Control of roof plate formation by Lmx1a in the developing spinal cord

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Summary

Numerous studies have identified the roof plate as an embryonic signaling center critical for dorsal central nervous system patterning, but little is known about mechanisms that control its formation and its separation from clonally related neural crest cells and dI1 sensory interneurons. We demonstrate that the LIM homeodomain transcription factor, *Lmx1a*, mutated in the *dreher* mouse, acts to withdraw dorsal spinal cord progenitors from the cell cycle and simultaneously direct their differentiation into functional roof plate cells. Lmx1a cell-autonomously represses the dI1 progenitor fate, distinguishing the roof plate and dI1 interneuron programs, two major developmental programs of the dorsal neural tube. Lmx1a is not directly involved in neural crest development. We establish that Bmp signaling from epidermal ectoderm is necessary and sufficient for inducing Lmx1a and other co-factors that also regulate the extent of roof plate induction. We conclude that Lmx1a controls multiple aspects of dorsal midline patterning and is a major mediator of early Bmp signaling in the developing spinal cord.

Supplemental data available online

Key words: Lmx1a, *dreher*, Roof plate, Bmp, Neural crest, Developing spinal cord, Chick, Mouse

Introduction

During development of the vertebrate central nervous system (CNS), distinct classes of neurons are generated at stereotypical positions in response to inductive signals provided by local cell groups that act as organizing centers (Harland and Gerhart, 1997; Goulding and Lamar, 2000; Shirasaki and Pfaff, 2002). In the developing dorsal neural tube, a small group of specialized non-neural cells, the roof plate, serves as a signaling center (Lee and Jessell, 1999). Although numerous experiments have clearly shown the importance of roof plate as a critical center for dorsal CNS patterning, little is known about molecular mechanisms involved in specification of the roof plate itself. Roof plate cells are generated from multipotent mitotically active precursors in the neural folds and early neural tube as a result of interaction between neural tissue and non-neural ectoderm. This region also gives rise to two other cell types: neural crest cells, which have been shown to share a common progenitor with roof plate, and later to dI1 dorsal interneuronal precursors (Bronner-Fraser and Fraser, 1988; Echelard et al., 1994; Lee and Jessell, 1999; Helms and Johnson, 2003). The close association of roof plate formation to the development of neural crest cells and dI1 interneurons has made it extremely difficult to identify the molecular pathways specifically involved in the specification of each cell fate.

The search for molecular signals involved in dorsal neural cell type specification has identified several secreted candidate molecules, including Bmp4/7 and Wnt1 (Liem et al., 1997; Garcia-Castro et al., 2002); however, their precise role in roof plate specification is poorly understood. Even less is known about intrinsic factors controlling the specification of roof plate cells. Only a few transcription factors, including Pax3, Pax7 and Msx1/2, have been implicated in roof plate development. Importantly, however, these molecules are also involved in the development of neural crest and dorsal interneurons (Lee and Jessell, 1999; Knecht and Bronner-Fraser, 2002). This suggests that other, roof plate-specific, intrinsic factors must separate the roof plate fate from other dorsal cell fates.

Recently we analyzed the spontaneous neurological mouse mutant *dreher*, which fails to develop roof plate, and identified inactivation of the LIM-homeodomain transcription factor *Lmx1a* as the cause of this phenotype (Millonig et al., 2000). This analysis demonstrated that Lmx1a is essential for either the induction or maintenance of roof plate cells. In this paper we demonstrate that Lmx1a is sufficient for withdrawing dorsal spinal cord progenitors from the cell cycle and simultaneously driving them to differentiate into functional roof plate cells in the dorsal neural tube. Interestingly, our data demonstrate that Lmx1a is not involved in suppression of neural crest cell fate. At the same time, Lmx1a represses Math1 and prevents generation of dI1 dorsal interneurons in a cell-autonomous fashion, thus distinguishing the roof plate and dI1 interneuronal programs in the dorsal neural tube. Finally, our results indicate that Bmps are necessary and sufficient for inducing Lmx1a and roof plate cells in the neural tube, and that Lmx1a is a major mediator of early Bmp signaling in developing spinal cord.

Materials and methods

Embryos

Fertilized White Leghorn eggs were incubated and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). *Dreher*^{J} mice (Lyons et al., 1996) were obtained from Jackson laboratory and maintained on a C3H/B6 mixed background.

Expression constructs

cDNAs encoding full-length mouse wild-type and *dreher*^{J} point mutation alleles of *Lmx1a* (Millonig et al., 2000), *Bmp4* and *Bmp7* were cloned upstream of IRES-EGFP in the pCIG expression vector (Megason and McMahon, 2002). *Bmp4* was also expressed using pMiwII-Bmp4 (Kishimoto et al., 2002). No differences between pCIG-Bmp4 and pMiw-Bmp4 were detected. Activated human *Bmp receptor Ia*, chick *Noggin*, human *dnTCF4* and mouse *dnWnt1* were expressed from pCAGGS-caBmpr-Ia (Timmer et al., 2002), a Noggin expression vector (L. Niswander, unpublished, a gift from L. Niswander), pCIG-dnTcf4 (Megason and McMahon, 2002) and pC1-neo-dnWn1 (Garcia-Castro et al., 2002). In all experiments an appropriate empty vector was used as a control.

In-ovo electroporation

Supercoiled plasmid DNA was injected into the lumen of the rostral neural tube of stage 10 embryos at a concentration of 1-2 mg/ml in water together with 50 μg/ml Fast Green dye (if the injected plasmid did not express EGFP, empty pCIG at a concentration of 0.2 mg/ml was added to the injected solution). Electroporation was performed as previously described (Megason and McMahon, 2002). The eggs were sealed and allowed to develop for a further 15-48 hours. Embryos with high levels of GFP fluorescence in the neural tube were processed for further analysis.

Neural explants

Intermediate neural plate explants from electroporated and control neural plates were isolated (Liem et al., 1995; Liu and Jessell 1998) and cultured in serum-free medium as described by Garcia-Castro et al. (2002). Explants of mouse neural tubes were dissected in dispase and cultured as described by Lee et al. (1998). Induction experiments were performed in the presence of 100 ng/ml human Bmp4 (R&D Systems).

Immunohistochemistry and in-situ hybridization

Immunohistochemistry was performed on frozen sections and in whole-mount as previously described (Helms and Johnson, 1998; Sela-Donenfeld and Kalcheim, 1999). Rabbit anti-Lmx1a antibody (M. German, unpublished) was a gift from M. German. Rabbit anti-LH2A/B (Lee et al., 1998) and rabbit anti-Maf B antibodies (Pouponnot et al., 1995) were provided by T. Jessell. The rabbit antibodies anti-Math1 (Helms and Johnson 1998), anti-Sox9 (Stolt et al., 2003) and anti-Lbx1 (Muller et al., 2002) were provided by J. Johnson, M. Wegner, and T. Muller and C. Birchmeier, respectively. Mouse anti-HNK-1 antibody was obtained from ATCC. Mouse anti-MitF and rabbit anti-GFP antibodies were purchased from Neomarkers and Biocompare, respectively. The following primary mouse antibodies were obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242): anti-Islet1 (51.4H9), anti-Lim1/2 (4F2), anti-Slug (62.1E6), anti-Ap2 (3B5), anti-RhoB (54.4H7), anti-Msx1/2 (4G1), anti-Pax7, anti-Pax6, anti-Math1 and anti-neurofilament (2H3). Secondary species appropriate antibodies with Texas Red, FITC or HRP conjugates were obtained from Jackson Immunological.

In-situ hybridization was performed on sections and in whole-mount as previously described (Kos et al., 2001; Helms and Johnson 1998) using digoxigenin-labeled riboprobes to chick *Gdf7*, *Bmp4*, *Wnt1* and rat *Isl1* provided by T. Jessell (*Gdf7* and *Isl1*), P. Brickell (*Bmp4*) and A. McMahon (*Wnt1*). Sections were digitally photographed with an AxioCam on a Zeiss AxioPlan 2 microscope and processed using Adobe Photoshop.

BrdU labeling

BrdU labeling was performed as previously described (Megason and McMahon 2002; Dickinson et al., 1994). For double labeling, frozen sections were first processed for immunostaining, then refixed and processed for BrdU staining with mouse anti-BrdU antibody (Zymed). Some sections were also counterstained with propidium iodine to visualize the tissue.

Measurements and statistical analyses

All sections analyzed were from the region between the forelimbs and hind limbs, except the section shown in Fig. 1A, which were taken at the hind limb level. All results described here were replicated in at least three independent embryos or explants. Analysis of the effect of ectopic expression of Lmx1a on the cellular distribution along the medio-lateral axis of the chick neural tubes was conducted as illustrated in Fig. 4C,D. The number of BrdU/Lmx1a double positive cells was counted using ten serial 12 μm transverse sections of the spinal cord of wild-type and *dreher* embryos. The Lmx1a positive area was calculated using these same BrdU/Lmx1a labeled sections by the method described by Dickinson et al. (1994). All quantitative data are expressed as the mean±s.e.m. Statistical significance was determined by two-tailed *t*-test. * indicates $P<0.01$.

