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The transcription factor, *Lmx1b*, promotes a neuronal glutamate phenotype and suppresses a GABA one in the embryonic trigeminal brainstem complex

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Abstract

Achieving an appropriate balance between inhibitory and excitatory neuronal fate is critical for development of effective synaptic transmission. However, the molecular mechanisms dictating such phenotypic outcomes are not well understood, especially in the whisker-to-barrel cortex neuraxis, an oft-used model system for revealing developmental mechanisms. In trigeminal nucleus principalis (PrV), the brainstem link in the whisker-barrel pathway, the transcription factor *Lmx1b* marks glutamatergic cells. In PrV of *Lmx1b* knockout mice ($^{-/-}$), initial specification of glutamatergic versus GABAergic cell fate is normal until embryonic day 14.5. Subsequently until the day of birth, glutamatergic markers (e.g. *VGLUT2*) stain significantly fewer PrV neurons, whereas, GABAergic markers (*Pax2* and *Gad1*) stain significantly more PrV cells, notably in *Lmx1b*-null PrV cells. These changes also occurred in *Lmx1b/Bax* double $^{-/-}$ mice, where PrV cells are rescued from *Lmx1b* $^{-/-}$ -induced apoptosis; thus, effects upon excitatory/inhibitory cell ratios do not reflect a cell death confound. Electroporation-induced ectopic expression of *Lmx1b* in an array of sites decreases numbers of neurons that express GABAergic markers, but increases *VGLUT2*⁺ cell numbers or stain intensity. Thus, *Lmx1b* is not involved in the initial specification of glutamatergic cell fate, but is essential for maintaining a glutamatergic phenotype. Other experiments suggest that *Lmx1b* acts to suppress *Pax2*, a promoter of GABAergic cell fate, in a cell-autonomous manner, which may be a mechanism for maintaining a functional balance of glutamatergic and GABAergic cell types in development.

Keywords

Principalis; Whiskers; Barrels; Neurotransmission; Bax; Homeodomain

INTRODUCTION

Trigeminal (V) primary afferent neurons project to several brainstem nuclei that integrate somatotopic information from the face and convey it to the thalamus and somatosensory

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(S1) cortex (reviewed in Woolsey, 1990; Henderson and Jacquin, 1995). These inputs are processed and relayed by excitatory or inhibitory neurons in the V brainstem complex which is comprised of the V nucleus principalis (PrV) and the spinal trigeminal subnuclei (SpV). PrV originates the V lemniscal pathway that is the essential conduit for transmission of a whisker-related pattern to thalamic barreloids in development (Killackey and Fleming, 1985; Ding et al., 2003). PrV is also known to consist mostly of glutamatergic cells, most of which project to the contralateral thalamus, and a smaller number of GABAergic local circuit neurons (Magnusson et al., 1987; Kaneko et al., 1987; Haring et al., 1990; Clements and Beitz, 1991; Bae et al., 2000). Recent studies have begun to reveal the molecular mechanisms that govern the development of the PrV-based lemniscal pathway (reviewed in Erzurumlu et al., 2006; Jacquin et al., 2008; Xiang et al., 2010; da Silva et al., 2011), but the molecular machinery that controls the determination of a glutamatergic versus GABAergic phenotype in developing PrV neurons is unclear. This has important system implications because inhibitory local circuits have a profound influence upon the response properties of whisker-sensitive PrV cells (Minnery et al., 2003; Furuta et al., 2008).

In the mammalian central nervous system, specification of a glutamatergic versus GABAergic cell fate has been shown to occur at multiple molecular levels. In the retina, cerebellum, forebrain and dorsal spinal cord, the transcription factors *Ngn1*, *Ascl1*, *Gsh1/2*, *Otx2* and *Ptf1a* are expressed in neuronal progenitor cells and influence whether a neuron becomes glutamatergic or GABAergic (Glasgow et al., 2005; Mizuguchi et al., 2006; Hoshino et al., 2005; Puelles et al., 2006; Dullin et al., 2007). In postmitotic cells, other transcription factors have also been shown to be necessary for the specification of neurotransmitter phenotype. In the developing midbrain, *Gata2* has been shown to be a necessary post-mitotic selector gene for GABAergic neurons (Kala et al., 2009). In the developing dorsal spinal cord, *Tlx1* and *Tlx3* serve as key selectors in defining the fate of glutamatergic neurons, whereas *Lbx1* and *Pax2* may function as selector genes to specify a GABAergic fate (Cheng et al., 2004, 2005; Batista and Lewis, 2008). While *Tlx3* may function to antagonize *Lbx1* to promote a glutamatergic cell fate, it is unclear whether a similar factor is required to suppress *Pax2* and, consequently, a GABAergic phenotype. In this regard, *Lhx1* and *Lhx5* are known to activate and maintain *Pax2* expression which is necessary for the differentiation of GABAergic cells in the spinal cord (Pillai et al., 2007). *Lmx1b*, a LIM homeodomain transcription factor whose expression is restricted to glutamatergic cells and not colocalized with *Pax2* in the dorsal horn, may have a role analogous to the *Tlx* genes noted above. To date, however, there is no evidence in support of *Pax2* upregulation in the dorsal horn of *Lmx1b*^{-/-} mice, nor is there evidence of upregulation of glutamatergic markers in the dorsal horn of *Pax2*^{-/-} mutants (Cheng et al., 2004; Ding et al., 2004).

