their smaller size, aggregate to form a separate layer, whereas laminae III-IV neurons also become distinct in being more loosely distributed (Fig. 2C). In *Lmx1b* mutants, these laminar boundaries were not recognizable (Fig. 2D) and the dorsal funiculus was smaller relative to wild-type controls (Fig. 2E,F).

We next used BrdU to determine the migratory behavior of the dorsal horn neurons in *Lmx1b* mutants. To do this, we labeled dorsal horn neurons of wild-type and *Lmx1b* mutant embryos with BrdU at E11.5 and E12.5, and examined their settling position at E14.5. Interestingly, neurons labeled with BrdU at E11.5 in the wild-type embryo did not migrate to the most superficial region of the dorsal horn (Fig. 2G). By contrast, neurons labeled at E12.5 migrated through earlier-born neurons and occupied the most superficial layer of the dorsal horn, consistent with previous studies (Nornes and Carry, 1978; Nornes and Das, 1974). However, unlike previous studies in which [^{3}H]thymidine autoradiography was used, our

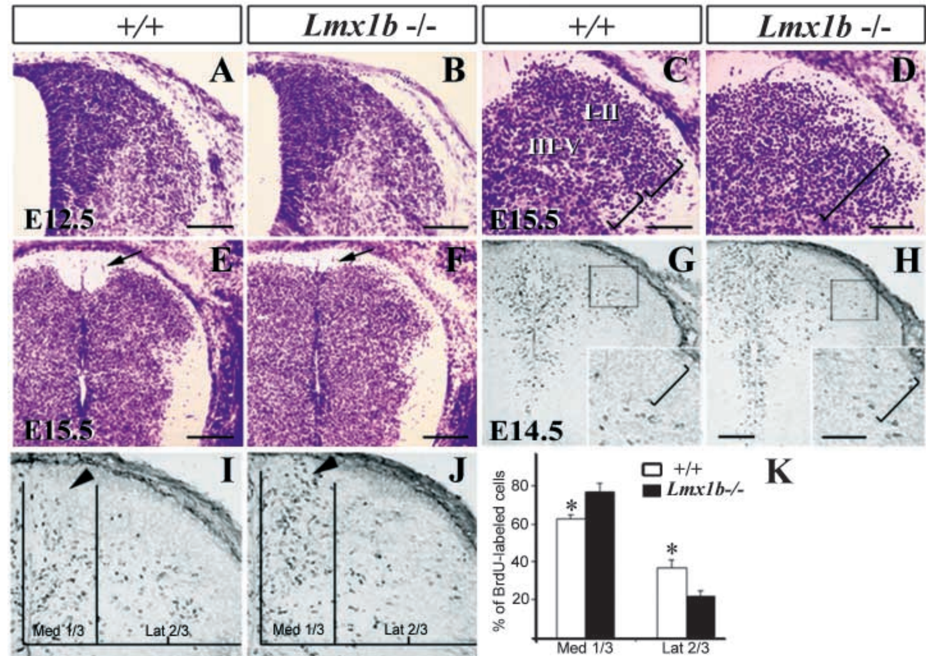

Fig. 2. Morphology, distribution and migration of wild-type and *Lmx1b* mutant dorsal horn neurons. (A,C,E) Nissl staining of wild-type embryos. (B,D,F) Nissl staining of *Lmx1b*$^{-/-}$ embryos. (A,B) At E12.5, no major difference was found between wild-type (A) and mutant embryos (B). (C,D) At E15.5, in wild-type, laminae I-II and laminae III-V are distinguishable, brackets outline the domain of laminae I-II and III-V, respectively. In *Lmx1b*$^{-/-}$ embryo, no clear boundary between laminae I-II and III-V exists (bracket outlines the whole domain). (E,F) Lower magnification of C,D. In wild-type embryo, the dorsal funiculus is enlarged (E, arrow), whereas in *Lmx1b* mutant it is smaller (F, arrow). (G) Wild-type embryo at E14.5: neurons labeled with BrdU at E11.5 are not detected in the outer aspect of laminae I-II (inset indicating a high magnification). Bracket outlines the most superficial layer. (H) *Lmx1b* mutant embryo at E14.5: neurons labeled with BrdU at E11.5 are detected in the most superficial layer (bracket in the insert). (I,J) Higher magnification view of G,H showing the dorsal horn that is divided into the medial one-third (Med 1/3) and lateral two-thirds (Lat 2/3) region by lines for quantitative analysis. (K) Quantitative comparison of BrdU-labeled neurons in the medial one-third region and lateral two-thirds region between wild-type (white bar) and *Lmx1b*$^{-/-}$ embryos (black bar). Asterisks indicate significant difference using Student's *t*-test $P<0.0001$. Scale bars: 100 μm in A-D,H; 200 μm in E,F; 60 μm in inset in H.

BrdU labeling clearly revealed an inside-out migration pattern for laminae I-II neurons. We next examined the migration of BrdU-labeled neurons in *Lmx1b* mutants. First, we did not find any significant change in the total number of BrdU-labeled neurons between wild-type and *Lmx1b* mutants (data not shown). However, some neurons labeled with BrdU at E11.5 were present in the most superficial region of the dorsal horn of E14.5 *Lmx1b*$^{-/-}$ embryos (Fig. 2H), while a significantly higher number of BrdU$^+$ neurons were accumulated near the midline region as compared with the control (Fig. 2I-K). In line with this observation, fewer neurons labeled with BrdU at E11.5 were located in the lateral region of the dorsal horn in *Lmx1b*$^{-/-}$ embryos (Fig. 2J). This altered distribution of dorsal neurons was not due to abnormal neuronal death, as the TUNEL staining pattern was similar in both *Lmx1b*$^{-/-}$ and wild-type embryos between E11.5 and E15.5 (data not shown). Together, these results indicate that there is a major defect in the migration of neurons in the dorsal horn of *Lmx1b*$^{-/-}$ embryos.

