Lmx1b-Controlled Isthmic Organizer Is Essential for Development of Midbrain Dopaminergic Neurons

Chao Guo,^{1*} Hai-Yan Qiu,^{1,2*} Ming Shi,¹ Ying Huang,¹ Randy L. Johnson,³ Marcelo Rubinstein,⁴ Sheng-Di Chen,² and Yu-Qiang Ding¹

¹Institute of Neuroscience, Key Laboratory of Neurobiology, Chinese Academy of Sciences, Shanghai 200031, China, ²Department of Neurology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China, ³Department of Biochemistry and Molecular Biology, University of Texas, Maryland Anderson Cancer Center, Houston, Texas 77030, and ⁴Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas and Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires 1428, Argentina

The LIM homeodomain transcription factor *Lmx1b* has been suggested to be required for the differentiation of midbrain dopaminergic (mDA) neurons. However, whether the loss of mDA neurons in *Lmx1b*^{-/-} mice is due to its intrinsic role in the mDA lineage or to a consequence of the malformations caused by the earlier mid/hindbrain patterning defects remains to be clarified. We report here that *Lmx1b* expression in mDA neurons is dispensable for their differentiation, maintenance, and the loss of mDA neurons in *Lmx1b*^{-/-} mice is due to the disruption of inductive activity of the isthmic organizer (IsO) in the absence of *Lmx1b* at the mid/hindbrain boundary (MHB). We found that mDA neurons revealed by tyrosine hydroxylase (TH), Pitx3, Nurr1, and dopamine transporter were indistinguishable from wild-type controls during embryonic development as well as adulthood in *TH-Cre;Lmx1b*^{lox/-} and *Dat*^{Cre/+}; *Lmx1b*^{lox/-} mice, in which *Lmx1b* was selectively deleted in differentiating mDA neurons. In addition, mDA neurons were recovered in *Lmx1b*^{-/-} mice, when IsO activity was restored by *Wnt1-Lmx1b* transgene at MHB. The restored IsO activity was evidenced by apparently normal tectum and cerebellum and recurrence of expression of *Wnt8* and *Wnt1* at MHB in *Wnt1*^{Lmx1b}; *Lmx1b*^{-/-}. Furthermore, when *Lmx1b* was deleted in the whole brain after the formation of IsO by Nestin-Cre, mDA neurons were normal, whereas serotonergic neurons displayed defective development phenocopying what observed in *Lmx1b*^{-/-} mice. Thus, our results indicate that the inductive activity of IsO is essential, but *Lmx1b* expression in mDA neurons is dispensable for their differentiation and maintenance.

Key words: *Lmx1b*; development; midbrain dopaminergic neurons; isthmic organizer; substantia nigra; transcription factor

Introduction

Midbrain dopaminergic (mDA) neurons are located in the ventral tegmental area and substantia nigra, and have been studied extensively owing to their involvement in Parkinson's disease, as well as in psychiatric and affective disorders. During development, mDA neurons are generated in the embryonic ventral midbrain, and numerous efforts have been made to characterize the molecular program that controls the specification, differentiation, and survival of mDA neurons in development. This line of research is likely to facilitate the engineering of mDA neurons

from stem cells that, in turn, may be critical for development of stem cell-based therapies for Parkinson's disease.

Molecular mechanisms underlying the development of mDA neurons are still poorly understood. The development of mDA neurons can be divided into three distinct stages: regional specification and early and late differentiation (Ang, 2006). The first postmitotic DA neurons are detected in the ventral midbrain at approximately embryonic day (E) 10.5 in mouse, and the induction of mDA depends on sonic hedgehog emanated from the floor plate (Hynes et al., 1995a,b; Hynes and Rosenthal, 1999) and fibroblast growth factor family member 8 (FGF8) secreted from mid/hindbrain boundary (MHB) (i.e., isthmus) (Ye et al., 1998; Lee et al., 2000). Secreted protein WNT1 in the midbrain is crucial for mDA development by the prevention of expansion of *Nkx2.2*-expression domain in the ventral midbrain (Prakash et al., 2006). In addition to these secreted proteins, several transcription factors are also required for proper development of mDA neurons (Simon et al., 2004; Simeone, 2005; Prakash and Wurst, 2006), such as *Lmx1a* and *Msx1* (Andersson et al., 2006a; Ono et al., 2007), *Foxa1* and *Foxa2* (Ferri et al., 2007), *Nurr1* (Saucedo-Cardenas et al., 1998; Sakurada et al., 1999), *Pitx3* (Smidt et al., 2004; Maxwell et al., 2005; Martinat et al., 2006), *Ngn2* and *Mash1* (Andersson et al., 2006b; Kele et al., 2006), *En*

Received July 14, 2008; revised Oct. 11, 2008; accepted Nov. 6, 2008.

This project was supported by grants from the National Natural Science Foundation of China (30721004, 30525014, 30628016, 30570637), "973" program and Key State Research Program from Ministry of Science and Technology of China (2006CB806601, 2006CB943903, 2006CB500706), K. C. Wong Education Foundation (Hong Kong), China Postdoctoral Science Foundation (20070420678), and Chinese Academy of Sciences (KSCX2-YW-R-100, KSCX2-YW-R-35). We thank Dr. A. P. McMahon for providing the pWexp2 construct in generation of transgenic mice, Dr. Xiaoxi Zhuang for providing *Dat*^{Cre/+} mice, Drs. A. Simeone, S. Aizawa, G. R. Martin, and P. Gruss for providing *in situ* probes, Dr. J. Peter H. Burbach for the antibody, and Dr. Yuichi Ono for providing the protocol for BrdU and *Lmx1b* double staining.