The present study addresses the function of *Lmx1b* in determining neurotransmitter phenotype in the PrV of embryonic mice. We (Xiang et al., 2010) have recently reported that *Lmx1b* is necessary for whisker-related pattern formation in PrV. Here, *Lmx1b* and *Pax2* expression in the PrV were found to be mutually exclusive. Loss-of-function and gain-of function approaches were then utilized to test the hypotheses that *Lmx1b* promotes a neuronal glutamatergic phenotype and suppresses a GABAergic phenotype and that *Lmx1b* accomplishes this by suppressing *Pax2* expression.

MATERIAL AND METHODS

Animals

The generation and genotyping of *Lmx1b*^{-/-} and *Lmx1b*^{+/-} mice were performed as previously described (Chen et al., 1998; Xiang et al., 2010). *Lmx1b*/Bax double null mice were generated by breeding *Lmx1b*^{+/-} and *Bax*^{-/-} mice (Jacquin et al., 2008); Bax mutants were on a C57BL/6 background. *Lmx1b*^{+/-} mice were maintained in the mouse facility according to protocols approved by the Division of Comparative Medicine at Washington University.

BrdU/Lmx1b double labeling

To assess the mitotic status of *Lmx1b* expressing cells, BrdU pulse-labeling was followed by *Lmx1b* immunohistochemistry. BrdU was injected into timed mated female mice on embryonic day (E) 11.5 and embryos were dissected out after 2 hrs for immunocytochemical analysis. The processing of the embryos and double staining for BrdU/*Lmx1b* were performed as previously described (Ding et al., 2004).

In situ hybridization and immunocytochemistry

In situ hybridization and immunocytochemical staining were performed as previously described (Chen et al., 2001; Ding et al., 2003; Ding et al., 2004). Sections were incubated with rabbit anti-*Lmx1b* (1:1500), goat anti-*Lmx1b* (1:200) or rabbit anti-Pax2 (1:300) in phosphate buffered saline (PBS) containing 2% normal donkey serum and 0.3% TritonX-100 overnight, followed by incubation in biotinylated donkey anti-goat or anti-rabbit (Jackson Immuno Research, 1:200) for 2 hrs, followed by FITC or cy3-conjugated streptavidin (Molecular Probes, 1:1000) for 1 hr. After washing with PBS, slides were coverslipped and observed under an Olympus fluorescent microscope (BX51). For immunostaining after *in situ* hybridization, protocols for incubation in primary antibodies and biotinylated donkey anti-rabbit IgG were identical to those described above. Following incubation with secondary antibody, ABC reagents (Vector) or TSA reagents were used. Controls for antibody and probe specificity were run in tandem with initial experiments and they included deletion of the primary antibody and substitution of a sense probe, respectively.

In utero electroporation

To introduce exogenous *Lmx1b* into the embryonic central nervous system, *in utero* electroporation was performed as previously described (Ding et al., 2004). Briefly, the uterine horns were exposed and 2–4 µl of plasmid solution was injected through the uterus into the lateral or third ventricles of the embryonic brain, or central canal of the embryonic spinal cord, with a glass micropipette. Embryos were then placed between the electrodes of an ECM830 electroporator, and five 50-msec pulses of 25–40 volts were delivered. The uterus was then placed back into the repaired abdominal cavity and embryonic development continued until appropriate later stages for phenotypic analyses.

Cell counting and statistical analysis

Changes in total numbers of labeled PrV cells were estimated in consecutive series of sections taken through the pons in each case. Four to six animals from each experimental and wild-type (or plasma negative) control group provided such data. The V motor nucleus and spinal tract served as cytoarchitectural landmarks to ascertain the PrV and adjoining structures, such as the supratrigeminal nucleus (SuV) and the SpV subnucleus interparialis (SpVi). Images used in these analyses were digitally stored. For cell counting after electroporation, at least 4 slides deemed to have the highest electroporation efficiency from

positive embryos were selected for analysis. Data are presented as means $\pm$ S.E.M, and Student's independent *t* tests were used for statistical analysis with $p < .05$ considered significant.