Lmx1b controls *Drg11, Rnx* and *Ebf1*, and *Ebf3* expression in the dorsal horn

To further characterize the dorsal horn defects in *Lmx1b* mutants, we examined the expression profile of dorsal horn neuron molecular markers *Drg11*, *Ebf1*, *Ebf2*, *Ebf3* and *Rnx*. In *Lmx1b*$^{-/-}$ embryos, *Drg11* expression was completely abolished from the normal onset of its expression (Fig. 3A,B; see Fig. S1 at http://dev.biologists.org/supplemental). At E11.5, *Ebf1, Ebf2, Ebf3* and *Rnx* appeared to be normally expressed in *Lmx1b*$^{-/-}$ embryos (see Fig. S1 at http://dev.biologists.org/supplemental). However, by E12.5, *Ebf3* and *Rnx* expression levels were lower in *Lmx1b* mutants (see Fig. S1) and by E15.5, *Ebf3* expression was completely absent in the *Lmx1b* mutants (Fig. 3C,D). At this stage, in wild-type embryos, *Ebf1* was most strongly expressed in laminae I-II neurons and weakly in laminae III-IV neurons. By contrast, *Ebf1* expression was markedly reduced in *Lmx1b* mutant embryos (Fig. 3E,F). Similarly, *Rnx* was dramatically downregulated in the *Lmx1b* mutants (Fig. 3G,H).

The complete absence of expression of *Drg11* and *Ebf3* expression in *Lmx1b* mutants promoted us to examine whether *Lmx1b* is able to induce the expression of these two genes in *Lmx1b* mutants. To address this question, *Lmx1b*-expression vectors were introduced into the dorsal spinal cord of *Lmx1b* mutant embryos by the use of in utero electroporation (Saba et al., 2003). Although the expression of *Drg11* and *Ebf3* was absent in the dorsal horn of *Lmx1b*$^{-/-}$ mutants electroporated with green fluorescent protein expression vectors (Fig. 3J,K; data not shown), *Drg11* and *Ebf3* expression was detected in the dorsal horn of *Lmx1b*$^{-/-}$ mutants electroporated with *Lmx1b*-expression vectors (Fig. 3L,M,O,P). The contralateral unelectroporated side of the dorsal horn showed no *Drg11* and *Ebf1* expression (Fig. 3L,N,O,Q). We observed that the induced expression was considerably weaker when compared with the endogenous expression, which we attribute to the inefficiency of electroporation. Because of the presence of the residual expression of *Rnx* and *Ebf1* in the dorsal horn of *Lmx1b* mutants, it was difficult to determine whether *Lmx1b* is capable of activating these two genes in the mutants. Nevertheless, these data demonstrate that *Drg11* and *Ebf3* act downstream of *Lmx1b* in the developing dorsal horn, and *Lmx1b* is required either for initiating or maintaining expression of these two genes in the dorsal horn.

