Development/Plasticity/Repair

Lmx1b, *Pet-1*, and *Nkx2.2* Coordinately Specify Serotonergic Neurotransmitter Phenotype

Leping Cheng,[1,2]* Chih-Li Chen,[1,2]* Ping Luo,[1,2] Min Tan,[3] Mengsheng Qiu,[3] Randy Johnson,[4] and Qiufu Ma[1,2]

[1]The Dana-Farber Cancer Institute, [2]Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115, [3]Department of Anatomical Sciences and Neurobiology, School of Medicine, University of Louisville, Louisville, Kentucky 40292, and [4]Program in Genes and Development, Department of Biochemistry and Molecular Biology, University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

Serotonergic (5-HT) neurons in the brainstem modulate a wide range of physiological processes and behaviors. Two transcription factor genes, *Pet-1* and *Nkx2.2*, are necessary but not sufficient to specify the 5-HT transmitter phenotype. Here we show that the Lim class homeobox gene *Lmx1b* is required for proper formation of the entire 5-HT system in the hindbrain, as indicated by the loss of expression of genes necessary for serotonin synthesis and transport in *Lmx1b* null mice. Lmx1b and Pet1 act downstream of Nkx2.2, and their expression is independently regulated at the time when 5-HT transmitter phenotype is specified. Ectopic expression of *Lmx1b* plus *Pet-1* is able to induce formation of 5-HT cells in the most ventral spinal cord, where *Nkx2.2* is normally expressed. Combined expression of all three genes, *Lmx1b*, *Pet-1*, and *Nkx2.2*, drives 5-HT differentiation in the dorsal spinal cord. Our studies therefore define a molecular pathway necessary and sufficient to specify the serotonergic neurotransmitter phenotype.

Key words: **Lmx1b; Pet-1; Nkx2.2; serotonergic; neurotransmitter specification; brainstem**

Introduction

The serotonergic (5-HT) system contains nine small clusters of neurons localized along the midline of the hindbrain (Dahlstrom and Fuxe, 1964; Steinbusch, 1981; Tork, 1990; Jacobs and Azmitia, 1992). These neurons innervate throughout the brain and the spinal cord and modulate a wide array of physiological processes and behaviors (Steinbusch, 1981; Jacobs and Azmitia, 1992; Lucki, 1998). Dysfunction of the 5-HT system is therefore associated with many neurological and psychiatric disorders, including anxiety, depression, aggression, obsessive–compulsive disorder, and schizophrenia (Lucki, 1998; Davidson et al., 2000; Nelson and Chiavegatto, 2001).

5-HT neurons develop from the most ventral hindbrain in proximity to the floor plate (Lidov and Molliver, 1982; Wallace and Lauder, 1983; Hendricks et al., 1999). Signals derived from the floor plate, particularly sonic hedgehog, are required for 5-HT neuron formation (Matise et al., 1998; Ye et al., 1998; Hynes and Rosenthal, 1999; Goridis and Rohrer, 2002). Recent studies have led to the identification of several transcription factors necessary for 5-HT neuron development. The homeobox gene *Nxk2.2* is expressed in the ventral precursor cells that first give rise to the visceral motor neurons (vMNs) and then to the 5-HT cells (Briscoe et al., 1999; Pattyn et al., 2003). In *Nkx2.2* null mice, prospective 5-HT neurons are transformed into vMNs (Briscoe et al., 1999; Pattyn et al., 2003). Nkx2.2 promotes 5-HT differentiation primarily by suppressing the expression of *Phox2b*, a paired class homeobox gene (Pattyn et al., 2003). Pet-1, an ETS class transcription factor, is expressed specifically in postmitotic 5-HT neurons (Hendricks et al., 1999, 2003). In *Pet-1* null mice, most 5-HT neurons fail to form, and the residual 5-HT neurons are also functionally compromised (Hendricks et al., 2003). Consequently, *Pet-1* mutant mice show anxiety and aggressive behaviors (Hendricks et al., 2003). *GATA3*, a zinc finger gene, is also expressed in postmitotic 5-HT neurons (Van Doorninck et al., 1999); however, the function of *GATA3* is still unclear, because the total number of 5-HT neurons is not changed in *GATA3* mutant mice (Van Doorninck et al., 1999).

Despite this progress, the molecular pathway capable of the specification of the 5-HT transmitter phenotype remains unknown. For example, *Nkx2.2* is also expressed in nonserotonergic neurons (Briscoe et al., 1999; Pattyn et al., 2003). As shown in this study, *Pet-1* alone is also insufficient to induce 5-HT cell fate, suggesting the existence of additional transcription regulators for 5-HT differentiation.

The *Lim* class homeobox gene *Lmx1b* was initially characterized as a key regulator controlling dorsoventral patterning in the developing limbs (Johnson and Tabin, 1997). Mutation of this gene causes the nail patella syndrome, showing abnormalities in limb and kidney development (Chen et al., 1998). In the nervous system *Lmx1b* is expressed in restricted populations of neurons, including midbrain dopaminergic (DA) cells (Smidt et al., 2000). *Lmx1b* is required for the maintenance but not the initial specification of the DA transmitter phenotype (Smidt et al., 2000).

Here we show that *Lmx1b* is required for the formation of the

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*L.C. and C.-L.C. contributed equally to this work.