*C.G. and H.-Y.Q. contributed equally to this work.

Correspondence should be addressed to Dr. Yu-Qiang Ding, Institute of Neuroscience, Chinese Academy of Sciences, Shanghai 200031, China. E-mail: dingyq@ion.ac.cn.

DOI:10.1523/JNEUROSCI.3267-08.2008

Copyright © 2008 Society for Neuroscience 0270-6474/08/2814097-10\$15.00/0

Figure 1. *Lmx1b* expression in the ventral midbrain. **A**, Double immunostaining shows *Lmx1b* and BrdU colocalized in the ventricular zone (VZ) at E10.5. **B**, Separate distribution of *Lmx1b* and BrdU in the ventral midbrain at E11.5. BrdU was injected before pregnant mice were killed. **C, D**, Double immunostaining shows coexpression of *Lmx1b* and TH at E12.5 (**C**) and E14.5 (**D**). Note that TH/*Lmx1b*-coexpressing neurons in the mantle layer are located ventral and ventrolateral to those expressing *Lmx1b* only at E12.5. Aq, Aqueduct. Scale bars: **A, B, D**, 50 μ m; **C**, 100 μ m.

genes (Simon et al., 2001, 2004; Alberi et al., 2004), and *Cx2* (Puelles et al., 2004).

Lmx1a and *Lmx1b* are in the same LIM class homeobox gene family, and *Lmx1b* is first identified as the key gene responsible for nail–patella syndrome, a dominant inherited disease in human (Chen et al., 1998; Dreyer et al., 1998). *Lmx1b* is widely expressed in developing CNS (Asbreuk et al., 2000; Dai et al., 2008), and it plays a key role in development of serotonergic neurons in the raphe nuclei (Cheng et al., 2003; Ding et al., 2003) and spinal dorsal horn (Ding et al., 2004). On the other hand, isthmus organizer (IsO) is the local signaling center for patterning and growth of the mid/hindbrain region (Joyner et al., 2000; Simeone, 2000; Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001; Nakamura et al., 2005), and *Lmx1b* expression at MHB is essential for the development of the tectum and cerebellum by controlling the initiation of *Fgf8*, the key protein for the IsO inductive activity, and the maintenance of expression of other IsO-related genes in mice (Guo et al., 2007).

A previous study has reported the loss of mDA neurons in *Lmx1b*^{−/−} embryo, and it was claimed that *Lmx1b* was essential for the differentiation of mDA neurons (Smidt et al., 2000; Burbach and Smidt, 2006). In the present study, we revisited this issue by selective deletion of *Lmx1b* in postmitotic mDA neurons as well as in their progenitors after the formation of IsO, and found that *Lmx1b* expression in mDA neurons is not required for their differentiation or maintenance. Our data also demonstrate that the loss of DA neurons in *Lmx1b*^{−/−} embryos is due to the loss of IsO inductive activity during early embryonic development.

Materials and Methods

Genotyping and maintenance of animals. *Lmx1b* gene deletion mutant (Chen et al., 1998), *Lmx1b*^{fllox/fllox} (Guo et al., 2007), *Nestin-Cre* (Isaka et

al., 1999), *TH-Cre* (Gelman et al., 2003), and *Dat*^{Cre/+} (Zhuang et al., 2005) mice were generated and genotyped as previously described. For inactivation of *Lmx1b* expression in postmitotic mDA neurons, we crossed heterozygous *Lmx1b* mice with *TH-Cre* or *Dat*^{Cre/+} mice, and their *TH-Cre*;*Lmx1b*^{+/-} and *Dat*^{Cre/+};*Lmx1b*^{+/-} offspring were mated with *Lmx1b*^{fllox/fllox} mice. The desired *TH-Cre*;*Lmx1b*^{fllox/-} (hereafter referred to as *Lmx1b*TH CKO) and *Dat*^{Cre/+};*Lmx1b*^{fllox/-} (*Lmx1b*^{Dat} CKO) progeny were obtained. To delete *Lmx1b* in mDA precursors, we crossed *Lmx1b*^{fllox/fllox} mice with *Nestin-Cre* mice, and *Nestin-Cre*;*Lmx1b*^{fllox/fllox} (*Lmx1b*^{Nestin} CKO) mice were obtained. The age of embryos was determined according to the plug date (considered to be E0.5), because the mating time among the mice may differ from the actual developmental stage of the embryos was further ascertained according to the criteria described previously (Kaufman, 1995). In each set of experiments, at least three mutant embryos and equal number of control embryos were used. Animal experiments were reviewed and approved by the Animal Studies Committee at the Institute of Neuroscience, Chinese Academy of Sciences.