RESULTS

Lmx1b marks glutamatergic cells and Pax2 marks GABAergic cells in the PrV

Expression of *Lmx1b* and *VGLUT2*, a vesicular glutamate transporter that is considered to mark all glutamatergic neurons (Freneau et al., 2001), were assessed. During development, *Lmx1b* is abundantly expressed in the PrV (Fig. 1a). Expression of *Lmx1b* was also detected to a lesser degree in the SpVi and SuV (Fig. 1a; data not shown). When considering all brainstem regions receiving primary afferent inputs, *VGLUT2* was found to be most robustly expressed in the PrV and SuV (Fig. 1b). To assess the extent to which *Lmx1b* and *VGLUT2* are coexpressed, *in situ* hybridization of *VGLUT2* followed by *Lmx1b* immunocytochemistry were performed. Double labeling for *Lmx1b* and *VGLUT2* showed that the vast majority (95%) of *Lmx1b*⁺ cells are *VGLUT2*⁺ in the PrV (Fig. 1d, h), indicating that *Lmx1b* expression is largely restricted to glutamatergic PrV cells.

Similar staining procedures were utilized to assess potential *Lmx1b* colocalization with Pax2, a transcription factor marking GABAergic neurons in the dorsal spinal cord (Cheng et al., 2004). On E14.5, Pax2⁺ cells were most numerous in the lateral regions of the PrV (Fig. 1e). Double staining for *Lmx1b* and Pax2 failed to reveal coexpression in PrV cells. Mutual exclusion of *Lmx1b* and Pax2 labeling in PrV cells was the case at all embryonic timepoints examined (Fig. 1e; data not shown). To determine whether Pax2 reliably marks GABAergic cells in the PrV, double-staining of Pax2 and *Gad1*, the rate-limiting enzyme that is essential for the synthesis of GABA (Mugnaini and Oertel, 1985; Erlander et al., 1991), was performed. Although Pax2⁺ cells were much more numerous than *Gad1*⁺ cells prior to E13.5 (data not shown), from E14.5 onwards, *Gad1*⁺ and Pax2⁺ staining usually occurred in the same cells (78%, Fig. 1f, h), indicating that Pax2 is a fairly reliable marker for GABAergic cells in the PrV during the last prenatal week. To determine whether *Lmx1b* expression is restricted to postmitotic cells, BrdU labeling was examined at E11.5 after 2 hours of BrdU exposure. No BrdU⁺ and *Lmx1b*⁺ cells were colocalized (Fig. 1g). Thus, in the hindbrain region where PrV cells are generated, *Lmx1b* is only expressed in postmitotic cells.

In Lmx1b mutant mice, glutamatergic and GABAergic markers are normally expressed until E14

An evaluation of the expression of neurotransmitter markers in *Lmx1b*^{-/-} mice failed to reveal a difference in *VGLUT2* expression between *Lmx1b*^{-/-} and wild-type mice until E14.5 (Fig. 2a, b; data not shown). Similarly, there was no apparent effect of the mutation upon expression of the GABAergic markers Pax2 and *Gad1* in the PrV of *Lmx1b*^{-/-} mice up to E14.5 (Fig. 2c–f). Thus, *Lmx1b* is not required for initial specification of glutamatergic versus GABAergic cells in the PrV when it is undergoing extensive mitosis.

After E14.5, in Lmx1b mutants, glutamatergic PrV cells are fewer than normal and GABAergic PrV cells are more numerous

Post-mitotic (post-E14.5) PrV cells are significantly less likely to express *VGLUT2* in the absence of *Lmx1b* as compared to wild-type controls. For example, on P0, Figures 3a and b illustrate marked downregulation of *VGLUT2* relative to that which occurs in the wild-type PrV. This conclusion was supported by estimates of labeled cell numbers. (Fig. 3e). Staining for another glutamatergic neuron-related marker, *Gria2*, which encodes the AMPA-class receptor for glutamate (Kerr et al., 1998), was completely absent in the PrV from *Lmx1b*^{-/-}

mice (Fig. 3c, d). These data suggest that the development of glutamatergic cells is severely compromised in the PrV of *Lmx1b*^{-/-} mice in the last week of embryonic development.