Correspondence should be addressed to Qiufu Ma, SM1022B, Dana-Farber Cancer Institute, One Jimmy Fund Way, Boston, MA 02115. E-mail: Qiufu_Ma@dfci.harvard.edu.

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entire 5-HT system in the hindbrain. Most significantly, we show that the combined function of *Lmx1b*, *Pet-1*, and *Nkx2.2* is sufficient to induce 5-HT cell fate.

Materials and Methods

Animals. The generation of *Lmx1b* and *Nkx2.2* mutant mice has been described previously (Chen et al., 1998; Sussel et al., 1998). The morning that vaginal plugs were observed was considered as embryonic day (E) 0.5. Wide-type *Lmx1b* allele was amplified with the following primers that produce a 0.27-kb product: 5′-GATAGGGCATTCAACCAGGACGAGC-AAAGA-3′ and 5′-AAACAGAAGCCACA-GAGAGCCAAGGAGAAG-3′. Wide-type *Nkx2.2* allele was amplified with the following primers that produce a 0.37 kb product: 5′-GAAGCGCCGAGTGCTCTTCTCC-3′ and 5′-GCCGAGCTGTACTGGGCGTTGT-3′. Mutant *Lmxb1* and *Nkx2.2* alleles were amplified with primers derived from *neo* gene.

In situ *hybridization and immunostaining.* Section *in situ* hybridization was performed as described (Ma et al., 1998), and detailed protocol is available on request. The following *in situ* probes, including *SERT* (0.78 kb) (Chang et al., 1996), *TPH1* (0.63 kb) (Stoll et al., 1990), *TPH2* (0.75 kb) (Walther et al., 2003), *VMAT2* (0.75 kb) (GenBank accession number AJ555564), aromatic amino acid decarboxylase (*AADC*) (0.81 kb) (GenBank accession number NM_016672), *Nkx2.2* (0.82 kb) (Price et al., 1992), *Pet-1* (0.71 kb) (Pfaar et al., 2002), *VGlUT3* (0.73 kb) (Schafer et al., 2002), and *GATA3* (1.2 kb) (George et al., 1994) were PCR amplified with cDNA prepared from E13.5 or adult mouse brain. Standard immunostaining was performed with 5-HT antibody (ImmunoStar, Hudson, WI), TPH antibody (Sigma, St. Louis, MO), and Lmx1b antibody (kindly provided by Dr. Tom Jessell, University of Columbia).

In ovo *electroporation.* The cDNA fragments, which encode the full-length mouse *Lmx1b* (GenBank accession number NM_010725) and *Pet1* (Pfaar et al., 2002) coding regions fused with the Myc epitope, were cloned into the *RCASBP* chick viral expression vector (Morgan and Fekete, 1996). The *Nkx2.2* expression construct, in which the full-length mouse *Nkx2.2* cDNA was cloned into the pcDNA3.1/Myc-His(B) (Invitrogen, San Diego, CA) vector, was provided by Dr. David Rowitch (Dana-Farber Cancer Institute). The purified plasmid DNAs were resuspended in sterile water. Hamburger and Hamilton (HH) stage 12 (E2) chick embryos were electroporated unilaterally (five 50 msec pulses at 25 V) with plasmid DNAs using an ECM830 electro-squareporator (BTX, San Diego, CA). After electroporation, the embryos were allowed to grow at 38°C for an additional 48–72 hr. For single plasmid electroporation, 3 μg/μl DNA was used. For *Lmx1b* plus *Pet1* electroporation, 2 μg/μl of each plasmid was used. For triple injections, 1.7 μg/μl *Pet1* plus 1.7 μg/μl *Lmx1b* plus 6.7 μg/μl *Nkx2.2* were used.

Results

Lmx1b is expressed in developing serotonergic neurons

To determine whether *Lmx1b* is expressed in developing 5-HT neurons, we compared *Lmx1b* expression with that of *Pet-1*, the prospective 5-HT neuron marker (Hendricks et al., 1999, 2003). Neither *Lmx1b* nor *Pet-1* is expressed in E10.5 ventral hindbrain (data not shown), consistent with the fact that 5-HT neurons develop after E10.75 (Pattyn et al., 2003). In rostral hindbrain of E11.5 embryos, *Lmx1b* and *Pet-1* are expressed in two patches of cells adjacent to the floor plate (Fig. 1*A*,*B*, arrows), an area normally giving rise to 5-HT neurons (Lidov and Molliver, 1982; Wallace and Lauder, 1983; Hendricks et al., 1999). By E12.5, formation of 5-HT neurons extends to the caudal hindbrain. At this stage, *Lmx1b* and *Pet-1* again show identical expression patterns in both the rostral (Fig. 1, compare *C*, *D*, arrows) and caudal (Fig. 1, compare *E*, *F*, arrows) hindbrain midline areas. Double immunostaining of Lmx1b and 5-HT confirmed that Lmx1b is expressed in all 5-HT neurons (Fig. 1*G–I*, arrows) (data not shown); however, a small subset of Lmx1b-positive neurons is 5-HT negative in E14.5 embryos (Fig. 1*I*, arrowhead), suggesting that Lmx1b may also be expressed in some nonserotonergic neurons within the raphe nuclei.