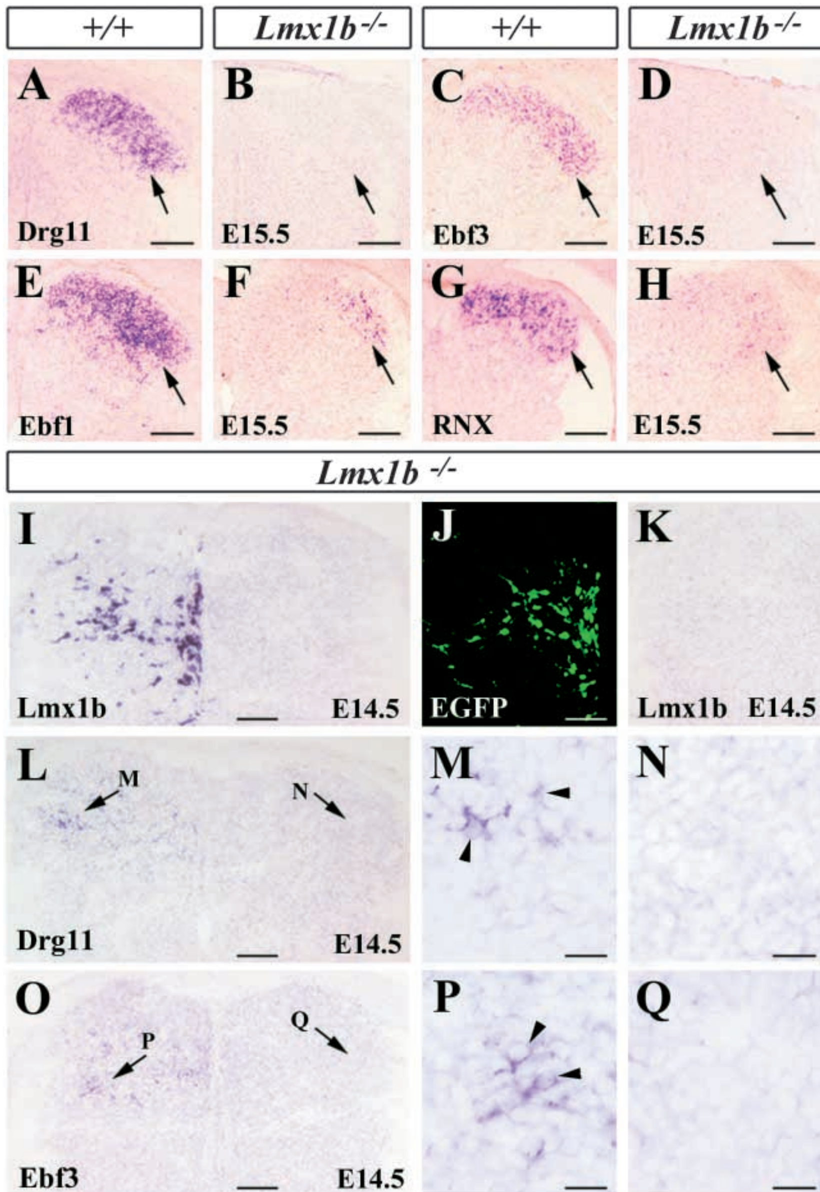

Fig. 3. Expression of laminae I-II markers and rescue of *Drg11* and *Ebf3* expression by forced expression of *Lmx1b* in the dorsal horn of *Lmx1b*$^{-/-}$ embryos. (A,B) *Drg11* expression in the dorsal horn of wild-type (A, arrow) and *Lmx1b*$^{-/-}$ (B, arrow) embryos. (C,D) *Ebf3* expression is restricted to laminae I-II neurons in wild-type dorsal horn (arrow in C), but is absent in *Lmx1b* mutant embryos (arrow in D). (E,F) In wild-type embryos (E) *Ebf1* is concentrated in laminae I-II (arrow in E,F), but its expression is markedly reduced in *Lmx1b* mutant embryos (F, arrow). (G,H) *Rnx* expression in laminae I-II neurons (arrows) of wild-type (G) and *Lmx1b* mutant embryos (H). (I,L-Q) Reactivation of *Drg11* and *Ebf3* expression in *Lmx1b*$^{-/-}$ spinal cord by exogenously introduced *Lmx1b*. (I) *Lmx1b* expression in *Lmx1b*$^{-/-}$ spinal cord after electroporation of *Lmx1b*-expression vectors. No *Lmx1b* was found in the contralateral side of the spinal cord. (J,K) Expression of EGFP control plasmids in *Lmx1b*$^{-/-}$ spinal cord. (K) No *Lmx1b* was detected in the electroporated side of *Lmx1b*$^{-/-}$ spinal cord after electroporation of EGFP. (L) Induction of *Drg11* (arrow) in *Lmx1b*$^{-/-}$ spinal cord electroporated with *Lmx1b* expression vectors. No *Drg11* staining was found in the contralateral side of the spinal cord (arrow). (M,N) Higher magnification of the regions indicated by arrows in L. Arrowheads indicate *Drg11*$^{+}$ cells. (O) Induction of *Ebf3* expression (arrow) in the dorsal horn of *Lmx1b*$^{-/-}$ embryos after electroporation. (P,Q) Higher magnification of the regions indicated by arrows in O. Arrowheads in P indicate *Ebf3*$^{+}$ cells. Scale bars: 100 μm in A-H,I-K,L,O; 30 μm in M,N,P,Q.

Lmx1b-independent dorsal horn-specific transcription factors

The expression of *Lbx1*, a lamina IIi-III marker which acts upstream of *Lmx1b* was examined in wild-type and *Lmx1b* mutant embryos (Fig. 4G,H). Although a few LBX1^{+} neurons were found in the most superficial layer of the *Lmx1b*$^{-/-}$ dorsal horn, no significant difference in the number of LBX1^{+} neurons was detected between the *Lmx1b* mutants and wild-type controls (Fig. 4I). The expression of *Brn3a*, a laminae III-V marker (Fig. 4J), was examined in *Lmx1b* mutants (Fig. 4K). Although a seemingly higher number of BRN3A^{+} neurons were located more medially in *Lmx1b* mutants (Fig. 4K), neuronal counts revealed no difference in the number of BRN3A^{+} neurons between wild-type and *Lmx1b* mutants (Fig. 4L).

In the dorsal horn, *Lmx1b* and *Pax2* expression is mutually exclusive (Gross et al., 2002). To determine whether the loss of *Lmx1b* affects *Pax2* expression, PAX2^{+} neurons in the dorsal horns of the wild-type and *Lmx1b*$^{-/-}$ embryos were counted. No significant change in the number of PAX2^{+} neurons was detected between wild-type and *Lmx1b* embryos (Fig. 4M-O).

Hox6 and *Hox8* are homeobox genes that are expressed in laminae I-II (Graham et al., 1991). In *Lmx1b* mutants, *Hoxb6* and *Hoxb8* expression domains appeared to have expanded (Fig. 4A,B,D,E). Although the expansion of the *Hoxb6* and *Hoxb8* domains could be attributed to an abnormal location of laminae I-II neurons in the deep dorsal horn, it is also possible that the expression of *Hoxb6* and *Hoxb8* is upregulated in laminae III-V neurons in the absence of *Lmx1b* function. If it were the latter case, the total number of *Hoxb6* and *Hoxb8* neurons would increase in the mutants. However, no significant difference in the number of *Hoxb6* and *Hoxb8* neurons was evident between wild-type and mutant animals (Fig. 4C,F). Our data thus suggest that abnormal distribution of *Hox6/8* neurons is due to migration defects.

Together, these results suggest that expression of *Hox6, Hox8*, *Lbx1, Brn3a* and *Pax2* is *Lmx1b* independent; however, the distribution pattern of neurons expressing these markers appears changed in *Lmx1b* mutants.