Conversely, the development of GABAergic PrV cells was significantly facilitated in the absence of *Lmx1b* after E14.5 when *Gad1*⁺ cells were more numerous in the PrV of *Lmx1b*^{-/-} mice relative to that of wild-type controls (Fig. 4a–c). Expression of another GABAergic marker, VGAT, which encodes the vesicular GABA transporter (Chaudry et al., 1998), was also significantly upregulated in *Lmx1b*^{-/-} mice when compared with wild-type controls (Fig. 4d–f). A final example pertains to Pax2, which marks GABAergic PrV cells at late embryonic times. In *Lmx1b*^{-/-} mice, Pax2 staining was normal prior to E14.5, but gradually increased to label about five times greater than normal numbers of PrV cells by the day of birth (Figs. 4g–i, Figs. 5a and b; data not shown). Taken together, these results are consistent with the notion that a significant *Lmx1b*^{-/-} phenotype is a switch from glutamatergic to GABAergic fate in PrV neurons. The latter also appears to have occurred in the nearby SupV and SpVi subnuclei (Fig. 4; data not shown).

Increased Pax2 staining occurs in those PrV cells lacking Lmx1b

The increased number of Pax2⁺ cells in the PrV of *Lmx1b*^{-/-} mice prompted an examination of the genotype of GABAergic PrV cells; namely, does Pax2 expression occur only in cells lacking *Lmx1b*? In the wild-type PrV on E17.5, Pax2⁺ cells were intermingled with *Lmx1b*⁺ cells, but they were rarely colocalized (Fig. 5a). To identify *Lmx1b*^{-/-} cells in the PrV, the *neo* gene was introduced into the *Lmx1b* locus as a positive selection marker (Chen et al., 1998) that mimics the endogenous *Lmx1b* expression, therein permitting double staining of Pax2 and *neo* in the PrV of *Lmx1b*^{-/-} mice. Because *neo* expression in *Lmx1b*^{+/-} heterozygotes was very weak, *Lmx1b*/Pax2 staining in wild-type mice served as the control group. Relative to *Lmx1b*^{+/+}Pax2⁺ cells in wild-type control, the number of *neo*^{+/+}Pax2⁺ cells was significantly increased in the PrV of *Lmx1b*^{-/-} mice (Fig. 5b,c). Moreover, the increased number of *neo*^{+/+}Pax2⁺ cells in the mutants was comparable to the increased number of Pax2⁺ cells in the PrV of *Lmx1b*^{-/-} mice. These data suggest that the increased number of Pax2⁺ cells reflects Pax2 staining in cells lacking *Lmx1b*. However, Pax2 staining was not observed in all *Lmx1b*^{-/-} cells, suggesting that other factors also regulate Pax2 expression in *Lmx1b*^{-/-} PrV cells. Nevertheless, it is clear that *Lmx1b* functions in a cell-autonomous manner in the regulation of Pax2 expression in the PrV.

Altered neurotransmitter phenotypic ratios in the *Lmx1b*^{-/-} do not reflect a mutation-induced PrV cell death confound

It has been documented that approximately half of all PrV neurons die by the day of birth in *Lmx1b* mutants (Xiang et al., 2010). The present study's main observation, that there are increased numbers of GABAergic cells and decreased numbers of glutamatergic cells in the *Lmx1b*^{-/-}, could simply reflect a less than interesting disproportionate cell death in the glutamatergic PrV cell class, as opposed to *Lmx1b*-induced specification of neurotransmitter phenotype. This possibility was tested by performing the exact same set of experiments in *Lmx1b*/Bax double knockouts, where currently studied mutants were bred with mice lacking the apoptosis promoting gene, *Bax*, therein rendering them resistant to apoptosis (see Jacquin et al., 2008, and Xiang et al., 2010, for details and baseline data). Should the same set of results be obtained in the *Lmx1b*/Bax double knockouts, then the presently described shift in transmitter phenotype cannot be attributed to a mutation-induced cell death confound. Indeed, this is exactly what was observed. As illustrated in Figure 6, the reduction in numbers of glutamatergic PrV cells observed in *Lmx1b* single null mutants was also observed in the *Lmx1b*/Bax double knockouts. Similarly, the increase in numbers of GABAergic PrV cells observed in *Lmx1b* null mutants was also observed in the *Lmx1b*/Bax

double knockouts. For estimates of total numbers of labeled PrV cells in both of these staining paradigms, single and double null mutants were statistically indistinguishable.

Lmx1b actions upon transmitter phenotype are not unique to the PrV

To test the generality of above described effects of *Lmx1b* deletion upon the developing PrV, additional gain-of-function experiments were carried out in the developing spinal dorsal horn, cerebral cortex and superior colliculus, where exogenous *Lmx1b* is conveniently introduced by electroporation, which is not the case for the PrV embedded deep within the ventral brainstem.