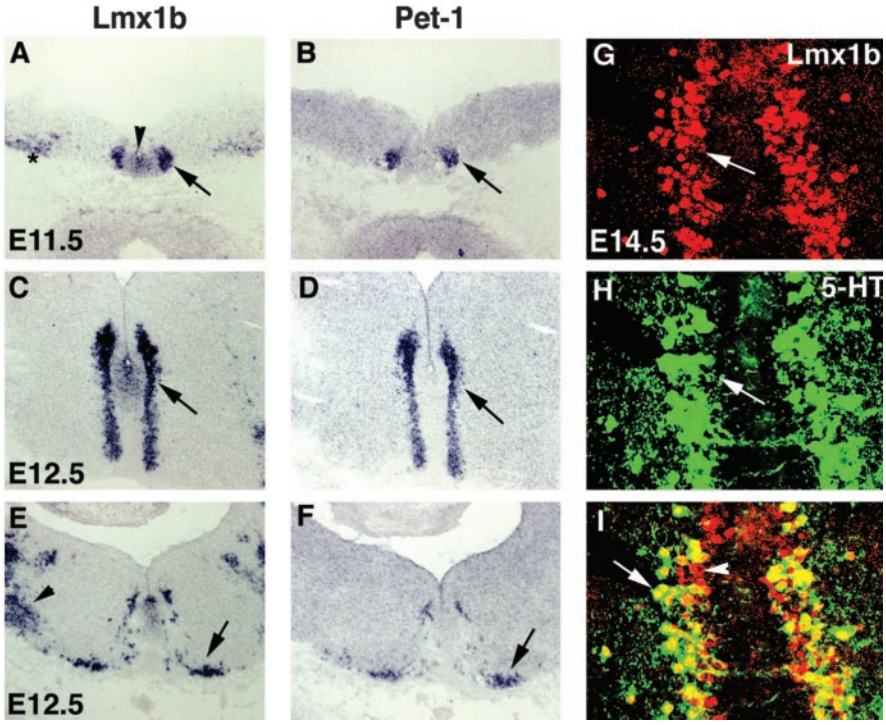

Figure 1. Lmx1b is expressed in serotonergic neurons. Transverse sections through E11.5 pons (*A*, *B*), E12.5 caudal pons (*C*, *D*), E12.5 medulla (*E*, *F*), and E14.5 caudal pons (*G–I*) are shown. *In situ* hybridization was performed with the indicated probes (*A–F*). *Lmx1b* and *Pet-1* are expressed in neurons derived from the ventral midline (*A–F*, arrows) that correspond to the primordial raphe nuclei. *Lmx1b* is also expressed weakly in the floor plate (*A*, arrowhead) and other areas in the medulla (*A*, star; *E*, arrowhead). *G–H*, Coronal sections through the E14.5 pons. Double immunostaining was performed with Lmx1b antibody (*G*, red) and 5-HT antibody (*H*, green). *I* is the superimposed image showing that Lmx1b is expressed in all 5-HT cells (with yellow nuclei) (*I*, arrow). A small subset of Lmx1b-positive cells is 5-HT negative (*I*, arrowhead).

Lmx1b is required for the proper specification of 5-HT transmitter phenotype

It has been reported that *Pet-1* is essential for the development of the 5-HT transmitter phenotype (Hendricks et al., 1999, 2003). The stringent correlation of *Lmx1b* and *Pet-1* expression suggests that *Lmx1b* might control 5-HT differentiation. To test this possibility, we examined the expression of a series of 5-HT markers in *Lmx1b* mutant embryos (Chen et al., 1998). Several enzymes are responsible for 5-HT synthesis. The tryptophan hydroxylases 1 and 2 (TPH1 and TPH2) convert tryptophan to 5-hydroxytryptophan (McGeer and McGeer, 1973; Walther et al., 2003); 5-hydroxytryptophan is then converted into 5-HT by AADC (McGeer and McGeer, 1973). At every stage examined (from E11.5 to P0), 5-HT immunostaining is entirely absent in the *Lmx1b* mutant hindbrain, including both the rostral areas (Fig. 2, compare *A*, *B* and *C*, *D*, arrows) and the caudal areas (Fig. 2, compare *E*, *F*, arrows). A complete loss of TPH immunostaining