Results

Lmx1a specifically marks roof plate progenitors and differentiated roof plate cells in both chick and mouse developing spinal cord

Extending previous studies (Millonig et al., 2000; Failli et al., 2002), we examined expression of Lmx1a protein in both chick and mouse developing spinal cord using rabbit polyclonal antibodies raised against a unique peptide in the amino terminal end of the hamster Lmx1a, CEENFQSAIETSASF (a gift from M. German). In both chick and mouse embryonic CNS, Lmx1a was first detected at the lateral edges of the closing neural tube (Fig. 1A and data not shown). Shortly after neural tube closure, Lmx1a-immunoreactive cells were specifically located in a restricted domain of the dorsal midline of the developing spinal cord (Fig. 1B-G) that gives rise to both roof plate cells and neural crest (Bronner-Fraser and Fraser, 1988; Echelard et al., 1994; Liem et al., 1997; Lee and Jessell, 1999; Helms and Johnson, 2003). Lmx1a expression persisted in the dorsal midline of chick and mouse embryos until at least stage 24 and E12.5, respectively, the latest stages investigated (Fig. 1I and data not shown). Co-immunohistochemistry showed that although chick prospective dorsal midline cells co-express Lmx1a and early neural crest markers such as Slug, Sox9, Ap2 and RhoB (Nieto et al., 1994, Mitchell et al., 1991; Liu and Jessell, 1998; Mori-Akiyama et al., 2003), Lmx1a expression was not detected in neural crest cells delaminating or migrating away from the dorsal neural tube (Fig. 1F and data not shown). At later stages, Lmx1a-immunoreactive cells at the dorsal midline lost expression of Slug, Sox9, Ap2 and RhoB and acquired typical morphology of roof plate cells (Fig. 1H and data not shown). Comparison of the expression of Lmx1a and an established roof plate marker, MafB (Liem et al., 1997), revealed that the expression domain of MafB completely overlaps with Lmx1a expression area in caudal dorsal midline, although MafB expression is not initiated in chick and mouse

until stage 15 and E9.75, respectively (Fig. 1D,G,I and data not shown). Finally, we compared expression of Lmx1a with bHLH transcription factors Math1 and Cath1 (chicken homolog of Math1), which mark progenitors of the dorsalmost interneurons, dI1 interneurons, in mouse and chick, respectively (Helms and Johnson, 1998; Gowan et al., 2001). This analysis revealed a complementary expression pattern of Lmx1a and these proteins in both chick and mouse (Fig. 6J and data not shown). Taken together, our data indicate that in developing spinal cord, expression of Lmx1a is restricted to roof plate progenitors and differentiated roof plate cells, a pattern that is conserved in vertebrate evolution.

Ectopic Lmx1a expression induces functional roof plate

To investigate if Lmx1a can induce roof plate in developing spinal cord, chick in-ovo electroporation was employed (Funahashi et al., 1999; Inoue and Krumlauf, 2001). We expressed mouse *Lmx1a* in chick caudal neural plate between presumptive forelimbs and hind limbs shortly before neural tube closure, at the time of initiation of endogenous *Lmx1a* expression.

Morphological analysis of cross sections of chick neural tubes expressing exogenous Lmx1a 48 hours after electroporation revealed expansion of the dorsal midline domain in 20/25 embryos (Fig. 2B,C and see Fig. S1 at http://dev.biologists.org/supplemental/). This phenotype has never been observed in embryos electroporated with EGFP alone (n=20) or unelectroporated embryos (n=15) (Fig. 2A,D,E). Roof plate marker analysis (Liem et al., 1997; Lee et al., 1998; Hollyday et al., 1995) revealed that there was an approximately fourfold increase in the number of MafB-positive dorsal cells in embryos electroporated with Lmx1a-IRES-EGFP (n=10 embryos) compared with embryos electroporated with EGFP alone (n=8 embryos) (Fig. 2B-F). Expression domains of *Gdf7*, *Bmp4* and *Wnt1* were also ectopically expanded on the *Lmx1a* electroporated side (13/15 embryos) (Fig. 2L-Q). Moreover, we found that markers of Bmp activity, the Msx1/2 proteins (Timmer et al., 2002), were shifted ventrally (13/15 embryos) (Fig. 2G-K) further indicating an expanded domain of Bmp signaling activated by exogenous Lmx1a in the dorsal neural tube. The ectopic expression of roof plate markers was limited to the most dorsal region of the neural tube. No expression of roof plate markers was detected in intermediate and ventral areas of the neural tubes, in spite of the high levels of exogenous Lmx1a expression (n=25 embryos) (Fig. 2C,I and data not shown). To demonstrate that *Lmx1a* was not simply expanding already specified roof plate, we conducted explant experiments. We determined that Lmx1a could induce roof plate cells in naive neural tissue in vitro, as defined by MafB/Pax7 double staining (Liem et al., 1997), when overexpressed in chick stage 10 intermediate neural plate explants (n=8 explants) (Fig. 2R-V). Analysis of dorsal neural tube or explants electroporated with Lmx1a-IRES-EGFP revealed extensive overlap of the GFP signal and the expression of the roof plate markers investigated (Fig. 2C,U and data not shown), suggesting that Lmx1a induces roof plate cell-autonomously.

To investigate if the ectopic roof plate induced by exogenous *Lmx1a* in vivo was functional, the specification of dorsal interneurons in Lmx1a electroporated neural tubes was analyzed. In particular, the numbers

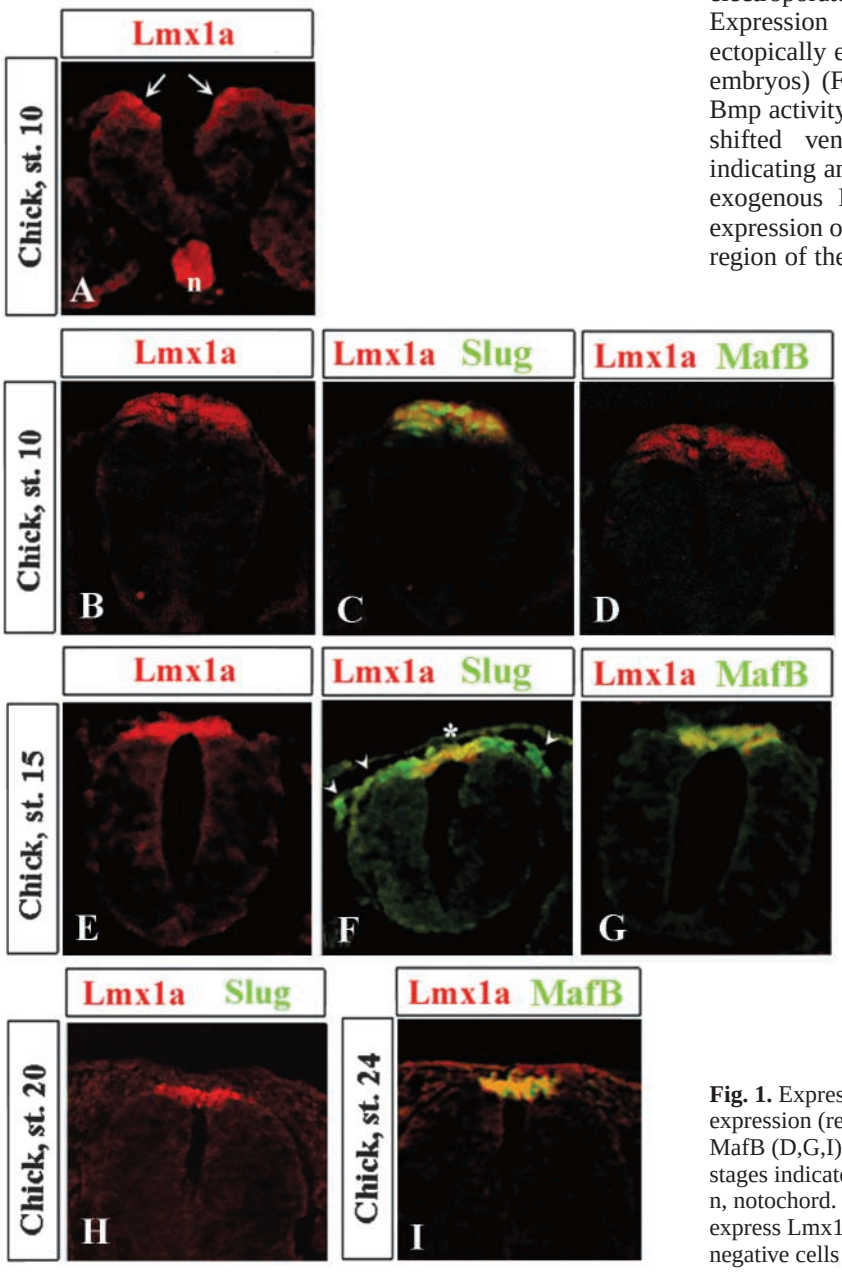


Fig. 1. Expression of Lmx1a in developing spinal cord. Lmx1a expression (red) alone (A,B,E) or together with Slug (C,F,H), and MafB (D,G,I) (both green) in chick developing spinal cord at the stages indicated. (A) Arrows point to neural folds expressing Lmx1a; n, notochord. (F) Asterisk indicates dorsal midline cells that co-express Lmx1a and Slug. Arrowheads point to Slug-positive/Lmx1a-negative cells delaminating and migrating away from the neural tube.