Generation of *Wnt1-Lmx1b* transgene. We generated *Wnt1-Lmx1b* transgene according to a reported method (Danielian and McMahon, 1996). We prepared the transgenic construct by blunt ligating a 1119 bp fragment containing the coding region of the mouse *Lmx1b* complementary DNA into the *EcoRV* site of the pExp2 construct. The construct was released from the plasmid by *Sall* digestion and used for pronucleus microinjection in FVB fertilized eggs. Transgenic embryos and mice were genotyped using PCR with primers specific for the full length of *Lmx1b*. To obtain *Wnt1*^{Lmx1b};*Lmx1b*^{−/−} mice, *Wnt1-Lmx1b* transgenic mice were mated with *Lmx1b*^{+/-} mice.

BrdU labeling. Pregnant mice were given a single injection of BrdU (60 μ g/g body weight) intraperitoneally at 10.5 and 11.5 d after coitus, and were allowed to survive for 2 h. Sections were processed for double immunostaining of *Lmx1b* and BrdU (see below) to localize *Lmx1b* expression in the ventral midbrain.

In situ hybridization, immunohistochemistry, and Nissl staining. Embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and either stored in methanol at −20°C (for whole-mount *in situ* hybridization only) or cryoprotected with 20% sucrose in 0.01 M PBS (pH 7.4). Postnatal mice were perfused with normal saline followed by the fixative. Brains were cryosectioned on a cryostat and mounted onto polylysine-coated glass slides. *In situ* hybridization was performed essentially the same as described previously (Guo et al., 2007). The following mouse antisense RNA probes were used: *Fgf8* (Wassarman et al., 1997), *Wnt1* (Danielian and McMahon, 1996), and *Aldh1a1* and *Lmx1b* (Ding et al., 2004). For immunostaining, the following primary antibodies were used: rabbit and mouse anti-TH (1:2000; Sigma), rabbit anti-*Lmx1b* (1:2000) (Dai et al., 2008), rabbit anti-serotonin (1:5000; Sigma), rabbit anti-Nurr1 (1:500; Santa Cruz), rat anti-Dat (1:1000; Chemicon), rabbit anti-Vmat2 (Chemicon), goat anti-Foxa2 (1:500; Santa Cruz), and rabbit anti-Pitx3 (gift from J. Peter H. Burbach, Rudolph Magnus Institute of Neuroscience, University Medical Center Utrecht, Utrecht, Netherlands). Sections were incubated overnight at 4°C with the appropriate primary antibody diluted in 0.1% Triton X-100 and 10% normal donkey serum in PBS. After being washed in PBS, the sections were incubated 2 h at room temperature with the corresponding biotinylated secondary antibody and then with avidin-Cy3 (Jackson ImmunoResearch Laboratories) or Cy2 (Jackson ImmunoResearch Laboratories) for 1 h. For double staining of TH with *Lmx1b* or Nurr1, and *Lmx1b* with BrdU, Cy3-labeled donkey anti-mouse IgG

Figure 2. Normal differentiation of mDA neurons in *Lmx1b*TH CKO embryos at E12.5. **A, B**, *In situ* hybridization shows great inactivation of *Lmx1b* expression in the ventral midbrain of *Lmx1b*TH CKO embryo (**B**) compared with wild-type control (**A**). **C–H**, Immunostaining shows normal expression of TH, Pitx3, and Nurr1 in *Lmx1b*TH CKO embryos compared with wild-type embryos (**C, E, G**). Aq, Aqueduct. Scale bar, 200 μ m.

(Jackson ImmunoResearch Laboratories) and Cy2-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were used. Nissl staining was performed to examine gross brain structure as described before (Guo et al., 2007).

Statistical analysis. For analysis of mDA neuron development, we counted numbers of TH- and Pitx3-positive neurons at E12.5, postnatal day 0 (P0), and adulthood (3 months) in the mice we examined. For embryos at E12.5, every third section (16 μ m) was collected; for P0 and for adult brains, every fifth section (30 μ m) was collected. Numbers of positive cells obtained from one brain were pooled and divided by numbers of sections counted. Comparisons were performed using an unpaired Student's *t* test, and for each set of comparison at least three mutant mice and wild-type littermate controls were included. The statistical significance level was set at *p* < 0.05. Data are expressed as mean $\pm$ SEM.

Results

Lmx1b is expressed in mDA neurons

We first examined *Lmx1b* expression in the ventral midbrain at different embryonic stages and in postnatal ventral midbrain. At E10.5, an initial stage for mDA progenitors, *Lmx1b* was expressed in the ventricular zone of the ventral midbrain where mDA neurons are generated, as shown by colocalization of *Lmx1b* with BrdU injected 2 h before mice were killed (Fig. 1A). At E11.5, a

stage when DA neurons are actively generated, *Lmx1b* expression in the ventral ventricular zone was extinguished, and it was only observed in the mantle layer (Fig. 1B). At E12.5, TH/*Lmx1b*-coexpressing neurons were found in the ventral midbrain, and its number was increased at E14.5 (Fig. 1C,D). The generation of mDA neurons from a neural progenitor cell can be divided into three distinct phases based on the expression of molecular markers and the state of differentiation: regional and neuronal specification, early differentiation, and later differentiation (Ang, 2006; Ferri et al., 2007). As shown in Figure 1C, TH/*Lmx1b*-coexpressing cells were located ventral and ventrolateral to those expressing *Lmx1b* only, showing *Lmx1b* expression in early differentiation stage of mDA neurons. In addition, coexpression of TH and *Lmx1b* was observed at later embryonic stages, and it persisted from early postnatal stage to adulthood (data not shown). Thus, we confirmed previous results (Asbreuk et al., 2002; Andersson et al., 2006; Ono et al., 2007; Dai et al., 2008) that *Lmx1b* is expressed in mDA neurons throughout all developmental stages as well as in matured mDA neurons.