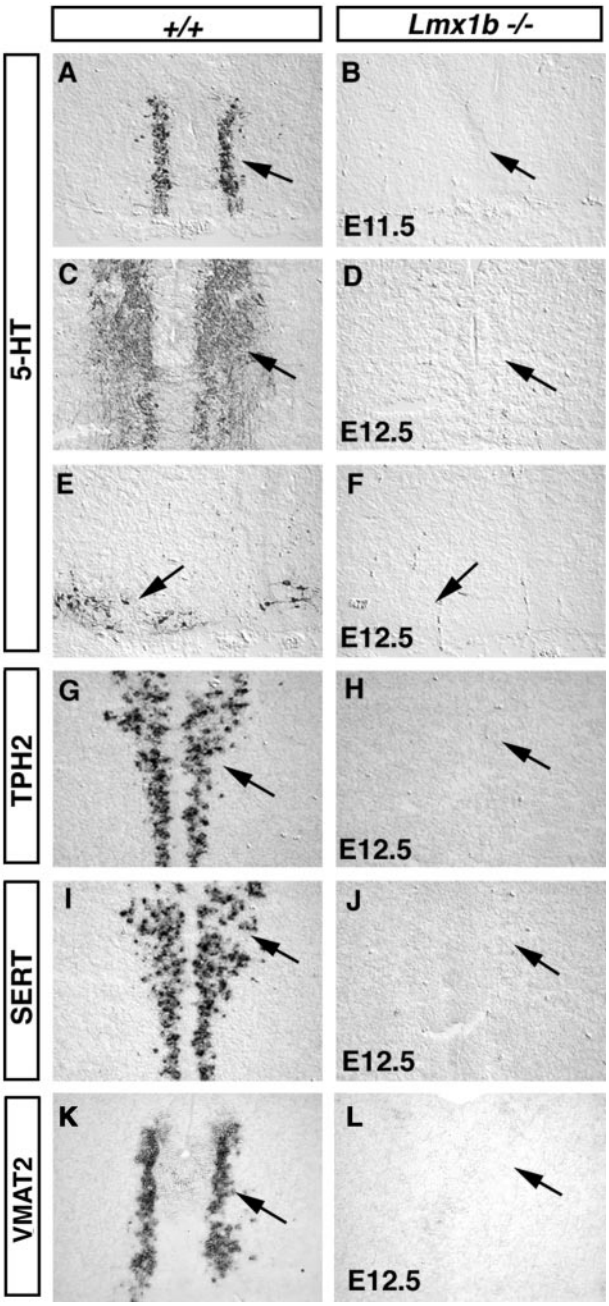

Figure 2. Development of 5-HT neurons is compromised in *Lmx1b* mutants. Transverse sections through the pons (*A–D*, *G–J*) and the medulla (*E*, *F*, *K*, *L*) of embryos with indicated stages and genotypes are shown. 5-HT immunostaining (*A–F*) and *in situ* hybridization (*G–L*) were performed with the indicated probes (*G–L*).

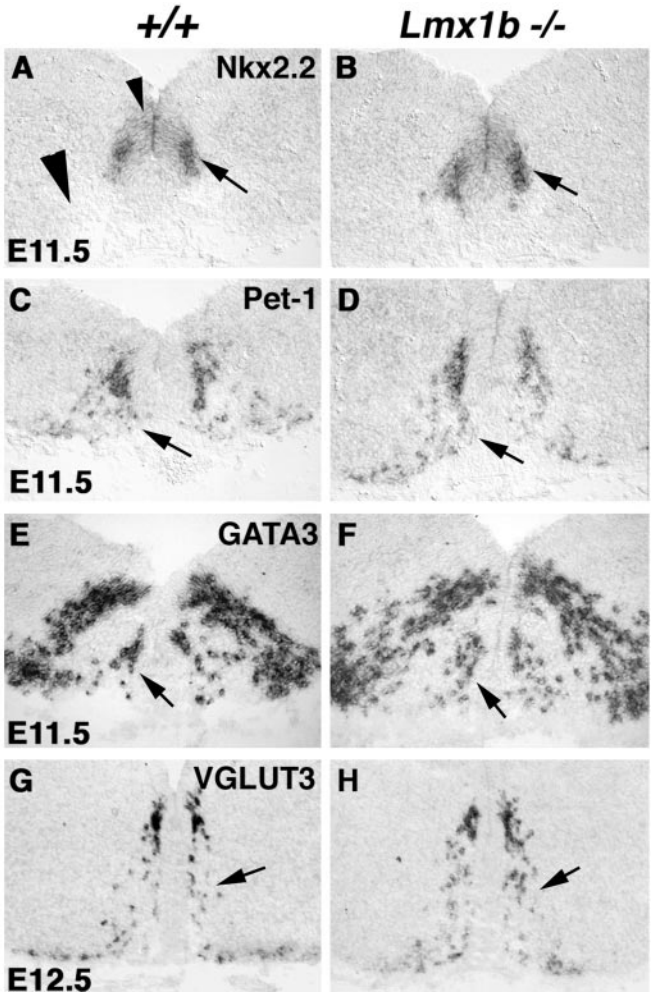

Figure 3. Prospective 5-HT neurons are formed in *Lmx1b* null embryos. Transverse sections through E11.5 medulla (*A–D*) and E12.5 pons (*E–H*) are shown. *In situ* hybridization was performed with the indicated probes.

was also observed in *Lmx1b* mutants (data not shown). It should be pointed out that in wild-type embryos, *TPH2* but not *TPH1* is expressed prominently in 5-HT neurons (Fig. 2*G*, arrow) (data not shown). Consistent with the loss of 5-HT and TPH immunostaining, expression of *TPH2* is absent in both the rostral (Fig. 2, compare *G*, *H*, arrow) and caudal (data not shown) hindbrain of *Lmx1b* mutants. Expression of *AADC* is also reduced significantly in the medulla area but is largely normal at the pons level (data not shown).

Proper function of 5-HT neurons also requires two transporter proteins, SERT and VMAT2 (Weihe and Eiden, 2000). SERT (plasma membrane serotonin transporter) is located in the presynaptic terminals and is responsible for the reuptake of 5-HT after synaptic release (Blakely et al., 1991). VMAT2 (the vesicular monoamine transporter) packages 5-HT into synaptic vesicles (Weihe and Eiden, 2000). In *Lmx1b* mutants, expression of both *SERT* (Fig. 2, compare *I*, *J*, arrows) and *VMAT2* (Fig. 2, compare *K*, *L*, arrows) in prospective 5-HT cells is absent. Therefore Lmx1b regulates a set of genes necessary for 5-HT synthesis and transport.