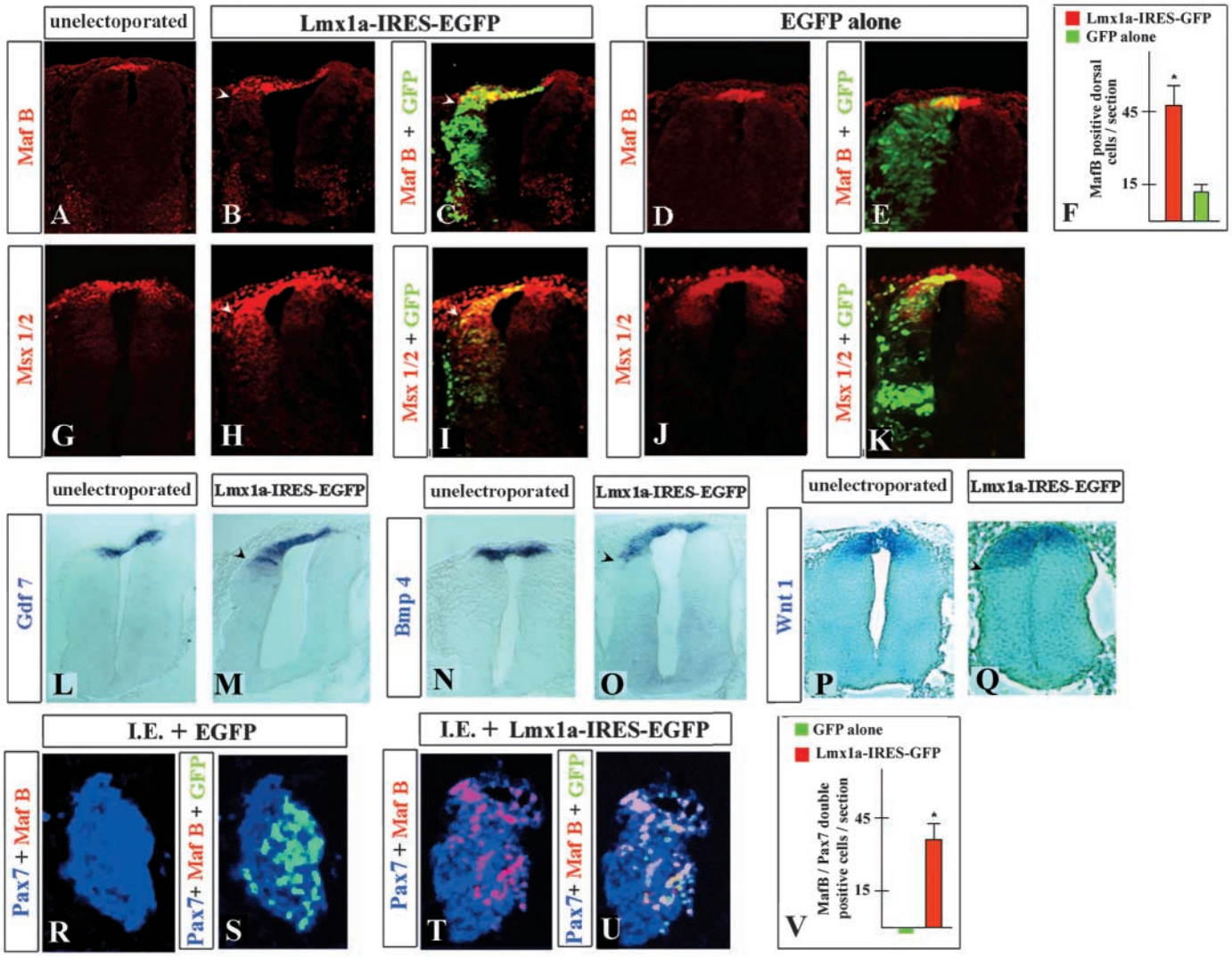


Fig. 2. Exogenous *Lmx1a* induces ectopic roof plate in the chick developing spinal cord. Expression of MafB (A-E), Msx1/2 (G-K) (both red), *Gdf7* (L,M), *Bmp4* (N,O), and *Wnt1* (P,Q) (all blue) in unelectroporated embryos (A,G,L,N,P) and embryos electroporated with either Lmx1a-IRES-EGFP (B,H,M,O,Q) or EGFP alone (D,E,J,K). All pictures are from stage-24 embryos, 48 hours after electroporation Arrowheads point to ventral boundary of ectopic domains of expression of roof plate markers induced by exogenous Lmx1a. (F) Quantitative analysis of dorsal MafB-positive cells in embryos electroporated with *Lmx1a* and EGFP alone. (R-U) Expression of MafB (red) and Pax7 (blue) (R,T) or MafB and Pax7 together with GFP (green) (S,U) in intermediate neural plate explants (I.E.), electroporated with Lmx1a-IRES-EGFP (T,U) or EGFP alone (R,S). (V) Quantitative analysis of MafB/Pax7 double-positive cells in intermediate explants electroporated with *Lmx1a* and EGFP alone.

of dI1, dI2 and dI3 interneurons were determined, identified by their expression of the LIM homeodomain proteins LH2A/B, Lim1/2 and Islet1, respectively (Muller et al., 2002; Gross et al., 2002), (Fig. 3A,E,H). To avoid the complications of any potential cell-autonomous effects of Lmx1a on dorsal interneuron cell fate, we limited our analysis to sections, with the expression of exogenous *Lmx1a* restricted to the most dorsal regions of the neural tube (initially detected by GFP fluorescence and confirmed by Lmx1a and GFP antibody staining) (Fig. 3C,G,J,L and data not shown). There was an approximately twofold increase in the number of dI1 interneurons in embryos electroporated with *Lmx1a* (*n*=9 embryos) compared with embryos electroporated with EGFP alone (*n*=8 embryos) (Fig. 3A-D). Additionally, there was a marked shift of the LH2A/B expression domain to more ventral regions of the neural tube (8/9 embryos), areas normally populated by dI2 and dI3 interneurons (Fig. 3B,C). Strong non-cell autonomous induction of dI1 interneurons was also observed in vitro when *Lmx1a* (9/9 explants) but not GFP alone (*n*=8 explants) was expressed in chick intermediate neural plate explants (Fig. 3M-O). Neither dI2 nor dI3 interneurons were found in regions of neural tubes demonstrating high levels of exogenous *Lmx1a* expression (*n*=9 embryos) (Fig. 3F,G,I,J). However, in samples with weak expression of exogenous *Lmx1a*, some Islet1 positive cells were generated at ectopic, more ventral, positions (6/9 embryos) (Fig. 3K,L). We show that in all these cases (6/6 embryos) intermediate neural tube cells adopted dorsal fates, as detected by Pax6/7 immunostaining (Timmer et al., 2002) (see Fig. S2 at http://dev.biologists.org/supplemental/). Notably, populations