Expression of *Lmx1b* in later-differentiating mDA neurons is not required for their differentiation

To study whether *Lmx1b* is required for the differentiation of mDA neuron, we first selectively deleted *Lmx1b* in later-differentiating mDA neurons by crossing *TH-Cre*;*Lmx1b*^{+/−} mice with *Lmx1b*^{lox/lox} mice. *Lmx1b*TH CKO mice survived postnatally with no obvious defect in the brain. Indeed, *Lmx1b* expression in *Lmx1b*TH

CKO embryos was largely inactivated in the ventral midbrain at E12.5 and completely at E13.5 (Fig. 2B, data not shown). On the other hand, its expression at MHB and other brain regions such as the raphe nuclei and dorsal spinal cord was remained unchanged (data not shown), indicating a specific deletion of *Lmx1b* in mDA neurons. Surprisingly, mDA neurons revealed by expressions of TH, Pitx3, and Nurr1 were observed in *Lmx1b*TH CKO embryos at E12.5 with no obvious difference from wild-type controls in terms of cell number (TH-positive cells/section: wild type 236 $\pm$ 49, CKO 246 $\pm$ 54; Pitx3-positive cells/section: wild type 251 $\pm$ 8, CKO 241 $\pm$ 28; *n* = 3 for each; *p* > 0.05, wild type vs CKO) and location (Fig. 2C–H). To study whether this deletion causes defective mDA neuron development at later embryonic stage, we examined mDA neurons in *Lmx1b*TH CKO at birth. mDA neurons were present in the ventral tegmental area and substantia nigra in *Lmx1b*TH CKO mice at P0, and their distribution pattern and cell number were essentially similar to those in wild-type controls (Fig. 3A–F,K). These results were confirmed by observation on *Lmx1b*^{Dat} CKO mice in which *Lmx1b* was specifically deleted in later-differentiating mDA neurons as well (Figs. 3H, 4B), showing no difference between *Lmx1b*^{Dat} CKO and wild-type controls (Fig. 3I–K). In addition,

Figure 3. Normal differentiation of mDA neurons in *Lmx1b*TH CKO and *Lmx1b*^{Dat} CKO mice. **A–F**, Expression of TH, Pitx3, and Nurr1 in the ventral tegmental area (VTA) and substantia nigra (SN) of *Lmx1b*TH CKO mice (**B, D, F**) is similar to that of wild-type mice (**A, C, E**). **G, H**, In situ hybridization shows *Lmx1b* expression in the ventral midbrain of wild-type (**G**) but not in *Lmx1b*^{Dat} CKO mice (**H**). **I, J**, Comparable expression of TH in the ventral tegmental area and substantia nigra of *Lmx1b*TH CKO and wild-type mice. **K**, Numbers of TH-positive neurons in *Lmx1b*TH CKO and *Lmx1b*^{Dat} CKO mice are similar to that of wild-type mice at P0 ($n = 4$ for each; $p > 0.05$). Filled nuclei. Scale bar, 200 μ m.

because *Lmx1b* expression is persistent in postnatal mDA neurons, mDA neurons in adult brain were also examined with markers (TH, Pitx3, Dat, and Vmat2) in *Lmx1b*TH CKO and *Lmx1b*^{Dat} CKO mice. Again, expression of these proteins in the ventral tegmental area and substantia nigra of these two CKO mice was similar to that of wild-type control (Fig. 3C, E, G, I and data not shown). Furthermore, expression of TH in the striatum, the major targeting area of mDA neurons in these CKO mice showed no detectable difference compared with wild-type control (Fig. 4D, F, H), suggesting normal axonal innervation of mDA neurons in the absence of *Lmx1b* expression. Together, our results demonstrate that *Lmx1b* is not required for later differentiation or survival of mDA neurons.

Wnt1-Lmx1b transgene restores IsO induction activity of *Lmx1b*^{-/-} mice

The above evidence clearly shows that *Lmx1b* expression in later-differentiating mDA neurons is not necessary for their development. We reasoned that the loss of mDA neurons in *Lmx1b*^{-/-} embryos is due, at least partly, to the loss of the IsO inductive activity (Guo et al., 2007). To testify this hypothesis, we generated *Wnt1-Lmx1b* transgene by which *Lmx1b* expression can be reactivated in *Lmx1b*^{-/-} embryos. *Wnt1* expression domain overlapped with *Lmx1b* at MHB from early somite stages to E11.5 (Fig. 5A, B) (Guo et al., 2007). *Wnt1-Lmx1b* transgenic mice were viable, fertile, and apparently normal, with no detectable defect in the CNS. *Lmx1b* expression at MHB was stronger in *Wnt1-Lmx1b* embryos compared with wild-type controls (Fig. 5A, C, arrowheads), and it was ectopically expressed in the neural crest where endogenous *Wnt1* is expressed (Fig. 5A–C, arrows). As expected,