The findings that *Lmx1b* and *Pax2* expression are mutually exclusive in the PrV, and that *Pax2* expression was promoted in *Lmx1b*^{-/-} cells, raise the possibility that *Lmx1b* may suffice to suppress *Pax2* expression. Because of the technical difficulties in introducing exogenous *Lmx1b* into the developing PrV by in utero electroporation, misexpression studies were carried out in the dorsal horn of the spinal cord. This site was chosen because of prior indications of mutual exclusivity in *Lmx1b* and *Pax2* expression there (Ding et al., 2004; Gross et al., 2002). Moreover, *Pax2* is abundantly expressed in the dorsal horn, which makes for more likely incorporation of exogenous *Lmx1b* into *Pax2*⁺ cells. Exogenous *Lmx1b*-EGFP plasmids (expressing *Lmx1b* and EGFP separately via IRES cassette insertion) were introduced into the dorsal horn at E12.5 and dorsal horn phenotype was analyzed a few days later. In the dorsal horn electroporated with EGFP plasmids only, EGFP/*Lmx1b*⁺ double labeled cells were observed (Fig. 7b, d). By contrast, when *Lmx1b*-EGFP plasmids were introduced into the dorsal horn, no EGFP⁺/*Pax2*⁺ co-stained cells were observed (Fig. 7a, c). While it is possible that *Lmx1b*-EGFP might have been incorporated by *Pax2*⁺ cells, this is likely to be rare because EGFP/*Pax2* double positive cells were always seen in electroporation control cases (Fig. 7b, d). These data suggest that exogenous *Lmx1b* is necessary and sufficient to suppress *Pax2* expression in the developing dorsal horn.

Whether *Lmx1b* is inherently capable of promoting a glutamatergic phenotype was then assessed by ectopic expression of *Lmx1b*-EGFP plasmids in the developing cerebral cortex, where numerous glutamatergic neurons exist and transcription factors are known to impact their genesis (Hevner et al., 2006). An increased expression of *VGLUT2* was observed in cortices electroporated with *Lmx1b*-EGFP plasmids, as compared to normal levels of *VGLUT2* staining in the contralateral cortices into which no plasmids were introduced (Fig. 8a, c). By contrast, *VGLUT2* expression was normal in cortices electroporated with EGFP only (Fig. 8b, d). Increased *VGLUT2* expression could reflect an increased number of *VGLUT2*⁺ cells or increased levels of *VGLUT2* expression. Insofar as there was no difference in numbers of *VGLUT2* labeled cells between *Lmx1b* electroporated cortices and contralateral controls (Fig. 8f), and EGFP/*VGLUT2* double-staining revealed many single- and double-labeled cells in cortices electroporated with *Lmx1b*-EGFP plasmids (Fig. 8e), *Lmx1b* alone did not activate cortical *VGLUT2* expression in an all-or-none manner. However, examination of the optical density of gene expression revealed that the quantity of *VGLUT2* expression increased relative to the control cortices (Fig. 8g). Similar results were obtained when *Lmx1b*-EGFP plasmids were introduced into the developing hippocampus (data not shown). Thus, exogenous *Lmx1b* does not appear to be sufficient to induce ectopic *VGLUT2* expression, yet it is sufficient to increase the level of expression of *VGLUT2*.

Lastly, whether *Lmx1b* is sufficient to suppress expression of GABAergic markers was tested by ectopic expression of *Lmx1b* in the superior colliculus. The latter contains many GABAergic neurons (Tsunekawa et al., 2005) and no endogenous *Lmx1b* expression. At E19.5, *Gad1* staining revealed numerous GABAergic cells, with the most intense staining in the intermediate zone (Fig. 9a, d). In colliculi electroporated with *Lmx1b*-EGFP at E14.5, *Gad1* expression was markedly reduced relative to the contralateral side into which no

Lmx1b-EGFP plasmids were introduced (Fig. 9b, e). An estimate of total numbers of labeled cells revealed a significant reduction in the number of *Gad1*⁺ cells as compared to the control side (Fig. 9c). By contrast, the colliculi electroporated with EGFP only showed no change in *Gad1*⁺ expression (data not shown). To assess possible coexpression of *Gad1* and *Lmx1b*, *Gad1* and EGFP (*Lmx1b* positive) double staining failed to reveal double labeled cells in the electroporated in the electroporated colliculi (Fig. 9f). These data suggest that ectopic *Lmx1b* will suppress *Gad1* expression in the superior colliculus.