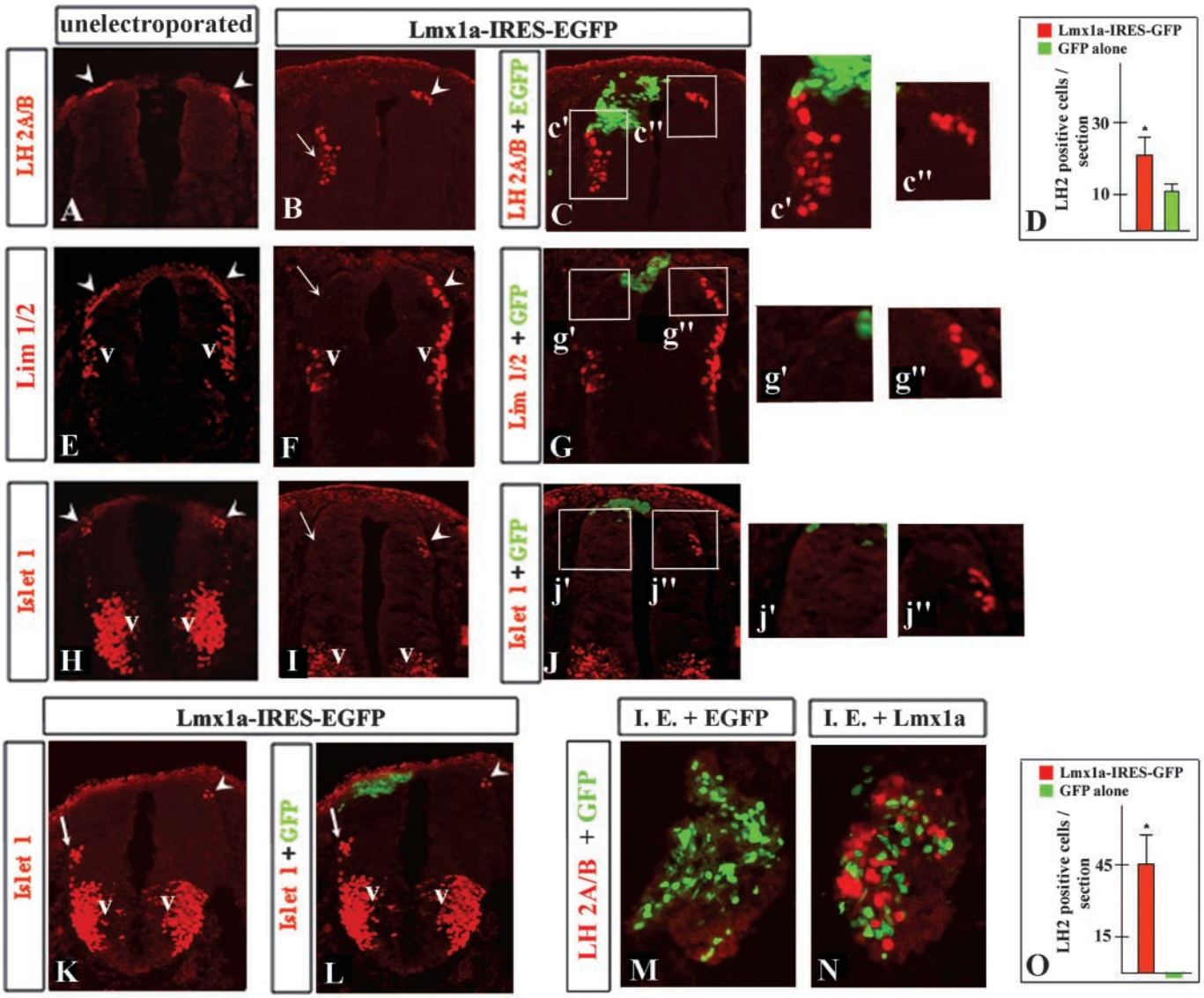


Fig. 3. Non-cell-autonomous effect of ectopic Lmx1a-generated roof plate on the specification of dorsal interneurons in chick neural tube. Expression of LH2A/B (A-C), Lim1/2 (E-G) and Islet1 (H-L) in neural tubes of unelectroporated embryos (A,E,H) and embryos electroporated with Lmx1a-IRES-EGFP (B,C,F,G,I-L) (all red). All pictures are taken at stage 24, 48 hours after electroporation Arrowheads and arrows point to non-affected and affected neuronal populations, respectively. Insets show higher magnifications of boxed regions. Lim1/2 and Isl1 expressing cells in the ventral half of the neural tube are marked by 'V'. (M,N) Visualization of expression of LH2A/B (red) together with GFP (green) in intermediate explants (I.E.) electroporated with Lmx1a-IRES-EGFP (N) or EGFP alone (M). (D,O) Quantitative analysis of LH2-positive cells in embryos (D) or I.E. (O) electroporated with Lmx1a and EGFP alone.

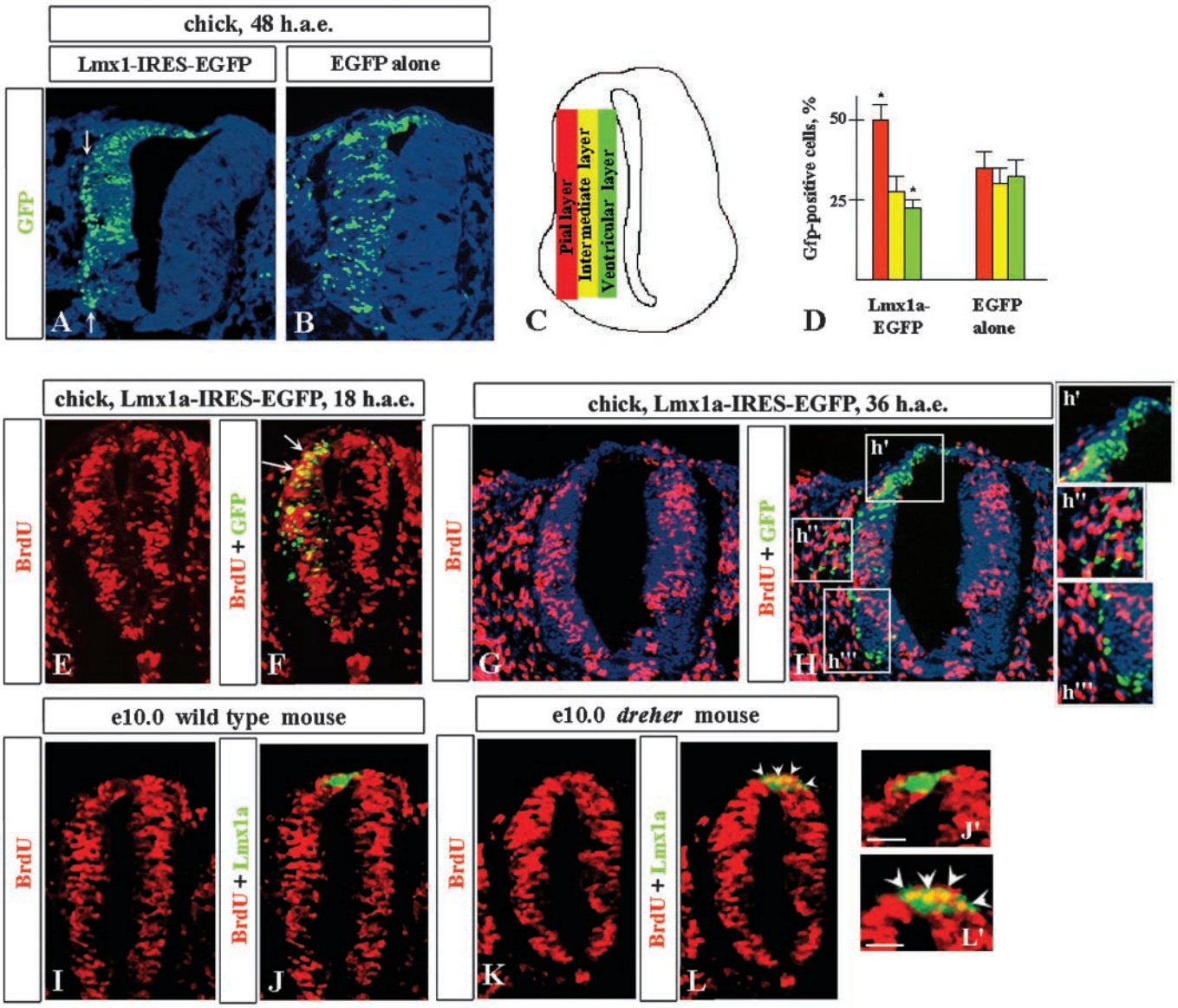
of Lim1/2 and Islet1 expressing cells in the ventral half of the neural tubes were unaffected (*n*=9 embryos) (Fig. 3E-L). No change in the number or position of either class of dorsal interneurons investigated was found in samples electroporated with EGFP alone (*n*=8 embryos; data not shown). These data show that ectopic roof plate induced by exogenous *Lmx1a* is functional, because it non-autonomously alters adjacent dorsal interneuron specification.

Lmx1a withdraws neural progenitors from the cell cycle

Another striking feature of embryos electroporated with *Lmx1a* was the nonrandom distribution of electroporated cells along the medio-lateral axis of the neural tube. At 48 hours after electroporation, the majority of cells electroporated with *Lmx1a* were found in the mantle layer of the neural tube, which consist mostly of differentiated neurons (*n*=25 embryos) (Fig. 4A, arrows), while in GFP controls, electroporated cells were randomly distributed along the medio-lateral axis (*n*=8 embryos) (Fig. 4B-D). This nonrandom distribution of *Lmx1a* electroporated cells along the medio-lateral axis indicates that Lmx1a may be involved in cell cycle regulation. This hypothesis is also consistent with the hypocellularity and thinning of the neural tube electroporated with *Lmx1a* (Fig. 4A).

Analysis of BrdU incorporation at 18 hours after

electroporation showed that many cells expressing exogenous *Lmx1a* were BrdU positive and were still randomly distributed along the medio-lateral axis of the neural tube (Fig. 4E,F). At 36 hours after electroporation, however, there was a marked reduction in the number of BrdU-incorporating cells in the *Lmx1a* electroporated regions compared with the control side of the neural tube (n=9 embryos). At this later time, the vast majority of cells expressing exogenous *Lmx1a* were BrdU-negative, including cells located in the neural tube and migrating neural crest cells (Fig. 4G,H). Cells electroporated with EGFP alone were still proliferating (n=8 embryos) (data not shown). TUNEL labeling to determine the number of cells