Lmx1b is also weakly expressed in the floor plate (Fig. 1*A*, arrowhead). To rule out the possibility that the defect of 5-HT neuron development is caused by a patterning change in the ventral hindbrain, we examined a series of markers expressed in the floor plate or ventral precursors. At the medulla level, expression of sonic hedgehog in the floor plate, which plays a central role in patterning the ventral neural tube (Hynes and Rosenthal, 1999), is not affected in *Lmx1b* mutants (data not shown). Expression of *Nkx2.2*, which is required for 5-HT differentiation, is also normal in *Lmx1b* mutant embryos at stages E9.5–E11.5 (Fig. 3, compare *A*, *B*, arrows) (data not shown). Consistent with a lack of a patterning defect, prospective 5-HT neurons are formed, as indicated by a normal expression of *Pet-1* (Fig. 3, compare *C*, *D*, arrows) and *GATA3* (Fig. 3, compare *E*, *F*, arrows) in E11.5 mutant embryos. A significant amount of *Pet-1* expression is also detected in E12.5 mutant embryos, although *Pet-1* expression is

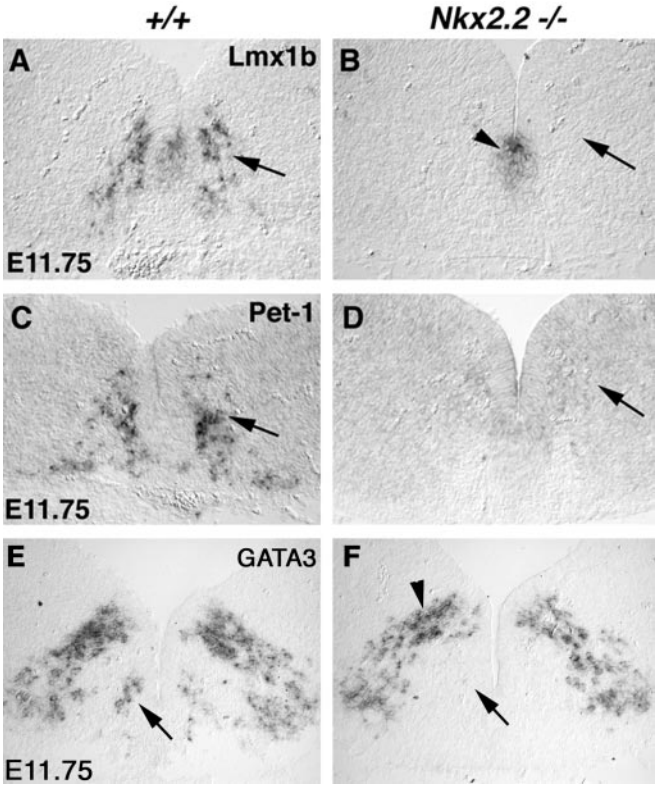

Figure 4. Nkx2.2 is necessary for the expression of *Lmx1b*, *Pet-1*, and *GATA3*. Transverse sections through medulla of E11.75 wild-type (*A*, *C*, *D*) and *Nkx2.2* mutants (*B*, *D*, *F*) are shown. *In situ* hybridization was performed with the indicated probes. Expression of *Lmx1b*, *Pet-1*, and *GATA3* in prospective 5-HT neurons is lost in *Nkx2.2* null medulla (*B*, *D*, *F*, arrows). Residual *Lmx1b* expression in the floor plate is still detected in *Nkx2.2* mutants (*B*, arrowhead). Also, *GATA3* expression in the more dorsally localized neurons is also unaffected in the mutants (*F*, arrowhead).

eventually reduced at prenatal stages (data not shown). 5-HT neurons also release another neurotransmitter (glutamate), and they express VGLUT3, the vesicular glutamate transporter (Gras et al., 2002). Expression of *VGLUT3* in prospective 5-HT neurons is largely unaffected in E11.5 and E12.5 *Lmx1b* mutants (Fig. 3, compare *G*, *H*, arrows) (data not shown). The normal expression of a set of markers for prospective 5-HT neurons suggests that *Lmx1b* is required neither for neurogenesis nor for an early neuronal survival. *Lmx1b* therefore specifically controls the development of the 5-HT transmitter phenotype.

Lmx1b and *Pet-1* act downstream of *Nkx2.2*

Pet-1 and *Nkx2.2* are also necessary for the specification of 5-HT cell fate (Briscoe et al., 1999; Pattyn et al., 2003). We next examined the functional relationship among these three genes. *Nkx2.2* is expressed in the precursor cells (Fig. 4*A*, small arrowhead) (Briscoe et al., 1999; Pattyn et al., 2003) and possibly in newly formed postmitotic 5-HT neurons in E11.5 medulla (Fig. 4*A*, arrow). By the time developing 5-HT neurons migrate to a more ventral position, however, *Nkx2.2* expression is turned off (Fig. 4*A*, large arrowhead). By contrast, *Lmx1b* and *Pet-1* are expressed in postmitotic cells, and their expression is maintained in mature 5-HT neurons (Fig. 1) (data not shown) (Hendricks et al., 1999). In line with this temporal order, expression of *Lmx1b* and *Pet-1* is absent in *Nkx2.2* mutant hindbrain, from caudal pons to the medulla (Fig. 4, compare *A*, *B*; *C*, *D*, arrows) (data not shown); however, *Lmx1b* and *Pet-1* expression in the most rostral 5-HT neurons is not affected in E11.75 *Nkx2.2* mutants (data not shown), consistent with the fact that development of the most rostral 5-HT neurons is independent of *Nkx2.2* (Briscoe et al., 1999). In addition, expression of the *GATA3* zinc finger gene, which is normally detected in 5-HT cells (Fig. 4*E*, arrow) (Van Doorninck et al., 1999), is also absent in E11.75 *Nkx2.2* mutants (Fig. 4, compare *E*, *F*, arrows), suggesting that *Lmx1b*, *Pet-1*, and *GATA3* all act downstream of *Nkx2.2* during caudal 5-HT neuron development.