Lmx1b, *Rnx* and *Drg11* repress *Zic1* and *Zic4* genes

We next asked whether there is a causal relationship between the expression of Zic genes and *Lmx1b* as *Zic1* appears to be

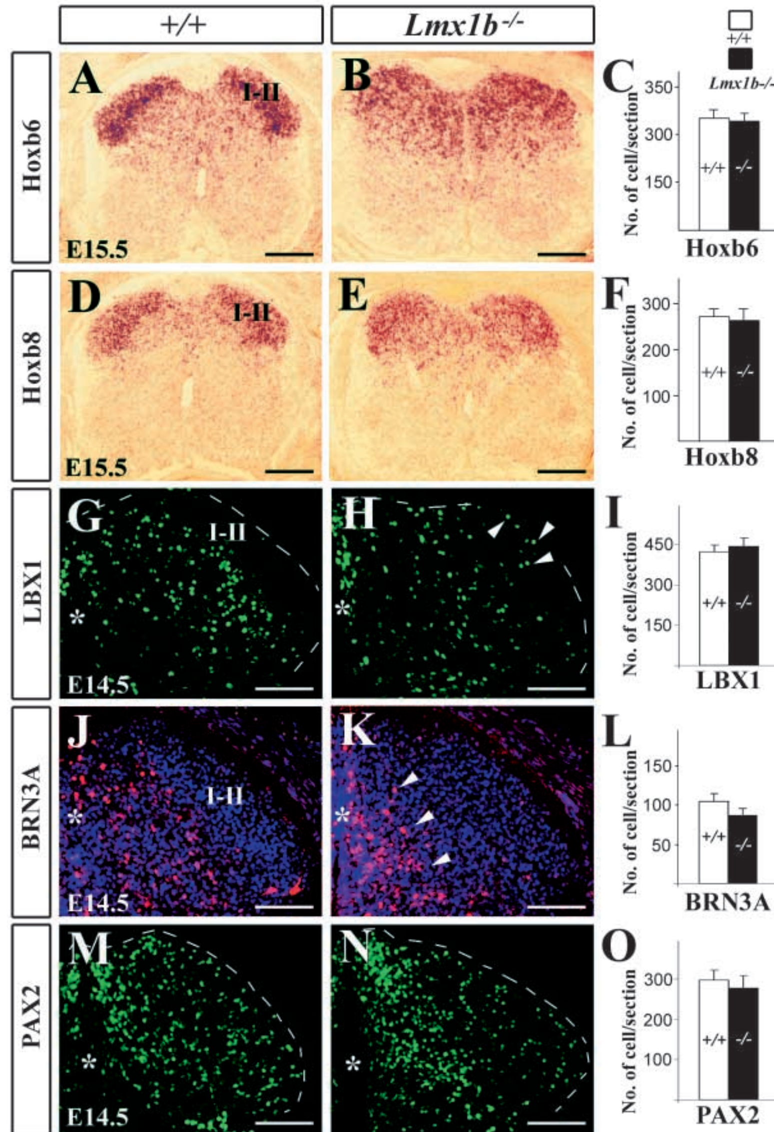

Fig. 4. Expression of dorsal horn-specific molecular markers in wild-type and *Lmx1b*$^{-/-}$ embryos at E14.5 (G-N) and E15.5 (A-E). (A,B) *Hoxb6* expression in wild-type (A) and *Lmx1b* mutant embryos (B). (D,E) Spinal cord *Hoxb8* expression is mainly evident in laminae I-II of wild type (D); however, in *Lmx1b* mutant embryo (E) its expression appears expanded ventrally. (C,F) Quantitative analysis of *Hoxb6*$^{+}$ (C) and *Hoxb8*$^{+}$ (F) neurons in wild-type (white bar) and *Lmx1b*$^{-/-}$ embryos (black bar). (G,H) Expression of *Lbx1* in wild-type (G) and *Lmx1b* mutant embryos (H). Broken white lines outline the dorsal horn and arrowheads indicate mispositioned LBX1^{+} neurons in *Lmx1b* mutant embryos. (I) Quantitative analysis of the number of LBX1^{+} neurons between wild-type and *Lmx1b* mutant embryos. (J,K) BRN3A^{+} expression in wild-type (J) and *Lmx1b*$^{-/-}$ embryos (K) at E14.5. Arrowheads indicate the accumulation of BRN3A^{+} neurons around the midline (asterisk). (L) Quantitative comparison of the total number of BRN3A^{+} neurons between wild-type and *Lmx1b*$^{-/-}$ embryos. (M,N) PAX2 expression in wild-type (M) and *Lmx1b* mutant embryos (N). (O) Numbers of PAX2^{+} neurons in wild-type and *Lmx1b* mutant embryos. Asterisks indicate the midline region. Scale bars: 200 μm in A,B,D,E; 100 μm in G,H,J,K,M,N.

required for maintaining the progenitor state of undifferentiated dorsal neurons by repressing the differentiation of spinal cord progenitors (Aruga et al., 2002b; Ebert et al., 2003).

In wild-type embryos, three Zic genes (*Zic1, Zic2* and *Zic4*) are expressed in the developing dorsal spinal cord (Fig. 5). Their expression is mainly concentrated around the midline region, suggesting that the Zic genes may play a role in modulating the differentiation of the dorsal horn neurons. Strikingly, in *Lmx1b*$^{-/-}$ mutants, while *Zic2* expression remained unaltered (Fig. 5E,F), expression of *Zic1* and *Zic4* was significantly upregulated (Fig. 5A-D). This was observed not only around the midline region, but also in the superficial layer of the dorsal horn where numerous laminae I-II neurons were present but failed to differentiate further at a later stage (Fig. 5B,D). An upregulation of *Zic1* and *Zic4* was also detected in *Rnx* mutants (Fig. 5H,J).

The maturation and differentiation of lamina I-II neurons is impaired in *Lmx1b*$^{-/-}$ mutants

The aberrant migratory behavior and altered gene expression profile of the dorsal horn neurons in *Lmx1b* mutants could also be viewed as the evidence of impaired maturation and differentiation of laminae I-II neurons in the absence of *Lmx1b*. To further examine the cellular property of laminae I-II neurons, immunocytochemical staining using anti-MAP2 antibody was performed. MAP2 is a microtubule-associated protein expressed in the dendrites, axons and somata of neurons (Lewis et al., 1986). During development, the onset of MAP2 expression concurs with the differentiation, maturation and lamination of laminae I-II neurons. In the dorsal horn of E15.5 wild-type embryos, MAP2 expression was prominent in laminae I-II neurons, especially in the dendrites (Fig. 6A,C). By contrast, MAP2 expression was much weaker in the superficial layer of the *Lmx1b* mutant (Fig. 6B,D). One possible explanation for the weak expression of MAP2 is that MAP2^{+} laminae I-II neurons could be aberrantly located in the deeper laminae of *Lmx1b* mutants. However, no obvious increase of MAP2 staining in these regions was found in *Lmx1b* mutants (Fig. 6A-D). Therefore, it is most likely that MAP2 expression is largely reduced in laminae I-II neurons of *Lmx1b* mutants.