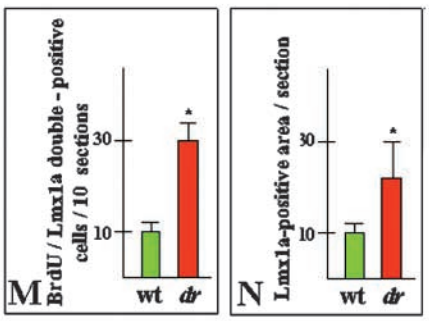


Fig. 4. Lmx1a withdraws neural progenitors from the cell cycle. GFP-fluorescence (green) in chick neural tubes 48 hours after electroporation (h.a.e.) with Lmx1a-IRES-EGFP (A) or EGFP alone (B). Arrows point to electroporated cells located in the pial layer of the neural tube (A). (C) Schematic representation of the neural tube divided into three layers to quantify distribution of electroporated cells along the medio-lateral axis. (D) Quantification of percent of electroporated cells found in each of these layers. (E-H). BrdU incorporation (red) in chick developing spinal cords 18 h.a.e. (E,F) and 36 h.a.e. (G,H) with Lmx1a-IRES-EGFP. Arrows point to BrdU-positive cells expressing exogenous Lmx1a. Insets show higher magnifications of boxed regions. (I-L). BrdU immunohistochemistry (red) alone (I,K) or together with Lmx1a visualization (green) (J,L) in E10 wild-type (I,J) and *dreher* (K,L) mouse embryos. BrdU/Lmx1a-double-positive cells (yellow) are indicated by arrowheads (L). Insets show higher magnifications of the dorsal midline regions. Bars equal 40 μm. (M,N) Quantification of proliferation (M) of Lmx1a-positive cells and measurement of Lmx1a-positive area (in arbitrary units) (N) in transverse sections of neural tubes taken from E10.0 wild type and dr^J/dr^J embryos.

undergoing apoptosis showed no differences between *Lmx1a* electroporated and control sides of the neural tube either at 18 hours after electroporation or 36 hours after electroporation (*n*=7 and 5 embryos, respectively, data not shown). Taken together, our data indicate that, when overexpressed in chick neural tube, Lmx1a withdraws neural progenitors from the cell cycle. This effect is not immediate but is delayed by at least 18 hours. Interestingly, expression of exogenous *Lmx1a* does not change the identity of intermediate and ventral neural cells, since they continue to express appropriate subtype-specific markers, including the dI4-6 interneuron marker Lbx1 (Muller et al., 2002; Gross et al., 2002) and the motoneuron marker Isl1 (Ericson et al., 1992) (data not shown).

To further investigate the role of Lmx1a in the cell cycle regulation of roof plate progenitors we returned to the *dreher* mouse model. Using Lmx1a-specific antibodies we could detect mutant Lmx1a protein in dorsal spinal cord of E9-10 dr^J/dr^J embryos (Fig. 4L). This observation allowed us to use BrdU/Lmx1a double labeling to compare number of proliferating cells on the dorsal midline of the neural tube in dr^J/dr^J and wild-type embryos. Our analysis revealed that at E9.25 dorsal Lmx1a-positive cells were proliferative, and no difference between dr^J/dr^J and wild-type embryos was detected at this stage (data not shown). At E10.0, however, the number of BrdU/Lmx1a-double-positive cells in spinal cord of dr^J/dr^J embryos (*n*=5 embryos) was threefold higher than that of wild-type littermates (*n*=8 embryos) (Fig. 4I-M). In addition, the mean area occupied by Lmx1a positive cells in dr^J/dr^J embryos at E10.0 was approximately 2.2 times larger then that observed in wild type littermates (Fig. 4J,L,N). This increase in Lmx1a-positive area correlates well with the increase in number of BrdU-positive cells in this region found in dr^J/dr^J embryos. Since no change in the rate of apoptosis has been detected in dorsal spinal cord of dr^J/dr^J embryos (Millen et al., 2004), this indicates that an increase in proliferation of Lmx1a-positive cells accounts for the increase in the area of Lmx1a-positive territory in dr^J/dr^J embryos. Thus, our gain-of-function experiments in chick and loss-of-function studies in mouse indicate that Lmx1a is necessary and sufficient for negatively regulating proliferation of dorsal neural progenitors in vivo.

Lmx1a is not critically involved in the early neural crest specification program

Since roof plate cells and neural crest cells derive from a common progenitor in the neural folds and the early dorsal neural tube (Bronner-Fraser and Fraser, 1988; Echelard et al., 1994), we investigated the role of *Lmx1a* in neural crest formation. Analysis of the chick embryos electroporated with *Lmx1a* 18 hours after electroporation showed that ectopic expression of this gene failed to significantly change numbers of early neural crest cells, as indicated by several markers staining including Slug, Sox9 and Ap2 (*n*=8-10 embryos for each marker) (Fig. 5A-C). Also, there was no change in the expression of migratory neural crest markers RhoB and HNK1 that were investigated 48 hours after electroporation (*n*=9 embryos) (Fig. 5D-G and data not shown). In addition, *Lmx1a* did not induce expression of any of these neural crest markers when ectopically expressed in intermediate neural plate explants in vitro (*n*=7 explants, data not shown). Finally, chick embryos electroporated with Lmx1a-IRES-EGFP revealed many GFP-positive cells migrating away from the neural tube (*n*=9 embryos) (Fig. 5E,G), suggesting that Lmx1a is insufficient for preventing neural crest migration.

To further assess the role of *Lmx1a* in neural crest development, we analyzed neural crest formation in *dreher* mouse embryos. Using molecular markers of the neural crest specification program, including Sox9 (*n*=5 wild-type and dr^J/dr^J E9.0 embryos) and Ap2 (*n*=6 wild-type and *n*=4 dr^J/dr^J E9.5 embryos), we showed that early neural crest formation is normal in dr^J/dr^J embryos along all developing spinal cord (Fig. 5H-L). In addition, we did not see a reduction of neural crest derivatives in dr^J/dr^J embryos at E10.5, as assayed by *Isl1* and neurofilament staining (Ikeya et al., 1997) (*n*=4 wild-type and dr^J/dr^J embryos for each marker) (Fig. 5M,N and data not shown). Finally, other neural crest derivatives, melanocytes, revealed by MitF immunostaining (Opdecamp et al., 1997), were also normal at the trunk level of E11.5 dr^J/dr^J embryos (*n*=4 wild-type and *n*=5 dr^J/dr^J embryos) (Fig. 5O). These results indicate that early neural crest differentiation is essentially normal at all axial levels of the developing spinal cord of dr^J/dr^J embryos and that *Lmx1a* is not critically involved in the early neural-crest-specification program.

Lmx1a distinguishes roof plate and dI1 interneuron programs in the dorsal spinal cord

Next we investigated the cell-autonomous effects of exogenous *Lmx1a* on development of dI1, dI2 and dI3 interneurons in chick dorsal spinal cord. Since we have demonstrated that roof plate induced by exogenous *Lmx1a* in the most dorsal regions of the neural tube non-autonomously affects adjacent dorsal interneuronal specification (Fig. 3), we limited our analysis to neural tubes expressing exogenous *Lmx1a* only in regions normally populated by dorsal interneurons (Fig. 6D,F). This revealed that many cells expressing exogenous *Lmx1a* still express Lim1/2 or Islet1 (*n*=4-6 embryos for each marker) (Fig. 6C-F), indicating that exogenous *Lmx1a* cannot cell-autonomously alter dI2 and dI3 interneuronal fates. By contrast, we very rarely observed cells co-expressing LH 2A/B and exogenous *Lmx1a* (*n*=10 embryos) (Fig. 6A,B), although many cells co-expressed LH2 A/B and EGFP when a GFP control was used (*n*=5 embryos) (data not shown). This indicates that Lmx1a can cell-autonomously suppress the dI1 interneuronal fate when overexpressed in the chick developing spinal cord. To gain insight into the mechanism of this suppression, dI1 interneuron progenitors characterized by expression of Cath1 (chicken homolog of Math1) (Helms and Johnson, 1998; Lee et al., 1998) were examined in electroporated samples (*n*=5 embryos). Again, no overlap between exogenous *Lmx1a* and Cath1 was detected (Fig. 6G,H). Thus, the cell-autonomous inhibition of dI1 interneuronal fate by Lmx1a has already occurred at the progenitor stage.

To further test the cell-autonomous inhibition of dI1 interneuron progenitors by Lmx1a, we investigated expression of Math1 in mice. In wild-type E10.0 mouse spinal cord, Math1-positive dI1 interneuron progenitors are located directly adjacent to the ventral boundary of the roof plate and there is no overlap between the Lmx1a and Math1 expression domains at cellular level (Fig. 6I,J). In dorsal spinal cord of dr^J/dr^J E10.0 embryos, however, Math1 positive dI1 dorsal interneuronal progenitors were located in the region normally