Combined expression of *Lmx1b*, *Pet-1*, and *Nkx2.2* is sufficient to specify 5-HT transmitter phenotype

We then asked whether *Nkx2.2*, *Lmx1b*, and *Pet-1* together are sufficient to specify the 5-HT transmitter phenotype, by ectopically expressing these genes in chick spinal cord. To do this, we cloned the mouse full-length *Lmx1b* and *Pet-1* cDNAs into the *RCASBP* viral expression vector. We also used an *Nkx2.2* expression construct driven by the cytomegalovirus (CMV) promoter, which is functional in chick neural tube (Sun et al., 2001). These plasmids, singly or in combination, were electroporated into E2 chick neural tube, and the embryos were analyzed 2 or 3 d later. Not surprisingly, singular ectopic expression of *Lmx1b*, *Pet-1*, or *Nkx2.2* is not sufficient to induce the formation of 5-HT neurons in the spinal cord (Fig. 5*A–D*) (data not shown). Because endogenous *Nkx2.2* is expressed in the most ventral precursor cells (Ericson et al., 1997), the lack of 5-HT induction after ectopic expression of *Lmx1b* or *Pet-1* also suggests that *Lmx1b* or *Pet-1* plus endogenous *Nkx2.2* is not sufficient to induce the 5-HT cell fate.

Remarkably, co-electroporation of *Lmx1b* plus *Pet-1* is sufficient to induce the formation of 5-HT cells in the most ventral spinal cord (Fig. 5*G*,*H*, arrows) but not in the dorsal spinal cord (Fig. 5*G*, arrowhead), despite the fact that the exogenous *Lmx1b* and *Pet-1* genes are expressed throughout the dorsal ventral axis (Fig. 5*E*,*F*, arrows). As mentioned above, *Nkx2.2* is endogenously expressed in the ventral precursors in the spinal cord. We then asked whether *Nkx2.2* confers the competence to allow *Lmx1b* and *Pet-1* to drive the 5-HT cell fate. We therefore electroporated all three genes, *Lmx1b*, *Pet-1* plus *Nkx2.2*, into E2 chick neural tubes. We note that *Nkx2.2* driven by the CMV promoter is expressed in fewer cells than *Lmx1b* and *Pet-1* driven by the *RCAS* viral promoter (Fig. 5, compare *K*, *I*, *J*, arrows). Nonetheless, with this combination, ectopic 5-HT neurons appear in both the ventral (Fig. 5*L*, arrow) and dorsal (Fig. 5*L*, arrowheads) spinal cord, indicating that combinatorial expression of *Nkx2.2*, *Pet-1*, and *Lmx1b* is sufficient to specify the 5-HT cell fate in the spinal cord.

Discussion

In this study, we demonstrated that *Lmx1b* is the third gene (besides *Pet-1* and *Nkx2.2*) identified thus far that is required for 5-HT cell fate specification. Lmx1b controls a set of molecules essential for serotonin synthesis (tryptophan hydroxylase), vesicular transport (VMAT2), and reuptake after synaptic release (SERT). Figure 6 summarizes a model regarding how 5-HT neurons are formed in the caudal hindbrain. Nkx2.2 is required for the expression of *Lmx1b*, *Pet-1*, and an unknown target gene. These downstream genes then work coordinately to drive the specification of the 5-HT transmitter phenotype (Fig. 6).

Recent studies show that Nkx2.2 promotes 5-HT cell fate primarily by repressing the expression of *Phox2b*, a paired class homeobox gene (Pattyn et al., 2003). Thus, it remains unclear whether Nkx2.2 directly or indirectly regulates the expression of *Lmx1b* and *Pet-1*. Regardless, Nkx2.2 needs a cofactor (Fig. 6,