Lmx1b expression was observed at MHB in *Wnt1*^{*Lmx1b*}; *Lmx1b*^{-/-} embryos, and more importantly expression of *Fgf8* and *Wnt1* was rescued at MHB (Fig. 5D–I). Furthermore, in *Wnt1*^{*Lmx1b*}; *Lmx1b*^{-/-} mice, the dorsal midbrain (i.e., tectum) and cerebellum showed apparently normal appearance compared with wild type, whereas their development are severely defective in *Lmx1b*^{-/-} mice (Fig. 5J–L). Given this evidence, we concluded that *Lmx1b* expression at MHB is not only required but also sufficient for the formation of IsO inductive activity in early embryogenesis.

mDA neurons develop normally in *Wnt1*^{*Lmx1b*}; *Lmx1b*^{-/-} mice

mDA neurons are lost in *Lmx1b*^{-/-} mice (Smidt et al., 2000; Guo et al., 2007). However, our present data obtained from selective inactivation of *Lmx1b* in later-differentiating mDA neurons indicate that *Lmx1b* is not required for their later differentiation or survival. As mentioned above, on the basis of our present and previous results, we hypothesized that the loss of mDA neurons in *Lmx1b*^{-/-} mice is due to the loss of the IsO inductive activity in the absence of *Lmx1b* at MHB. Therefore, mDA neurons were examined in *Wnt1*^{*Lmx1b*}; *Lmx1b*^{-/-} mice with normal IsO inductive activity restored by *Wnt1-Lmx1b* transgene (Fig. 5). We found that mDA neurons indicated by TH, Pitx3, and Nurr1 expression were observed in *Wnt1*^{*Lmx1b*}; *Lmx1b*^{-/-} mice, and its number and distribution pattern were similar to wild-type controls at E12.5 (Fig. 6D–F, P, Q), E14.5 (Fig. 6G–O), and P0 (Fig. 7). Based on both loss- and gain-of-function data, we concluded that IsO-inductive activity is essential for the development of mDA neurons in mice.

Figure 4. Normal expression of *Lmx1b* in adult *Lmx1bTH* CKO and *Lmx1b^{Dat}* CKO mice. **A, B.** *In situ* hybridization shows *Lmx1b* expression in the ventral tegmental area (VTA) and substantia nigra (SN) of adult wild-type (**A**) but not in *Lmx1b^{Dat}* CKO mice (**B**). **C, E, G.** Expression of TH-positive neurons in the ventral tegmental area and substantia nigra of *Lmx1bTH* CKO (**E**) and *Lmx1b^{Dat}* CKO mice (**G**) was similar to that of wild-type mice (**C**). **D, F, H.** Expression of TH-positive fibers in the striatum of *Lmx1bTH* CKO (**F**) and *Lmx1b^{Dat}* CKO mice (**H**) was also similar to that of wild-type mice (**D**). **I.** Numbers of TH-positive neurons in *Lmx1bTH* CKO and *Lmx1b^{Dat}* CKO mice show no significant difference (wild type, $n = 3$; *Lmx1bTH* CKO, $n = 4$; *Lmx1b^{Dat}* CKO, $n = 4$; $p > 0.05$, wild type vs *Lmx1bTH* CKO; $p > 0.05$, wild type vs *Lmx1b^{Dat}* CKO). ac, Anterior commissure; IP, interpeduncular nucleus; LV, lateral ventricle; R, red nucleus. Scale bars: **A, B**, 200 μ m; **C, E, G**, 500 μ m; **D, F, H**, 600 μ m.

Lmx1b in mDA progenitors is also dispensable for mDA neuron differentiation

Lmx1b is not only expressed in differentiating mDA neurons, but also in their precursors (Fig. 1). With the advantage TH-Cre- and Dat-Cre-mediated specific inactivation of *Lmx1b*, we demonstrate that *Lmx1b* expression in later-differentiating mDA neurons is not required for their differentiation or maintenance. We next asked whether *Lmx1b* expression in mDA precursors and early differentiating mDA neurons plays a role in their development, and therefore contribute to the loss of mDA neurons in *Lmx1b^{-/-}* mice. In addition, in *Wnt1^{Lmx1b};Lmx1b^{-/-}* embryos, weak *Lmx1b* expression was transiently observed in the ventricular zone of the ventral midbrain at E12.5 (Fig. 6C), raising the possibility that mDA neurons were rescued in *Lmx1b^{-/-}* em-

bryos by the transient expression of *Lmx1b* in mDA progenitors rather than by the recovered IsO activity. To address these possibilities, we generated *Lmx1b^{Nestin}* CKO mice. The *cre* gene is driven by the *Nestin* promoter and enhancer, and Cre is expressed in the precursor cells of developing CNS (Isaka et al., 1999). The onset of Nestin-Cre activity occurs around E10.5, as indicated by X-gal staining in *Nestin-cre;Rosa26* mice (data not shown), which is consistent with previous results (Vernay et al., 2005). In *Lmx1b^{Nestin}* CKO embryos, *Lmx1b* transcripts were not detectable in the ventral midbrain at E10.5 (Fig. 8B), a stage of *Lmx1b* initiation in mDA precursors, but its expression in the roof plate and rhombencephalon were not affected (data not shown). At E11.5, *Lmx1b* expression was not found in the whole midbrain or other regions of the CNS in *Lmx1b^{Nestin}* CKO mice (Fig. 8D, data not shown). *Lmx1b* is essential for the serotonergic neuron development in the raphe nuclei of the brainstem (Cheng et al., 2003; Ding et al., 2003) and spinal dorsal horn (Ding et al., 2004). Thus, to confirm the effects of Nestin-Cre-mediated *Lmx1b* deletion in the brain, we examined serotonin-expressing neuron in *Lmx1b^{Nestin}* CKO embryos at E12.5 (Fig. 8T), which is consistent with the previous results (Cheng et al., 2003; Ding et al., 2003); the spinal dorsal horn also showed similar phenotype as what found in *Lmx1b^{-/-}* embryos (Ding et al., 2004). Thus, *Lmx1b^{Nestin}* CKO embryos allow us to study the functions of *Lmx1b* in mDA precursors during neurogenesis.