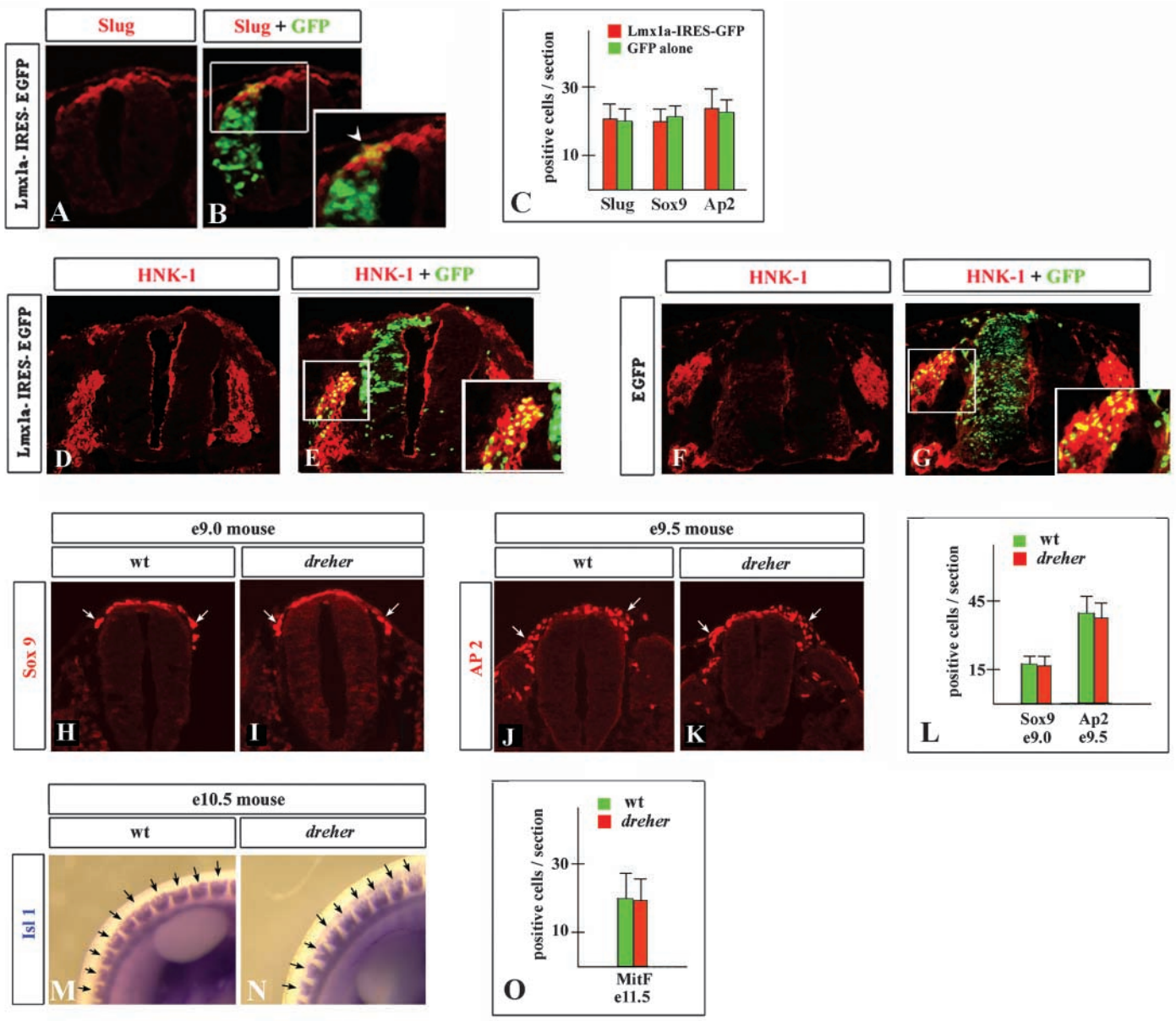


Fig. 5. Lmx1a is not involved in neural crest development. Chick neural tubes electroporated with Lmx1a-IRES-EGFP (A,B,D,E) or EGFP alone (F,G) 18 hours after electroporation (A,B) or 48 hours after electroporation (D-G). (A,B) Visualization of the early neural crest cell marker Slug (red). (D-G) Visualization of migratory neural crest cell by HNK-1 staining (red). Insets show higher magnification of boxed regions. Arrowhead points to yellow cells co-expressing exogenous Lmx1a and Slug. (C) Quantitative analysis of Slug, Sox9 and Ap2-positive cells in chick embryos electroporated with *Lmx1a* and EGFP alone. Visualization of early neural crest cells by Sox9 (H,I) and Ap2 (J,K) and neurogenic derivatives of dorsal root ganglia revealed by *Isl1* staining (M,N) in neural tubes of wild-type (wt) (H,J,M) and *dreher* (I,K,N) mouse embryos at indicated stages. Quantitative analysis of Sox9 and Ap2 (L), and MitF-positive cells (O) in wild-type and *dreher* mouse embryos.

occupied by the roof plate, and many of them were also Lmx1a-positive (Fig. 6K,L). Taken together with our chick overexpression studies, these data indicate that Lmx1a cell-autonomously represses Math1, preventing roof plate cells from adopting the dI1 interneuronal progenitor cell fate.

The role of Bmp and Wnt signaling in activation of Lmx1a expression and roof-plate development in vivo

We next addressed which signal might mediate activation of *Lmx1a* expression and roof plate formation in the developing chick spinal cord. Our studies were focused on Bmp and Wnt proteins since they are expressed in epidermal ectoderm at the time of roof plate formation and can induce specification of several dorsal cell types in neural plate explants when added to culture medium (Liem et al., 1997; Muroyama et al., 2002; Garcia-Castro et al., 2002). First, we expressed *Noggin*, a Bmp4-secreted inhibitor (Liem et al., 1997), to downregulate endogenous Bmp signaling in chick stage 10 neural plate. Marked inhibition of Lmx1a expression, as well as inhibition of other roof plate markers, including MafB and *Bmp4*, was detected 18 hours after

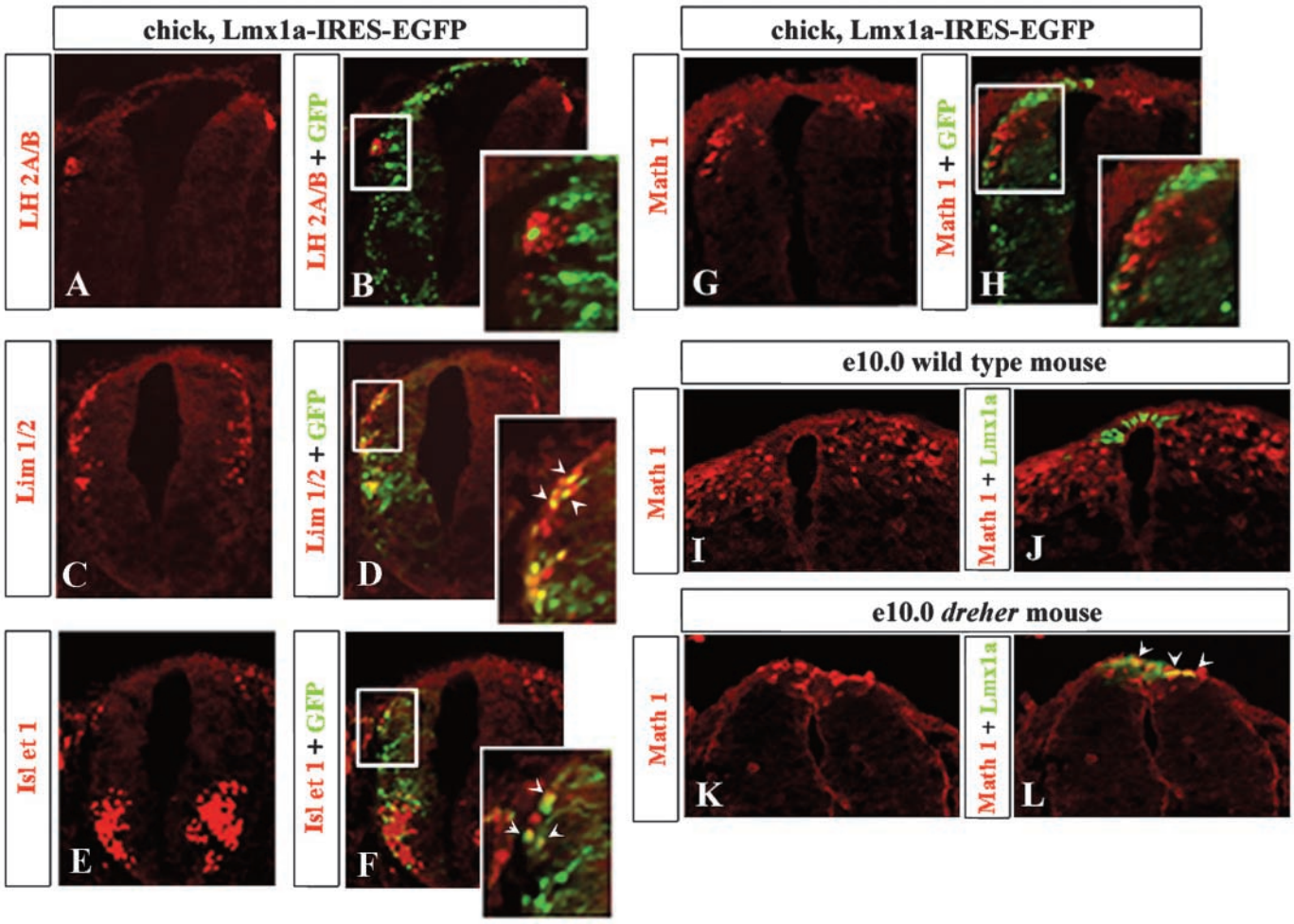


Fig. 6. Lmx1a cell-autonomously inhibits formation of dI1 interneurons. (A-H) Chick spinal cord expressing Lmx1a-IRES-EGFP 48 hours after electroporation. Visualization of expression of LH2A/B (A), Lim 1/2 (C), Islet 1 (E) and Cath1 (G) (all red) alone or together with GFP (green) (B,D,F,H). Insets show higher magnifications of boxed regions. Arrowheads point to yellow cells co-expressing exogenous Lmx1a and Lim1/2 (D) or Islet1 (F). (I-L) Visualization of expression of Math1 (red) alone (I,K) and together with endogenous Lmx1a (green) (J,L) in E10.0 wild-type (I,J) and dr^J/dr^J embryos (K,L). In dr^J/dr^J, embryos, numerous Lmx1a/Math1-double-positive cells (yellow) are indicated by arrowheads.

electroporation with Noggin-expressing plasmid (5/7 embryos) (Fig. 7A-E).