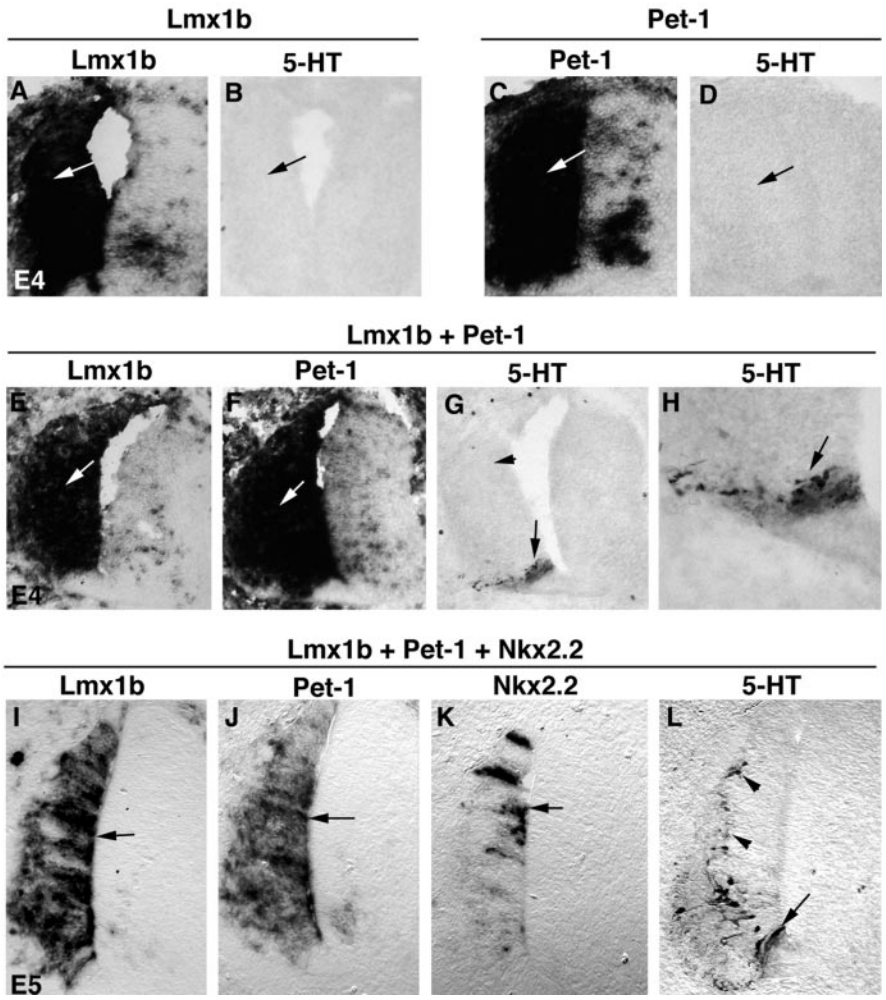

Figure 5. Lmx1b, *Pet-1*, and *Nkx2.2* in combination are sufficient to induce 5-HT cell fate in the chick spinal cord. Transverse sections through the spinal cord are shown. Plasmids were electroporated at E2 and analyzed at E4 (*A–H*) or E5 (*I–L*). The plasmids used for electroporation are shown above the lines. Expression of the transgenes (the names of which are shown at the top of the panels and below the lines) was detected by *in situ* hybridization. Also, 5-HT immunostaining was performed (*B*, *D*, *G*, *H*, *L*). *H* is the high magnification of the positive area shown in *G*.

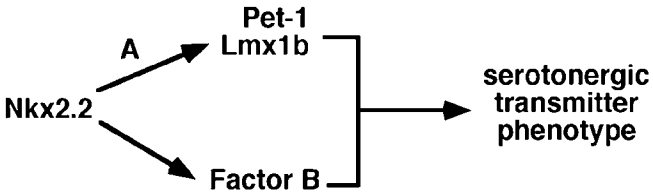

Figure 6. A molecular pathway controlling 5-HT cell fate specification. Nkx2.2 plus an unknown cofactor (A) directly or indirectly activate the expression of *Pet-1* and *Lmx1b* in prospective 5-HT neurons. Nkx2.2 might need a different cofactor to activate another downstream factor (B), or it removes an unknown inhibitor (data not shown) to allow Lmx1b and Pet-1 to promote 5-HT cell fate.

factor A) expressed in the 5-HT precursor cells to activate *Lmx1b* and *Pet-1* expression, because *Lmx1b* and *Pet-1* are not expressed in all cells derived from *Nkx2.2*-positive precursor cells, for instance, the visceral motor neurons in the hindbrain and the V3 interneurons in the spinal cord (Hendricks et al., 1999; Qian et al., 2002; Pattyn et al., 2003) (data not shown).

Lmx1b and *Pet-1* are sufficient to induce formation of 5-HT-positive cells in the most ventral spinal cord, where *Nkx2.2* is expressed, but they need ectopic *Nkx2.2* expression to do so in the dorsal spinal cord (Fig. 6). Nkx2.2 either activates another downstream factor (Fig. 6, Factor B) or removes an unknown inhibitor to allow Lmx1b and Pet-1 to specify the 5-HT transmitter phenotype. The putative factor B remains to be identified; however, the fact that 5-HT transmitter phenotype can be induced in the spinal cord suggests that factor B might be expressed throughout the ventral hindbrain and spinal cord. This would be in contrast to the restriction of *Lmx1b* and *Pet-1* expression to the ventral hindbrain. In other words, although Nkx2.2 needs a hindbrain-specific coregulator (Fig. 6, factor A) to activate *Lmx1b* and *Pet-1*, it may use a more generic coactivator to regulate factor B. Such model would explain why *Nkx2.2* alone or *Lmx1b* plus *Pet1* are insufficient to specify 5-HT cell fate in the spinal cord, whereas a combination of all three genes can. Previously, the *GATA3* zinc finger gene has been suggested to be necessary for 5-HT neuron development in the caudal hindbrain (Van Doorninck et al., 1999); however, although the raphe nuclei are disorganized, the total number of 5-HT neurons is not changed in *GATA3* null mutants (Van Doorninck et al., 1999). Also, *GATA3* is not expressed in the most ventral spinal cord (Pata et al., 1999). Thus, *GATA3* is an unlikely candidate for the missing factor B, although it remains a formal possibility that a *GATA3*-like family member might be involved in 5-HT cell fate specification.