Rnx and Drg11 mutants exhibit an aberrant migration and differentiation of laminae I-II neurons

The downregulation of *Rnx* and the loss of *Drg11* expression in *Lmx1b* mutants suggest that *Lmx1b* may control some aspects of the migration and differentiation via *Rnx* and *Drg11*. A previous study using BrdU labeling detected no migration deficit in the dorsal horn of *Rnx* mutants (Qian et al., 2002). We revisited this issue by examining the inside-out pattern of the dorsal horn neurons in *Rnx* and *Drg11* mutants and found that neurons labeled with BrdU at E11.5 were detected in the superficial region of the dorsal horn of *Rnx* and *Drg11* mutants (Fig. 7A,B,D,E). Quantitative analysis indicated that in *Rnx*

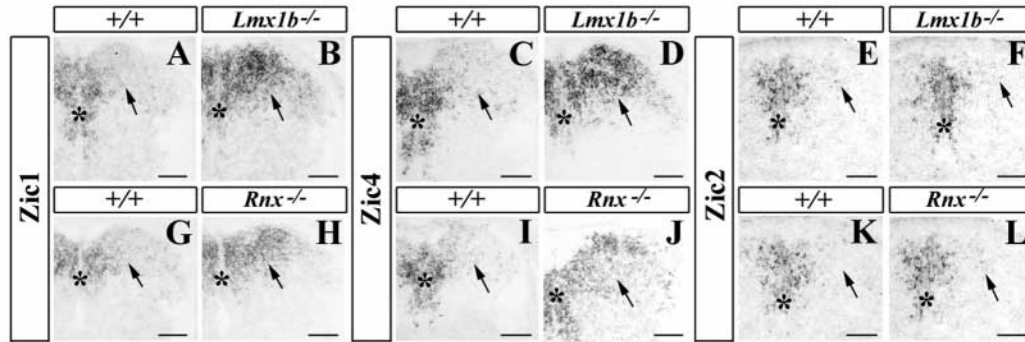

Fig. 5. Comparison of *Zic1, Zic2* and *Zic4* expression in the dorsal horns of wild-type, *Lmx1b*$^{-/-}$ and *Rnx*$^{-/-}$ embryos at E14.5. (A,B,G,H) *Zic1* expression in wild-type (A,G), *Lmx1b* mutant (B) and *Rnx* mutant embryos (H). (C,D,I,J) *Zic4* expression in wild-type (C,I), *Lmx1b* mutant (D) and *Rnx* mutant embryos (J). (E,F,K,L) *Zic2* expression in wild-type (E,K), *Lmx1b* mutant (F) and *Rnx* mutant (L) embryos. Arrows indicate the dorsal horn regions. Asterisks indicate the midline region. Scale bars: 100 μm.

and *Drg11* mutants there were more BrdU^{+} neurons accumulating in the medial one third of the dorsal horn when compared to controls, whereas fewer BrdU^{+} neurons were found in the lateral two-thirds of the dorsal horn (Fig. 7C,F). Immunocytochemical staining shows that MAP2 expression was also reduced in the superficial dorsal horn of both mutants (Fig. 7G,H,I,J). Nevertheless, the abnormalities detected in *Drg11* mutant are less severe than those in *Rnx* mutants.

Aberrant projection of TrkA^{+} afferent fibers into the dorsal horn of *Lmx1b* mutants

We reasoned that the impaired development of laminae I-II neurons in *Lmx1b* mutants could influence the projections of the primary cutaneous afferents which innervate the dorsal horn (Sharma and Frank, 1998). To address this possibility, we examined the projections of TrkA^{+} fibers into laminae I-II (Huang et al., 1999). At E14.5, in wild-type mice, TrkA^{+} afferents had begun to ramify within laminae I-II of the dorsal horn, and immunostaining showed densely stained laminae I-II, an indication of innervation by primary cutaneous afferents (Ozaki and Snider, 1997) (Fig. 8A). Strikingly, although *Lmx1b* mutants did not show apparent defects in projections

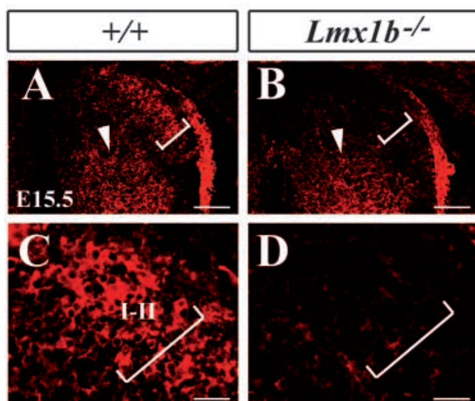

Fig. 6. MAP2 expression in laminae I-II neurons in the dorsal horn of wild-type and *Lmx1b*$^{-/-}$ embryos at E15.5. (A,B) MAP2 staining in laminae I-II of wild-type (bracket in A) and *Lmx1b* mutant embryos (bracket in B). (C,D) Higher magnification of A,B, respectively. Scale bars: 100 μm in A,B; 20 μm in C,D.

of muscle afferents, as examined by anti-Peripherin antibody staining (Goldstein et al., 1991) (Fig. 8C,D), no TrkA^{+} afferents were detected within the gray matter of the dorsal horn of *Lmx1b* mutants (Fig. 8B).