Next, we downregulated Wnt signaling by overexpressing dominantly negative *Wnt1* (*dnWnt1*) and *dnTCF4* (Garcia-Castro et al., 2002; Megason and McMahon, 2002) to investigate the role of Wnts in roof plate development. In accordance with previous studies, inhibition of Wnt signaling caused a decrease of cellular proliferation of the neural progenitors (Megason and McMahon, 2002) and affected expression of early neural crest markers (Slug and Sox9) (data not shown) (Garcia-Castro et al., 2002). Importantly, however, it had no effect on roof plate expression of Lmx1a, MafB or *Bmp4* (n=5 embryos) (Fig. 7F-J). Taken together, our data demonstrate that Bmp signaling is necessary for *Lmx1a* expression and roof plate specification or maintenance. Downregulation of Wnt signaling has no significant effect on early roof plate development.

Induction of Lmx1a and roof plate by Bmp signaling

To investigate if Bmp signaling is sufficient for inducing *Lmx1a* expression and ectopic roof plate formation, we expressed mouse *Bmp4*, *Bmp7* or an activated Bmp receptor, *BmprIa*, in chick developing spinal cords using the same parameters and conditions as described for *Lmx1a*. Ectopic expression of either construct resulted in a similar phenotype, including significant hypocellularity and increased apoptosis (as detected by TUNEL) within the electroporated regions (8/8 embryos). At the same time, induction of Lmx1a along the whole dorsoventral axis of the neural tube, excluding only the most ventral regions, was observed (Fig. 8A,B and data not shown). Surprisingly, activation of Bmp signaling induced expression of both MafB and Lmx1a to the same broad extent along the dorsoventral axis of the neural tube (compare adjacent sections shown on Fig. 8A,B and Fig. 8C,D). This was in contrast to exogenous Lmx1a, which could induce MafB only in a restricted dorsal domain of the neural tube (n=8 embryos). These data suggest that Bmps may not only induce *Lmx1a* but also induce co-factors required to make neural progenitors competent to Lmx1a roof plate inducing activity. An alternative hypothesis is that Bmps can induce roof plate via Lmx1a-independent mechanisms.

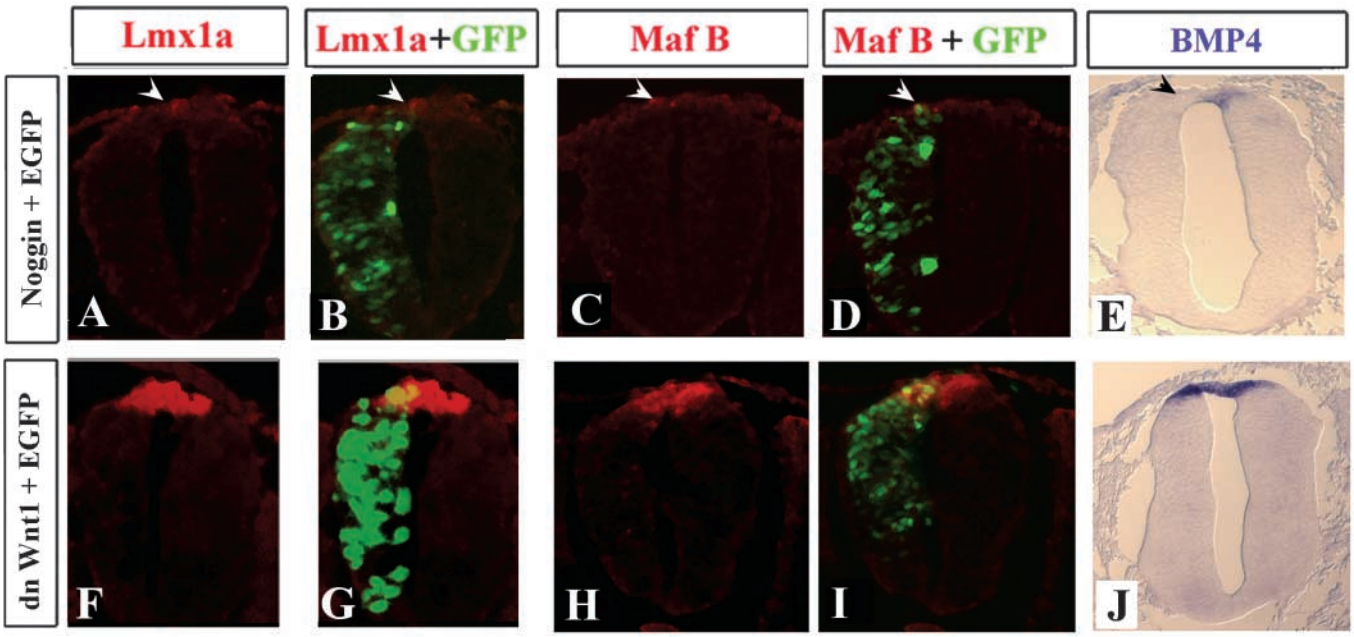


Fig. 7. Effect of downregulation of Bmp and Wnt signals on early roof plate development. Chick neural tubes expressing exogenous *Noggin* (A-E) and *dnWnt1* (F-J) 18 hours after electroporation. Visualization of expression of roof plate markers Lmx1a (A,F) and MafB (C,H) (both red), *Bmp4* (E,J) (blue) alone or together with GFP (green) (B,D,G,I). Arrowheads point to expression domains affected by overexpression of *Noggin*.

We performed in-vitro explant experiments to distinguish these two possibilities. We isolated E9.25 neural tubes of wild-type and dr^J/dr^J littermates and cultured them in vitro for 18 hours. Both wild-type and dr^J/dr^J explants revealed Lmx1a expression in the roof plate domain at the time of analysis (Fig. 8E,I). As expected, wild-type embryos also demonstrated expression of MafB in the roof plate region, while no MafB expression was detected in the dorsal domain of dr^J/dr^J embryos (Fig. 8F,J). When Bmp4 was added into the culture medium, strong induction of Lmx1a along almost all dorsoventral axis of the neural tube was observed in both wild-type and dr^J/dr^J explants (n=5 explants of each type) (Fig. 8G,K). However, only wild-type explants activated expression of MafB in the same domain, while dr^J/dr^J explants remained MafB-negative (Fig. 8H,L). These data show that Bmp4 cannot induce roof plate in the absence of Lmx1a and strongly argue that Bmp signaling induces Lmx1a and other co-factors that also regulate the extent of roof plate induction.

Discussion

In this study, we have demonstrated that Lmx1a is sufficient for withdrawing dorsal neural progenitors from the cell cycle and simultaneously drive them to differentiate into functional roof plate cells. Our results indicate that Lmx1a cell-autonomously represses the dI1 interneuron progenitor fate, thus distinguishing two major developmental programs in dorsal spinal cord – the roof plate and dI1 interneuron programs. At the same time, loss or overexpression of *Lmx1a* is not sufficient for preventing neural crest formation, indicating the existence of other factors necessary for the divergence of roof plate cells and neural crest cells from their common progenitors. We provide evidence that Bmps are necessary and sufficient for inducing *Lmx1a* and that Lmx1a is a major mediator of early Bmp signaling in developing spinal cord.

Lmx1a initiates a regulatory cascade that leads to formation of functional roof plate being a major mediator of early Bmp signaling in dorsal spinal cord

Our previous analysis of the *dreher* mouse established that *Lmx1a* is necessary for roof plate development (Millonig et al., 2000). In the current study, we have demonstrated that *Lmx1a* is sufficient for inducing ectopic roof plate when overexpressed in developing chick spinal cord and in neural explants in vitro. We have demonstrated functional activity of this ectopic roof plate by showing that it can non-autonomously induce dI1 and dI3 dorsal interneurons at ectopic positions and suppress specification of dI2 dorsal interneurons in chick developing spinal cord.

In this study we have shown that *Lmx1a* can induce the expression of members of the Bmp and Wnt families proteins, which are known to be important components of roof plate signaling in developing spinal cord (Liem et al., 1997; Lee et al., 1998; Muroyama et al., 2002). We have also conducted analysis of dorsally expressed genes in the *dreher* mouse and determined that loss of *Lmx1a* completely abolishes Bmp expression in embryonic spinal cord, while Wnt expression is maintained (Millen et al., 2004). These data identify Bmps as important components of Lmx1a-depedndent roof plate signaling. Also they indicate existence of other, Lmx1a-independent mechanisms that support Wnt expression in developing spinal cord and that Wnt signaling may account for residual dI1 interneurons generated in the *dreher* spinal cord.