It is noteworthy that although *Lmx1b* is required for the specification of the 5-HT transmitter phenotype throughout the hindbrain, the most rostral cluster of 5-HT neurons still develops in *Nkx2.2* mutants, and residual 5-HT cells are also formed in *Pet-1* mutants (Briscoe et al., 1999; Hendricks et al., 2003). Thus, besides working together with Pet-1 and Nkx2.2 to specify the vast majority of 5-HT neurons, Lmx1b may act with other factors to control the formation of the remaining subset of 5-HT neurons. Lmx1b likely acts autonomously in the caudal hindbrain, because *Lmx1b* is expressed in postmitotic 5-HT neurons (Fig. 1), and no patterning defect is detected in *Lmx1b* mutant medulla (Fig. 3); however, *Lmx1b* is essential for proper formation of the isthmic organizer in the junction of midbrain and hindbrain (Adams et al., 2000; Matsunaga et al., 2002). Thus, it remains a formal possibility that *Lmx1b* might nonautonomously control the formation of the most rostral clusters of 5-HT neurons. Indeed, other structures adjacent to the isthmic organizer fail to develop properly in *Lmx1b* mutants, including the locus ceruleus noradrenergic center (indicated by loss of expression of β-dopamine hydroxylase) and motor nuclei III and IV (indicated by loss of *Phox2b* expression) (data not shown).

While this manuscript was being reviewed, Ding and colleagues (2003) reported an independent analysis of *Lmx1b* mutants, and they also showed an essential role of *Lmx1b* in 5-HT specification; however, the model that they proposed is quite

different from ours (Fig. 6). In their model (Ding et al., 2003), Pet-1 (and GATA3) acts downstream of Lmx1b, whereas in our model *Pet-1* and *Lmx1b* act in combination to specify 5-HT cell fate (Fig. 6). The discrepancy most likely arrived from the stages chosen for their analysis. For instance, they analyzed E14.5 mutant embryos and showed a significant reduction of *Pet-1* expression at this stage. We found, however, that Pet1 expression is not affected in E11.5 *Lmx1b* mutants (Fig. 3), and significant *Pet1* expression is also observed at E12.5 (data not shown). In the case of *GATA3*, although we show a normal expression in E11.5 *Lmx1b* mutants (Fig. 3), Ding et al. (2003) reported a small reduction at E11. Because caudal 5-HT neurons just start to form at E11 (Pattyn et al., 2003), subtle difference of developmental stages among littermates might cause small variability of *GATA3* expression. Thus, our data suggest that at the time when 5-HT neurotransmitter phenotype is specified, expression of *Pet-1* (and *GATA3*) is largely independent of *Lmx1b*. Interestingly, *Lmx1b* expression is also unaffected in *Pet-1* null mice (Ding et al., 2003). Therefore, Lmx1b and Pet-1 act in combination, rather than in series to promote 5-HT cell fate (Fig. 6). Our model is also consistent with the finding that only combined (but not singular) expression of *Lmx1b* and *Pet-1* is able to induce formation of 5-HT neurons in the ventral spinal cord (Fig. 5); however, the loss of *Pet-1* expression in *Lmx1b* null embryos at E14.5 (Ding et al., 2003) (data not shown) suggests a possible role of *Lmx1b* in maintaining *Pet-1* expression at late embryonic stages.

Lmx1b is also required for proper development of the midbrain DA neurons (Smidt et al., 2000); however, the roles of *Lmx1b* in these two monoaminergic systems are different. Although *Lmx1b* is required for the specification of 5-HT transmitter phenotype, *Lmx1b* is necessary only for the maintenance but not the initial specification of the DA transmitter phenotype (Van Doorninck et al., 1999). Ontogenetically, both DA and 5-HT neurons develop from the ventral precursors adjacent to the floor plate, and their development is regulated by some common signals from the floor plate, such as sonic hedgehog (Hynes and Rosenthal, 1999; Goridis and Rohrer, 2002). Thus, it is not surprising that DA and 5-HT neurons share some overlapping molecular pathways. It will be interesting to determine whether Lmx1b acts in combination with DA-specific transcription factors, such as Nurr1 (Zetterstrom et al., 1997) and Ptx3 (Asbreuk et al., 2002), to control DA neuron development. Lmx1b may also control some common features between DA and 5-HT neurons. Indeed, we found that in *Lmx1b* mutants, expression of *VMAT2*, the vesicular transporter for both DA and 5-HT (Weihe and Eiden, 2000), is apparently lost both in prospective 5-HT neurons (Fig. 2) and in midbrain DA neurons (data not shown).

In summary, our studies show that *Lmx1b* plays an essential role in the specification of the 5-HT transmitter phenotype. Dysfunction of the 5-HT system has been associated with a wide range of neurological and psychiatric disorders (Lucki, 1998; Davidson et al., 2000; Nelson and Chiavegatto, 2001). The demonstration of the sufficiency of *Lmx1b*, *Pet-1*, and *Nkx2.2* to specify the 5-HT transmitter phenotype might eventually pave the way for *in vitro* preparation of 5-HT neurons that could be used for cell transplantation-based therapy.

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