We next analyzed the sensory afferent projection by DiI labeling of DRG neurons. At E14.5, in wild-type controls, projections to laminae I-IV were intensely labeled with DiI after its application to DRG cell bodies (Fig. 8E). The muscle sensory afferents that project towards the ventral horn were also labeled (Fig. 8E). In *Lmx1b* mutants, while the presumptive muscle sensory afferent projections appeared normal, there was a dramatic decrease in the intensity of DiI labeling in laminae I-II, although some DiI-labeled DRG afferents appeared to be present in laminae III-IV (Fig. 8F). Together, these results suggest that DRG afferent projection defects in *Lmx1b* mutants are specific to TrkA^{+} sensory afferents.

Stereotyped expression of attractive or repulsive axonal guidance molecules is crucial to guiding the precise axonal growth of neurons in the developing nervous system (Tessier-Lavigne and Goodman, 1996). To determine whether there was a change of expression of axonal guidance cues/molecules in the dorsal horn of *Lmx1b* mutants, we examined their expression by in situ hybridization. *Sema3a* is a secreted cell-surface protein that functions as a chemorepellent in the projection of cutaneous afferents in the spinal cord of the chick and the mouse (Luo et al., 1993; Messersmith et al., 1995). Its expression, however, was normal in *Lmx1b* mutants (data not shown). We also found that *Sema3c* (Feiner et al., 2001) was completely lost in the dorsal horn of *Lmx1b* mutants (Fig. 8G,H). By contrast, expression of the receptors for *Sema3a*, neuropilin 1 (*Nrp1*) and plexin A2 (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Takahashi et al., 1999), were markedly reduced in the mutants (Fig. 8I-L). We also examined the expression of the members of the Slit family, which have been implicated in axonal guidance and neuronal migration (Brose and Tessier-Lavigne, 2000; Wong et al., 2002). *Slit1* and *Robo2* were most strongly expressed in laminae I-II (Fig. 8M,O). In the mutants, *Slit1* and *Robo2* expression was dramatically reduced (Fig. 8N,P). In addition, netrin 1 expression was largely lost in the medial region of the deep dorsal horn of *Lmx1b* mutants (Serafini et al., 1994) (Fig. 8Q,R). Thus, the altered expression of multiple axonal

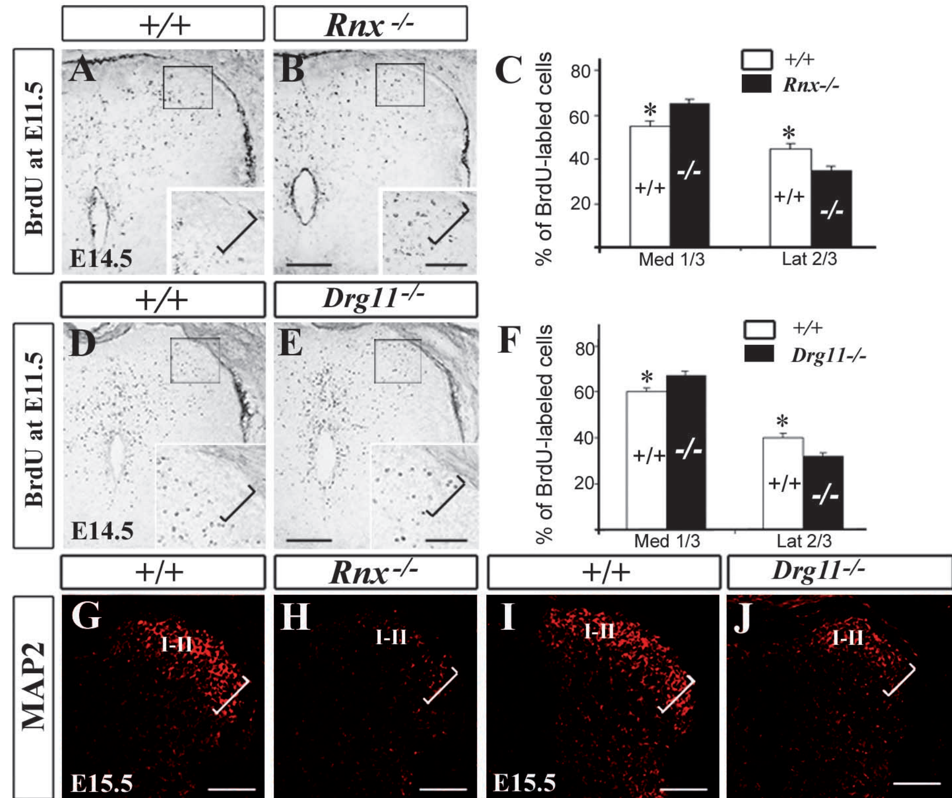

Fig. 7. Aberrant migration and defective differentiation of the dorsal horn neurons in wild-type and *Rnx*$^{-/-}$ and *Drg11*$^{-/-}$ mutant embryos. (A) Neurons labeled with BrdU at E11.5 are not detected in the outer layer of laminae I-II (bracket) in wild-type embryo at E14.5 (inset). (B) Neurons labeled with BrdU at E11.5 are detected in the outer layer of laminae I-II in *Rnx*$^{-/-}$ embryo (inset, bracket). (C) Quantitative comparison of the number of BrdU$^+$ neurons in the medial one-third (Med 1/3) and lateral two-thirds (Lat 2/3) regions between wild-type and *Rnx*$^{-/-}$ embryos (**P*<0.001). (D,E) Comparison of the distribution pattern of neurons labeled with BrdU at E11.5 in the dorsal horn of E14.5 wild-type (D) and *Drg11* mutants (E). (F) Quantitative analysis of the numbers of BrdU-labeled neurons in the medial and lateral regions of wild-type and *Drg11* mutant embryos (**P*<0.001). (G,H) MAP2 expression in the dorsal horn of wild-type (G, bracket) and *Rnx* mutant embryos (H, bracket). MAP2 expression of the dorsal horn in wild-type (I, bracket) and *Drg11*$^{-/-}$ mutant embryos (J, bracket). Scale bars: in B and C, 100 µm for A and D, respectively; 50 µm in insets in B and E; 100 µm in G-J.