As the Nestin-Cre activity turns on in the neural tube at E10.5, a stage after the formation of the IsO, and therefore Nestin-Cre-mediated gene inactivation is a novel mouse model to study a role of genes in the mid/hindbrain development, independently of its involvement in the IsO inductive activity (Vernay et al., 2005). Accordingly, in *Lmx1b^{Nestin}* CKO embryos, we found that IsO activity was indeed

remained intact as evidenced by the normal expression of *Fgf8* and *Wnt1* at MHB (Fig. 8I–L) and normal appearance of the tectum and cerebellum (Fig. 8U,V). Therefore, *Lmx1b* expression at MHB is essential for the formation of IsO inductive activity by E10.5 and therefore important for the development of the tectum and cerebellum.

We found that mDA neurons were present in the ventral midbrain of *Lmx1b^{Nestin}* CKO embryos at E12.5 (Fig. 8M–R), and ventral tegmental area and substantia nigra at P0, with no difference from wild-type controls (TH-positive cells/section: wild type, 1406 ± 156 ; CKO, 1666 ± 256 ; wild type, $n = 3$; CKO, $n = 4$; $p > 0.05$, WT vs CKO) (Fig. 9). *Lmx1a* expression in the ventral midbrain is critical for the specification of mDA neurons (Andersson et al., 2006a; Ono et al., 2007). To study whether

normal mDA neurons in *Lmx1b*^{Nestin} CKO mice is due to the compensation from *Lmx1a*, we examined the expression of *Lmx1a*. It showed that *Lmx1a* expression in *Lmx1b*^{Nestin} CKO embryos is similar to that in wild-type embryos with no obvious upregulation at E10.5 and E12.5 (Fig. 8E–H). In addition, expression of *Foxa2*, *Nurr1*, and *Aldh1a1* (*Raldh1*), which are also molecular markers for mDA progenitors in the ventral midbrain (Wallén et al., 1999; Andersson et al., 2006b; Ferri et al., 2007), remained unchanged in the ventral midbrain of *Lmx1b*^{Nestin} CKO embryos at E10.5 (see supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Thus, our data indicated that *Lmx1b* expression in the mDA precursors is dispensable for their differentiation and maintenance.

Discussion

Consistent with a previous report (Smidt et al., 2000), our previous and present data also showed the loss of mDA neurons in *Lmx1b*^{-/-} mice. However, the present study further demonstrates that the loss of mDA neurons in *Lmx1b*^{-/-} mice is secondary to the disruption of the IsO inductive activity in the absence of *Lmx1b* at MHB. We also provided evidence for the conclusion that *Lmx1b* expression in mDA neurons is dispensable for their differentiation and maintenance. The present data also argue for a notion that the IsO is not only essential for the morphogenesis of the dorsal midbrain and cerebellum but also for the development of mDA neuron in the ventral midbrain.

Lmx1b is not required for the differentiation or maintenance of mDA neurons

To study the role of *Lmx1b* in mDA neuron development, we selectively inactivated *Lmx1b* expression in later-differentiating mDA neurons by Dat-Cre and TH-Cre as well as in mDA precursors by Nestin-Cre. To our surprise, mDA neurons were present in the ventral midbrain at embryonic stages and in the ventral tegmental area and substantia nigra in P0 and adult brains (Figs. 2–4, 8, 9). Thus, the present findings indicate that *Lmx1b* expression in mDA neurons is not required for their differentiation or maintenance.

Lmx1b is also expressed in DA progenitors at E9.5, and it remains unclear whether *Lmx1b* is involved in the initial specification of mDA neuron progenitors before E10.5. mDA neurons are first present in the ventral midbrain in mouse at E10.5 (Bayer et al., 1995), and TH-positive mDA neurons in *Lmx1b*^{-/-} embryo show no difference from wild-type embryo in terms of cell numbers and distribution at this stage (see supplemental Fig. 2, available at www.jneurosci.org as supplemental material);