Using an inhibitor approach, we have shown that *Lmx1a* activation and early roof plate development is dependent on Bmp signaling and not affected when Wnt signaling

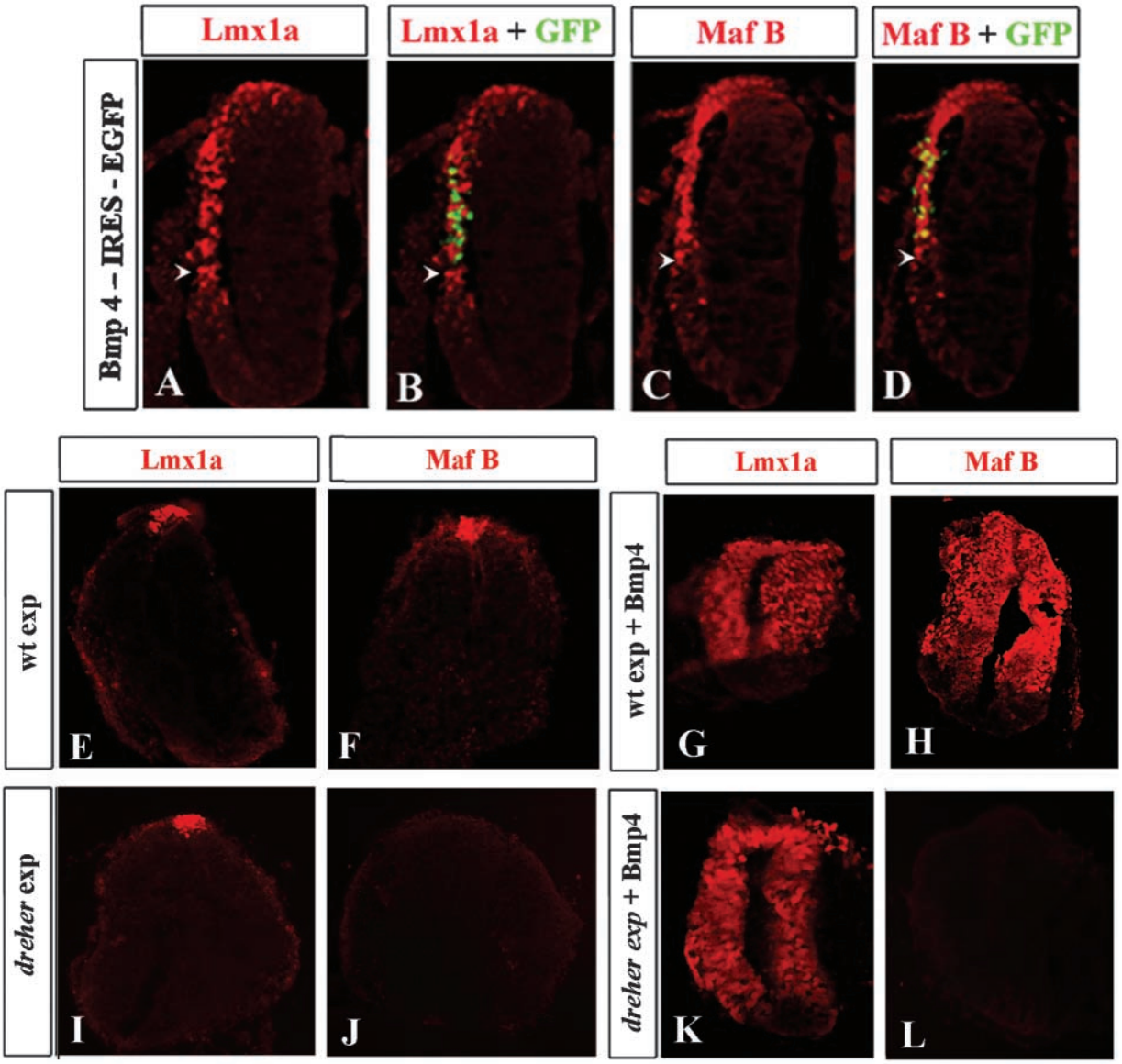


Fig. 8. Effect of *Bmp4* overexpression on Lmx1a expression and roof plate development. (A-D) Chick neural tubes expressing exogenous Bmp4 24 hours after electroporation. Visualization of expression of Lmx1a (A) and MafB (C) (both red) alone or together with GFP (green) (B,D). Arrowheads show the ventral boundary of expanded expression domains of both Lmx1a and MafB in neural tubes electroporated with Bmp4. (E-L) Wild-type (wt exp) (E-H) and *dreher* (*dreher* exp) (I-L) explants cultured without Bmp4 (E,F,I,J) or with Bmp4 (G,H,K,L). Visualization of expression of Lmx1a (E,G,I,K) and MafB (F,H,J,L).

is downregulated. Surprisingly, when Bmp signaling is ectopically activated, the domain of roof plate induction expands to include almost the entire dorsoventral axis of the spinal cord. This is in contrast to *Lmx1a*, which can induce roof plate only in the most dorsal region of the neural tube. Importantly, our *dreher* mouse in-vitro explant experiment data indicate, however, that Bmp4 cannot induce roof plate in the absence of *Lmx1a*. Thus, Lmx1a must be major mediator of the roof plate inducing activity of Bmp in the developing spinal cord. These data also support the conclusion that in addition to Lmx1a Bmps induce other factors that regulate the extent of roof plate induction.

The phenotype that we observed upon activation of Bmp signaling is different from the findings of Timmer et al. (2002). In particular, we observed roof plate induction when we expressed exogenous Bmp in chick stage 10 neural plates, while Timmer et al. found induction of dI1 interneurons when ectopic Bmp signaling was activated at later stages, 14-16. Taken together, these experiments indicate that timing of Bmp activation is critical in dorsal cell fate decisions, providing in-vivo evidence for the model previously proposed in vitro (Liem et al., 1997).

Lmx1a couples the program regulating cell cycle withdrawal and the program controlling cell fate specification during roof plate development

Building a CNS involves the generation of different neuronal and glial cell types in correct numbers and at appropriate

positions. Numerous studies have demonstrated that this is achieved by the activation of programs that commit neural progenitors to cell cycle exit and differentiation, and of programs directing cellular subtype identity. Recent studies suggest that during neurogenesis the action of specific proteins, including the products of bHLH proneural genes, is required to couple these two programs (Bertrand et al., 2002). Our data show that Lmx1a is sufficient not only for specifying roof plate fate when expressed in chick and mouse neural progenitors but also for promoting the arrest of their division and therefore playing a comparable role during roof plate development. Interestingly, unlike roof plate competence, neural progenitors are competent to the cell-cycle withdrawal activity of Lmx1a independent of their location along the dorsoventral axis of the spinal cord. Indeed, differentiated cells expressing exogenous Lmx1a within these regions still express appropriate neuronal subtype identity markers. This clearly indicates that different activities of Lmx1a, probably executed through cooperation with different partners, are required for the activation of the program regulating proliferation of the dorsal progenitors and the program responsible for the acquisition of the roof plate fate.

The role of Lmx1a in segregation of roof plate cells from neural crest and dI1 dorsal interneuron lineages

Roof plate cells are generated from uncommitted progenitors in the neural folds and early neural tube that also give rise to neural crest cells and dI1 interneuron precursors (Bronner-Fraser and Fraser, 1988; Echelard et al., 1994; Liem et al., 1997; Lee and Jessell, 1999; Helms and Johnson, 2003). We show that Lmx1a is specifically expressed by roof plate progenitors and differentiated roof plate cells. Surprisingly, however, overexpression of *Lmx1a* was not sufficient to alter the specification of neural crest cells either in vivo or in vitro. *dreher* mice have been reported to have some defects in a small number of neural crest derivatives, including abnormal pigmentation and improper formation of spinous processes of dorsal vertebrae (Manzanares et al., 2000) (this study). Although *dreher* mice lack a roof plate at all axial levels of the spinal cord, neural crest phenotypes are restricted to the posterior thoracic region, a small domain along the anterior–posterior axis of the spinal cord. Using multiple molecular markers, we found that early neural crest development is essentially normal along the developing spinal cord of dr^J/dr^J embryos, even in the affected region. Thus, abnormal pigmentation and improper fusion of the vertebra detected in the thoracic region of the *dreher* mice are probably late secondary defects, caused by loss of roof plate signaling rather then direct involvement of Lmx1a in neural crest generation. Taken together, our data suggest that Lmx1a is not directly involved in neural crest development. Further studies are necessary to dissect the mechanisms involved in segregation of roof plate and neural crest lineages.

Importantly, our data indicate that Lmx1a is both necessary and sufficient for segregating roof plate and dI1 dorsal interneuron progenitors. Thus, our experiments reveal a dual role for Lmx1a in dI1 dorsal interneuron formation. First, Lmx1a induces dI1 dorsal interneurons through non-autonomous roof plate signaling. At the same time, it cell-autonomously inhibits expression of Math1 (Cath1), preventing dorsal midline cells from adopting dI1 dorsal interneuronal fate. Cross-inhibitory regulation has recently been shown to play a key role in boundary formation between different classes of dorsal interneuron progenitors (Gowan et al., 2001). Our data show that negative regulation of Math1 expression by Lmx1a plays a similar role in boundary formation between the dI1 dorsal interneuron domain and the roof plate.

In the current study, we have demonstrated that Lmx1a controls multiple steps of dorsal spinal cord development. Elucidation of the downstream components of the Lmx1a regulatory network will be critical to further understanding of the molecular mechanisms underlying the diverse actions of Lmx1a as an important regulator of dorsal CNS patterning.

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