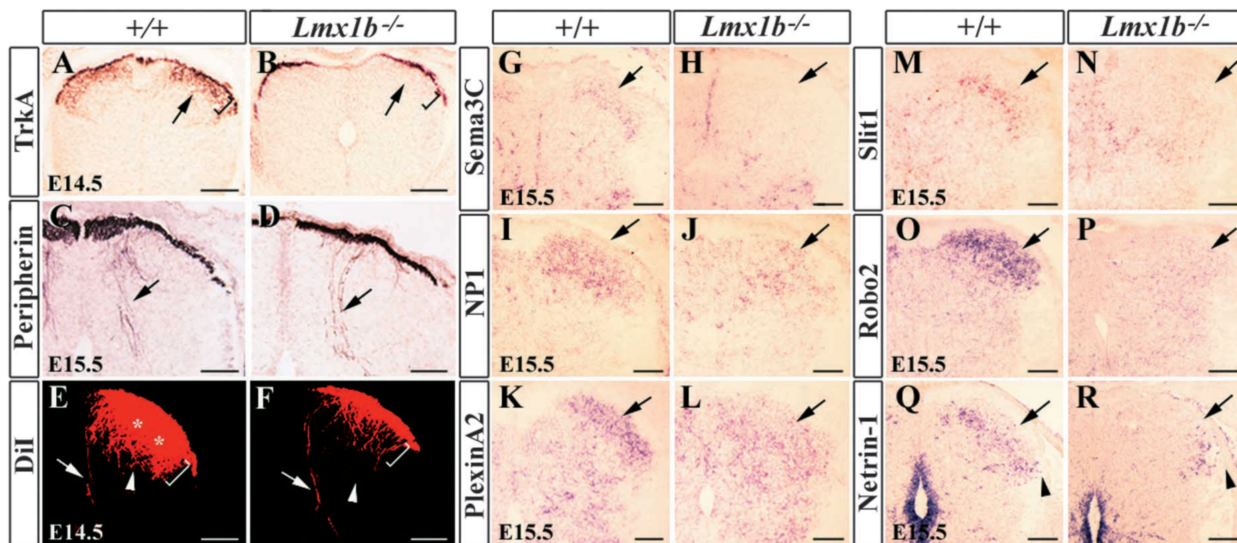

Fig. 8. Selective block of the ingrowth of cutaneous afferents in the dorsal spinal cord of *Lmx1b*$^{-/-}$ mutant embryos at E14.5. (A,B) Immunocytochemical detection of TrkA in the dorsal horn of wild-type (A) and *Lmx1b*$^{-/-}$ mutant (B) embryos. Arrows indicate the superficial laminae region. Brackets outline laminae I-II region. (C,D) Peripherin detection in the dorsal horn of wild-type (C) and *Lmx1b* mutant embryos (D) appears unchanged. Arrows indicate peripherin-labeled presumptive proprioceptive afferents. (E,F) DiI labeling of primary afferents in the dorsal horn of wild-type (E) and *Lmx1b* mutant embryos (F). Arrowheads indicate presumptive mechanoreceptor afferents and arrows indicate presumptive muscle proprioceptive afferents. Asterisks and brackets outline laminae I-II region. (G,H) *Sema3c* expression in wild-type (G) and *Lmx1b* mutant embryos (H). (I,J) *Nrp1* expression in wild-type (I) and *Lmx1b* mutant embryos (J). (K,L) Plexin A2 expression in wild-type (K) and *Lmx1b* mutant embryos (L). (M,N) *Slit1* expression in wild-type (M) and *Lmx1b* mutant embryos (N). (O,P) *Robo2* expression in wild-type (O) and *Lmx1b* mutant embryos (P). (Q,R) *Netrin 1* expression in wild-type (Q) and *Lmx1b* mutant embryos (R). Arrows in G-S indicate laminae I-II. Arrowheads in Q and R indicate the lateral region of laminae I-II. Scale bars: 200 µm in A, B; 100 µm in C-R.

guidance molecules could account for the failure of cutaneous sensory axon ingrowth in *Lmx1b* mutants.

Discussion

Lmx1b is required for the specification of the dI5 neuronal fate

Recent studies have begun to identify important players such as *Math1*, *Lbx1* and *Rnx* in the development of early-born postmitotic dorsal neurons (Bermingham et al., 2001; Gross et al., 2002; Muller et al., 2002). In this study, we find that dI5 interneurons can be further divided into two subgroups: one that expresses *Phox2a* and one that does not. *Phox2a* is a homeobox gene and has been shown to be an important determinant of several neuronal phenotype in the nervous system (Brunet and Pattyn, 2002). The seemingly normal expression of *Lbx1* and *Brn3a* expression in dI5 neurons of *Lmx1b* mutants supports the idea that these two genes may act upstream of *Lmx1b,* whereas the partial loss of *Phox2a* indicates that *Phox2a* lies either downstream of or in parallel to *Lmx1b*. It will be interesting to examine whether *Phox2a* plays a role in the development of dI5 neurons, and whether *Lbx1* and *Rnx* may specify the fate of dI5 neurons through an *Lmx1b-Phox2a* pathway.

Lmx1b controls the ingrowth of cutaneous afferents into the dorsal horn

In *Lmx1b* mutants, the selective blocking of cutaneous afferent ingrowth raises the possibility that *Lmx1b* may coordinate the projection of TrkA$^+$ afferents into the dorsal horn by regulating local axonal guidance cue(s). Altered ingrowth of TrkA$^+$ afferents is also found in *Lbx1* mutants and *Rnx/Tlx1* double mutants (Gross et al., 2002; Muller et al., 2002; Qian et al., 2002). Nevertheless, unlike *Lbx1* and *Rnx*, *Lmx1b* is not expressed in DRG neurons, therefore, the TrkA guidance defect most probably resides in the dorsal horn.