Figure 5. *Wnt1-Lmx1b* transgene restores IsO inductive activity in *Lmx1b*^{-/-} mice. **A–C**, *Wnt1*-driven *Lmx1b* expression at the MHB. Whole-mount *in situ* hybridization shows endogenous expression of *Lmx1b* (arrowhead in **A**) and *Wnt1* (arrowhead in **B**) at MHB of wild-type embryos at E9.5. In *Wnt1-Lmx1b* transgenic embryo, *in situ* signals for *Lmx1b* at MHB (arrowhead in **C**) are stronger than wild-type embryo (arrowhead in **A**), and *Lmx1b* is ectopically present in the neural crest (arrow in **C**) where *Wnt1* is expressed (arrow in **B**). **D–I**, Analysis of *Fgf8* and *Wnt1* expression at MHB of *Wnt1*^{Lmx1b};*Lmx1b*^{-/-} embryos at E9.5. *Fgf8* (arrowhead in **E**) and *Wnt1* expression (arrowhead in **H**) at MHB of *Lmx1b*^{-/-} embryo is not observed, whereas it is present in *Wnt1*^{Lmx1b};*Lmx1b*^{-/-} embryo (arrowheads in **F**, **I**). **J–L**, Parasagittal Nissl-stained sections of P0 brains. In *Wnt1*^{Lmx1b};*Lmx1b*^{-/-} mice (**L**), the superior colliculus (SC), inferior colliculus (IC), and cerebellum (Cb) display normal appearance compared with wild-type mice (**J**), whereas they are severely defective in *Lmx1b*^{-/-} mice (**K**). Scale bar: **J–L**, 500 μ m.

their reduction in *Lmx1b*^{-/-} embryo occurs by E11.5. mDA neurons develop normally in *Lmx1b*^{Nestin} CKO embryo, in which *Lmx1b* expression in the ventral midbrain is inactivated at E10.5 (Figs. 8, 9). Thus, further studies are needed to study whether *Lmx1b* plays an essential role in the early specification of mDA progenitors.

Lmx1a, a paralogue of *Lmx1b*, is a key instructor in the specification of mDA neurons, based on the data from loss- and gain-of-function experiments in chick embryos and from *in vitro* differentiation of mouse ES cells (Andersson et al., 2006a), and a

Figure 6. mDA neurons develop normally in *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} mice. **A–C**, *In situ* hybridization for *Lmx1b* in coronal sections of the ventral midbrain at E12.5. No *in situ* signal for *Lmx1b* is observed in *Lmx1b*^{-/-} embryo (**B**), and very weak signals are detected in the ventricular zone but not mantle layer of the ventral midbrain in *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} embryo (**C**) compared with wild-type control (**A**). **D–F**, Coronal sections of embryo at E12.5, showing that TH and Pitx3 expression in *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} embryo (**F**) is similar to that of wild-type control (**D**), whereas it is largely reduced in *Lmx1b*^{-/-} embryo (**E**). **G–O**, Immunostaining for *Lmx1b* (**G–I**), Pitx3 (**J–L**), and TH (**M–O**) in coronal sections of the ventral midbrain at E14.5. Note that expression of Pitx3 and TH in *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} embryos (**L**, **O**) is similar to that in wild-type control (**J**, **M**), whereas they are almost completely absent in *Lmx1b*^{-/-} embryo (**K**, **N**). **P**, **Q**, Cell counts of TH- and Pitx3-positive neurons in the ventral midbrain at E12.5 indicate no significant difference between wild-type control and *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} embryos, although they are nearly completely lost in *Lmx1b*^{-/-} embryos ($n = 4$ for each; $*p < 0.01$, wild type vs *Lmx1b*^{-/-}; $*p < 0.01$, *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} vs *Lmx1b*^{-/-}; $p > 0.05$, wild type vs *Wnt1*^{Lmx1b}*Lmx1b*^{-/-}). Scale bars: **A–F**, 100 μ m; **G–O**, 200 μ m.

recent study (Ono et al., 2007) shows that mDA neurons is reduced in *drher* mutant mice, which carry a mutation in the *Lmx1a* locus (Millonig et al., 2000), as a consequence of the reduced neurogenesis in mDA progenitors and misspecification of mDA neurons in some of mDA neurons. However, the phenotype is not as severe as that found by siRNA for *Lmx1a* in chick embryos (Andersson et al., 2006a), and most mDA neurons differentiated

normally in *drher* mice, suggesting residual *Lmx1a* activity in *drher* mutants or the existence of compensating factor(s) in the mice (Ono et al., 2007). Because *Lmx1a* and *Lmx1b* are homolog genes and coexpressed in mDA neurons, it is possible that *Lmx1a* and *Lmx1b* control the mDA neuron development in a combinational manner. This may account for the normal development of most mDA neurons in *drher* mice and mDA neurons in the three CKO mice and *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} mice. Nevertheless, *Lmx1b* alone is not crucial for mDA neuron differentiation or maintenance in mice.

IsO activity is essential for mDA neuron development

The present data indicate that the loss of mDA neurons in *Lmx1b*^{-/-} embryo is due to the loss of IsO inductive activity in the absence of *Lmx1b* expression at MHB. IsO is the local signaling center for the morphogenesis of the mid/hindbrain region (Joyner et al., 2000; Simeone, 2000; Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001; Nakamura et al., 2005). The position of the IsO controlled by *Gbx2* and *Otx2* determines the location and size of mDA neurons and hindbrain serotonergic cell population during development (Brodski et al., 2003). Our previous study shows that *Lmx1b* expression is required for the IsO inductive activity by control of initiation of *Fgf8* and maintenance of expression of *Wnt1* and other IsO-related transcription factors (Guo et al., 2007), and the present study further indicates that *Lmx1b* at MHB is sufficient for the formation of IsO activity, because transgenic expression of *Lmx1b* at MHB in *Lmx1b*^{-/-} embryos restored its inductive activity, as indicated by the recovered expression of *Fgf8* and *Wnt1* and normal appearance of the tectum and cerebellum (Fig. 5). Importantly, mDA neurons were recovered in *Wnt1*^{Lmx1b}*Lmx1b*^{-/-} mice (Figs. 6, 7). Furthermore, when *Lmx1b* was inactivated in the whole brain by E10.5 by Nestin-Cre, IsO remained intact in the CKO mice and mDA neurons differentiated normally, providing additional evidence for the requirement of IsO in mDA neuron development (Figs. 8, 9). Therefore, IsO is not only essential for the morphogenesis of the dorsal midbrain but also for the development of