DISCUSSION

The present study offers data in support of the following conclusions. *Lmx1b* marks glutamatergic cells, but not GABAergic cells, in the developing PrV. In the absence of *Lmx1b*, initial specification of glutamatergic versus GABAergic fate appears normal until E14.5. Over the next week, glutamatergic cell number decreases in the PrV of *Lmx1b*^{-/-} embryos. Conversely, the number of GABAergic PrV cells increases, consistent with the notion that, in the absence of *Lmx1b*, many cells initially specified as glutamatergic become GABAergic. In addition to such a fate alteration in *Lmx1b*^{-/-} cells, additional evidence supports the idea that *Lmx1b* is both necessary and sufficient for suppressing expression of a GABAergic phenotype. Namely, misexpression of *Lmx1b* in the superior colliculus reduces the number of *Gad1*⁺ cells there, and exogenous *Lmx1b* marked by EGFP is capable of suppressing *Pax2* expression in the dorsal spinal cord. Together with the observation that *Pax2* expression is upregulated in *Lmx1b*^{-/-} cells in the PrV, these data strongly suggest that *Lmx1b* also functions to suppress the GABAergic phenotype.

How does *Lmx1b* maintain transcriptional suppression of GABAergic-specific gene expression? One possible mechanism is that *Lmx1b* suppresses *Pax2*, whose expression is mutually exclusive with that of *Lmx1b*. This conclusion is prompted by the following observations: 1) *Lmx1b* deletion produces an upregulation of *Pax2* in the PrV in a cell-autonomous manner; 2) *Lmx1b* misexpression in the dorsal horn suppresses *Pax2* expression; 3) upregulation of *Pax2* also occurs in other brainstem V regions, such as the SpVi and SuV, in *Lmx1b*^{-/-} mice. These results strongly support the notion that *Pax2*/*Lmx1b* mutual exclusivity is a general transcriptional mechanism used in multiple developing hindbrain and spinal cord regions to maintain an effective ratio of glutamatergic versus GABAergic cell fates.

The present study also suggests that not all glutamatergic PrV cells adopt a GABAergic cell fate in the absence of *Lmx1b* because *Gad1* expression was suppressed in only a minority of *Lmx1b*^{-/-} cells. Such an impression of selective suppression may reflect the use of only a limited number of GABAergic markers in examining *Lmx1b*^{-/-} cells, therein resulting in our failure to visualize more GABAergic cells. Alternatively, other yet to be revealed GABAergic suppressors may have retained their function in these cells. The latter interpretation is favored in light of the observation here that, prior to E14.5, *Lmx1b* deletion had no effect upon the ratio of stained neurotransmitter phenotypes in PrV. Other unknown transcription factors may serve to maintain the glutamatergic phenotype at particular embryonic timepoints. Because the timing of *VGLUT2* downregulation begins at E15.5, coinciding with the onset of GABAergic marker upregulation, it may also be the case that GABAergic phenotype suppression is the default signaling pathway, though further study is required to test this idea.

An important conclusion derived from the present study is that *Lmx1b* is essential for maintaining or strengthening the glutamatergic phenotype. In the PrV of *Lmx1b*^{-/-} mice, *VGLUT2* staining is significantly reduced during the final embryonic week. Conversely, introducing *Lmx1b* to the late prenatal cerebral cortex enhances the intensity of *VGLUT2*

staining. These results demonstrate that *Lmx1b* imposes maintenance or promotion of the glutamatergic phenotype during development. However, because ectopic induction of *Lmx1b* expression in the cortex does not induce glutamatergic expression, but rather enhances such in specific locations, *Lmx1b* must work in concert with other existing glutamatergic-specific factors to sculpt excitatory circuits in the developing cortex. Moreover, it remains unclear whether *Lmx1b* regulates *VGLUT2* expression directly or indirectly. Other transcription factors may also be involved in the specification and/or maintenance of the glutamatergic fate decision. One likely candidate is *Tlx3* which has been shown to be expressed in glutamatergic cells in the dorsal spinal cord (Cheng et al., 2004; Xu et al., 2008). Inasmuch as *Tlx3* is also expressed in the developing PrV (data not shown), it may function similarly there, perhaps acting synergistically with *Lmx1b*.