The specific block of the entry of TrkA$^+$ afferents suggests that certain attractants may be missing in the mutants. Alternatively, the expression level of some repulsive molecules may be increased to repel the cutaneous afferents. Although these possibilities exist, no evidence for increased expression of any repellants examined in *Lmx1b* mutants was found. Given that multiple axonal guidance cues are expressed in the dorsal horn and their expression is lost in *Lmx1b* mutants, it is possible that multiple axonal guidance molecules could work synergistically to coordinate the projections of cutaneous afferents. Future analysis of mice lacking multiple axonal guidance cues may be required to reveal the identity of the cues responsible for the ingrowth of TrkA$^+$ afferents in the dorsal spinal cord.

Lmx1b guides the differentiation and migration of the dorsal horn neurons

Our study suggests that *Zic1, Zic4, Ebf1* and *Ebf3* function downstream of *Lmx1b* and *Rnx*. Forced expression of *Zic1* in chicks represses the differentiation of *Math1*-expressing neurons in the dorsal neural tube (Ebert et al., 2003). Transgenic mice overexpressing *Zic1* exhibit inhibited neuronal differentiation with extension of the progenitor state in the dorsal spinal cord (Aruga et al., 2002b). Loss of *Zic1* in mice also leads to premature expression of the βIII tubulin in the dorsal spinal cord (Aruga et al., 2002b). In the context of these findings, the Zic gene expression upregulation is interesting, and consistent with the migration and differentiation defects in *Lmx1b* mutants. *Zic1* could be an important component of the dorsal spinal cord neuron differentiation pathway.

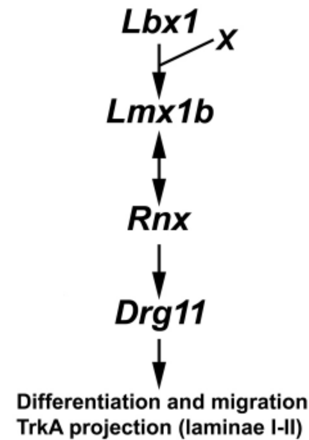

Fig. 9. Schematic diagram summarizing the transcriptional cascade that controls the development of laminae I-II circuitry. In this cascade, we hypothesize that an unidentified transcription factor X, cooperating with *Lbx1*, activates and maintains *Lmx1b* expression, which in turn maintains *Rnx* expression. *Lmx1b* most probably activates *Drg11* expression directly, whereas *Rnx* helps to maintain *Drg11* expression. *Rnx* is also required for maintaining *Lmx1b* expression. *Lmx1b* acts in part through *Rnx* and *Drg11*, and in part through other unknown genes to repress *Zic1* and *Zic4* expression (not shown). *Lmx1b* and *Rnx* maintain *Ebf1* and *Ebf3* expression in laminae I-II neurons (not shown). Together these transcription factors coordinate the migration and differentiation of the superficial dorsal horn neurons, and subsequent innervation of TrkA$^+$ afferents.

The role of *Zic4, Ebf1* and *Ebf3* in the development of the dorsal horn is unknown. Whether dysregulation of these genes plays a causal role in aberrant development of the dorsal horn also remains to be determined. Together with previous studies, we hypothesize that the *Lbx1/Lmx1b/Rnx/Drg11* pathway represents a major pathway to control the differentiation and migration of laminae I-II neurons and subsequent projection of cutaneous afferents (Fig. 9). The effects of *Lmx1b* and *Rnx* may be mediated in part through the *Drg11,* Ebf and Zic genes and in part via other unidentified transcription factors to control the assembly of the dorsal horn circuits.

The present study reveals the complicated relationship between *Lmx1b* and other transcription factors in the development of the dorsal spinal cord. For example, *Lmx1b* and *Rnx* may depend on each other for their normal expression. Although *Lmx1b* mutants recapitulate many aspects of the phenotype of *Rnx* mutants, there are noted differences in the level of regulation of downstream genes such as *Zic1* and *Drg11*. Thus, in addition to the common sets of downstream targets, *Lmx1b* and *Rnx* may control additional distinct set of target genes.

The finding that an inside-out migration pattern and a normal differentiation program are disrupted in the dorsal horn of *Lmx1b* mutants raises the question of whether aberrant migratory behavior could be attributed to aberrant differentiation of the dorsal horn neurons or vice versa.

Although we are not able to determine when the migration deficit first occurs in *Lmx1b* mutants, studies of the possible downstream targets of *Lmx1b* shed light onto this issue. For example, an alteration of axonal guidance cues such as netrin 1 in *Lmx1b* mutants might have contributed to aberrant neuronal migration (Brose and Tessier-Lavigne, 2000). Moreover, some of the transcription factors downstream of *Lmx1b* have also been shown to be required for neuronal migration. In mice, *Ebf1* is important for the migration of facial branchiomotor neurons (Garel et al., 2000). In chicks, *Ebf1* and *Ebf3* appear to control migration and differentiation of the dorsal neurons independently (Garcia-Dominguez et al., 2003; Garel et al., 2000). Thus, *Lmx1b* may have a unique role in neuronal migration in the developing spinal cord. However, this does not exclude the possibility that aberrant migration reflects some aspects of impaired differentiation of the dorsal horn neurons in the absence of *Lmx1b.* In fact, in addition to the dorsal horn cells, *Lmx1b* has also been implicated in neuronal differentiation of dopaminergic neurons and serotonergic neurons in the developing brain (Cheng et al., 2003; Ding et al., 2003; Pressman et al., 2000). Thus, it is likely that *Lmx1b* could play an important role in both cellular events.

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