Prior studies have revealed the *Tlx1* and *Tlx3* genes as postmitotic determinants of the glutamatergic phenotype, possibly via selective suppression of *Lbx1* function (Cheng et al., 2004, 2005). Here, *Lmx1b* is revealed to do the same in PrV, possibly via *Pax2*, a promoter of GABAergic cell fate, in a cell-autonomous manner. Yet, one important distinction between *Tlx1/3* and *Lmx1b* is that the latter is not required for the initial specification of glutamatergic versus GABAergic cell fate, which is also the case for *Lmx1b* in the dorsal horn (Ding et al., 2004). And, *Lmx1b* is unique in its duality, facilitating glutamatergic phenotype at the expense of the GABAergic phenotype. Taken together, information to date suggests that *Lmx1b* is a third class of transcription factors that is neither expressed in the progenitor cells nor essential for the initial specification of glutamatergic cell fate. This class of transcription factors is essential for maintaining glutamatergic phenotype at late embryonic stages and does so by suppressing aGABAergic phenotype in nascent glutamatergic cells. Thus, *Lmx1b* is a gene that is necessary for development of excitatory and inhibitory circuits in the developing brainstem.

A final implication of the present study pertains to a potential mechanism underlying *Lmx1b*'s essential role in PrV pattern formation. PrV normally develops sufficiently by the day of birth to view a whisker-related barrel-like pattern there, thus obviating the experimental limitations imposed by perinatal lethality in the *Lmx1b*^{-/-}. We (Xiang et al., 2010) have previously shown that PrV patterning of whiskers (barrelettes) fails to form in the newborn *Lmx1b*^{-/-}. While there are compelling data (Henderson et al., 1992) that fail to support a necessary role for neuronal impulse activity in the development of such patterns in PrV, there are indications (reviewed in Erzurumlu et al., 2010) that excitatory synaptic transmission is necessary for barrelette formation in PrV. If the latter is the case, then the dramatic diminution of glutamatergic cells in the *Lmx1b*^{-/-} PrV would be sufficient to disrupt the formation of this pattern. Selective transcriptional suppression of *Pax2* by *Lmx1b* may, by switching the balance of PrV circuitry in favor of requisite excitatory neurotransmission, be a patterning mechanism in the developing V system. The receptive field and electrophysiological properties of PrV cells in *Lmx1b*^{-/-}s are, therefore, important topics for future study.

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Figure 1.

Expression of molecular markers in transverse sections through the PrV. (a–c) On the day of birth (P0), *Lmx1b* (a) and *VGLUT2* (b) are abundantly expressed in the PrV, whereas *Gad1* staining occurs in a smaller number of PrV cells (c). (d) Double staining of *VGLUT2* (blue) and *Lmx1b* (yellow) reveals robust colocalization (arrows) of *Lmx1b* and *VGLUT2* in P0 PrV cells. (e) *Lmx1b* (red cells, arrowhead) is not colocalized with Pax2 (green cells, arrow) in PrV on E14.5. (f) A majority of Pax2⁺ cells (red) also express *Gad1* (green, arrows indicate double labeled cells) in PrV on E14.5, while almost all *Gad1*⁺ cells co-express Pax2. (g) *Lmx1b* (red, arrowheads) is not colocalized with BrdU staining (green, arrows). (h) 95.2% of *Lmx1b*⁺ cells coexpress *VGLUT2*, while 78.5% of Pax2⁺ cells coexpress *Gad1*. Mo: V motor nucleus, t: spinal V tract, SuV: supratrigeminal nucleus. Dotted lines outline the PrV. Scale bars: 100 μ m (a–c) or 50 μ m (d–f).

Figure 2.

Expression of *VGLUT2*, *Gad1* and *Pax2* in transverse sections through the PrV of wild-type and *Lmx1b*^{-/-} mice at E14.5. (a, b) normal expression of *VGLUT2* in *Lmx1b*^{-/-} mice. (c, d) *Gad1* expression in *Lmx1b*^{-/-} mice is similar to that of wild-type controls. (e, f) *Pax2* expression is not altered in *Lmx1b*^{-/-} mice. WT: wild-type. Scale bars: 100 μ m (a-f).

Figure 3.

Expression of glutamatergic markers is reduced or absent in the *Lmx1b*^{-/-} PrV at P0. (a,b) *VGLUT2* is down-regulated in the mutant (b), as compared to controls (a). (c,d) *Gria2* staining is absent in the *Lmx1b*^{-/-} PrV (d). (e) Estimates of total cell numbers revealed a significant reduction of the number of *VGLUT2*⁺ cells in the *Lmx1b*^{-/-} PrV (201.60±10.78), as compared to wild-type controls (459.83±22.73) ($p < 0.001$). Scale bar: 100 μ m (a–d).

Figure 4.

Increased numbers of PrV cells expressing GABAergic markers in *Lmx1b*^{-/-}. Expression of *Gad1* increased in *Lmx1b*^{-/-} mice (**b**) relative to wild-type control (**a**). *Gad1*⁺ cells were more numerous in the PrV of *Lmx1b*^{-/-} mice compared with the control (**c**, $p < 0.001$). (**d-f**) VGAT is similarly up-regulated in the *Lmx1b*^{-/-} PrV ($p < 0.001$). (**g-i**) A significant increase in the number of Pax2⁺ cells in the *Lmx1b*^{-/-} PrV, as compared to controls ($P < 0.001$). WT: wild-type, Mo: V motor nucleus, t: V spinal tract, SuV: supratrigeminal nucleus. Scale bar: 100 μ m (**a**, **b**, **d**, **e**, **g**, **h**).

Figure 5.

Pax2 upregulation in the PrV of *Lmx1b*^{-/-} mice is cell autonomous. (a) Pax2 (red, arrow in the frame) is not colocalized with *Lmx1b* mRNA (green, arrowhead in the frame) in the wild-type PrV. (b) Many Pax2/*Neo* double labeled cells (arrow in the frame) in *Lmx1b*^{-/-} mice are present among numerous *Neo*⁺ only cells (green, arrowhead in the frame). (c) Estimates of labeled cell numbers indicate that 11.6% of Pax2⁺ cells in the *Lmx1b*^{-/-} PrV are *Neo*⁺, whereas only 3.4% of Pax2⁺ cells in wild-type mice are *Lmx1b*⁺, indicating that a disproportionate number of *Lmx1b*⁺ cells become Pax2⁺ in the PrV of *Lmx1b*^{-/-} mice. t: V spinal tract. Scale bar: 100 μm.

Figure 6. Relative to wild-type (WT) controls (A, E), reduced numbers of glutamatergic, and increased numbers of GABAergic, PrV cells occur in both the *Lmx1b* null mutant and the *Lmx1b/Bax* double null mutant, as revealed by staining for *VGLUT2* (B, C) and *VGAT* (F, G) respectively. Estimates of total numbers of labeled PrV cells (ordinate) in these two staining conditions are shown in D and H. Bars containing an X indicate statistically significant differences from controls, at the $p < .001$ level. Arrows highlight equivalent locations in PrV in transverse sections from different cases.

Figure 7.

Electroporation of exogenous *Lmx1b* suppresses endogenous Pax2 expression in the dorsal spinal cord. (a,b) Transverse sections through the right E18.5 mouse spinal cord electroporated on E12.5 with *Lmx1b*-EGFP (which expressed *Lmx1b* and EGFP separately via insertion of an IRES cassette) or EGFP only plasmids. (c,d) EGFP (green) and Pax2 (red) double staining in the same sections as in a,b. The dorsal horn electroporated with *Lmx1b*-EGFP rarely displays Pax2/EGFP double labeling, whereas many cells do so in the EGFP plasmid control cases. Examples of single and double labeled cells are shown in the insets in c and d. Scale bar: 50 μ m applies to all panels.

Figure 8.

Ectopic expression of *Lmx1b* increases the intensity of *VGLUT2* expression in transverse sections of the embryonic cerebral cortex. (a,b) *Lmx1b*-EGFP and EGFP only plasmids were electroporated into the forebrain at E12.5 and analyzed here at E15.5. (c,d) Expression of *VGLUT2* in the cortex; EGFP immunocytochemistry followed *in situ* hybridization with a *VGLUT2* probe. (e). Higher magnification epifluorescence of the frame shown in c. Arrows indicate EGFP and *VGLUT2* double-stained cells; arrowhead highlights EGFP only cell. (f) The number of *VGLUT2*⁺ cells in the *Lmx1b* electroporated cortex does not differ from the control side, whereas ectopic *Lmx1b* produces a statistically significant increase in staining intensity in *VGLUT2*⁺ cells (g). EP: electroporation; Con: contralateral side. Scale bar: 100 μ m, applies to panels A–D.

Figure 9.

Ectopic expression of *Lmx1b* suppresses *Gad1* expression in transverse sections through the E19.5 superior colliculus. (**a,b**) **a** is the control side and **b** shows the side where Coronal sections through the midbrain at superior colliculus level; **a** indicates the contralateral side, whereas **b** indicates the side where *Lmx1b*-EGFP plasmids were electroporated into the midbrain at E14.5. (**c**) Total numbers of *Gad1*⁺ cells in the colliculus was significantly reduced after *Lmx1b* electroporation. (**d,e**) *Gad1* expression in adjacent sections as revealed by *in situ* hybridization and brightfield optics. (**f**) EGFP immunostaining was performed following *Gad1 in situ* hybridization. **f** shows with epifluorescence and at higher magnification the field boxed in **e**; EGFP (green, arrows) did not occur in *Gad1* labeled cells (red, arrowheads). Scale bar: 100 μ m.