mDA neurons in the ventral midbrain. In fact, FGF8 and WNT1, the major secreted proteins for the IsO activity, are known to be involved in the induction and differentiation of mDA neurons (Hynes and Rosenthal, 1999; Simeone, 2005; Burbach and Smidt, 2006; Prakash and Wurst, 2006). Therefore, it is possible that the loss of mDA neurons in *Lmx1b*^{-/-} mice is due to, at least partly, the loss of *Fgf8* and *Wnt1* expression in the absence of *Lmx1b*.

In summary, we provided evidence *in vivo* that *Lmx1b* expression in mDA neurons is not required for their difference or maintenance in mice. The loss of mDA neurons in *Lmx1b* mutant mice is due to the disruption of the IsO activity in the absence of *Lmx1b* expression at MHB. Our data also suggest that a special caution should be paid in studying the role of a gene in mDA neuron development when it probably participates or has been known to be involved in the regulation of IsO inductive activity.

References

- Albéri L, Sgadò P, Simon HH (2004) Engrailed genes are cell-autonomously required to prevent apoptosis in mesencephalic dopaminergic neurons. *Development* 131:3229–3236.
- Andersson E, Tryggvason U, Deng Q, Friling S, Alekseenko Z, Robert B, Perlmann T, Ericson J (2006a) Identification of intrinsic determinants of midbrain dopamine Neurons. *Cell* 124:393–405.
- Andersson E, Jensen JB, Parmar M, Guillemot F, Björklund A (2006b) Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development* 133:507–516.
- Ang SL (2006) Transcriptional control of midbrain dopaminergic neuron development. *Development* 133:3499–3506.
- Asbreuk CHJ, Vogelaar CF, Hellemans A, Smidt MP, Burbach JP (2002) CNS Expression pattern of *Lmx1b* and coexpression with *Ptx* genes suggest functional cooperativity in the development of forebrain motor control systems. *Mol Cell Neurosci* 21:410–420.
- Bayer SA, Wills KV, Triarhou LC, Ghetti B (1995) Time course of origin and gradients of neurogenesis in midbrain dopaminergic neurons in the mouse. *Exp Brain Res* 105:191–199.
- Brodski C, Weisenborn DMV, Signore M, Sillanpää I, Gasterlund M, Broccoli V, Acampora D, Simeone A, Yancopoulos G (2006) Location and size of dopaminergic and serotonergic cell populations are controlled by the position of the midbrain–hindbrain organizer. *J Neurosci* 23:4199–4207.
- Burbach JP, Smidt MP (2006) Molecular patterning of stem cells into mesodiencephalic dopaminergic neurons. *Trends Neurosci* 29:601–603.
- Chen H, Lun Y, Ovchinnikov D, Kozlov H, Oberg KC, Pepicelli CV, Gan L, Lee B, Johnson RL (1998) Limb and kidney defects in *Lmx1b* mutant mice suggest an involvement of *LMX1B* in human nail patella syndrome. *Nat Genet* 19:51–55.
- Cheng L, Chen CL, Luo P, Tan M, Qiu M, Johnson R, Ma Q (2003) *Lmx1b*, *Pet-1*, and *Nkx2.2* coordinately specify serotonergic neurotransmitter phenotype. *J Neurosci* 23:9961–9967.
- Dai JX, Hu ZL, Shi M, Guo C, Ding YQ (2008) Postnatal ontogeny of the transcription factor *Lmx1b* in the mouse central nervous system. *J Comp Neurol* 509:341–355.
- Danielian PS, McMahon AP (1996) Engrailed-1 as a target of the Wnt-1 signaling pathway in vertebrate midbrain development. *Nature* 383:332–334.
- Ding YQ, Marklund U, Yuan W, Yin J, Wegman L, Ericson J, Deneris E, Johnson RL, Chen ZF (2003) *Lmx1b* is essential for the development of serotonergic neurons. *Nat Neurosci* 6:933–938.
- Ding YQ, Yin J, Kania A, Zhao ZQ, Johnson RL, Chen ZF (2004) *Lmx1b* controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. *Development* 131:3693–3703.
- Dreyer SD, Zhou G, Baldini A, Winterpacht A, Zabel B, Cole W, Johnson RL, Lee B (1998) Mutations in *LMX1B* cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. *Nat Genet* 19:47–50.
- Ferri ALM, Lin W, Mavromatakis YE, Wang JC, Sasaki H, Whitsett JA, Ang SL (2007) *Foxa1* and *Foxa2* regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. *Development* 134:2761–2769.
- Gelman DM, Noain D, Avale ME, Otero V, Low MJ, Rubinstein M (2003) Transgenic mice engineered to target Cre/loxP-mediated DNA recombination to catecholaminergic neurons. *Genesis* 36:196–202.
- Guo C, Qiu HY, Huang Y, Chen H, Yang RQ, Chen SD, Johnson RL, Chen ZF, Ding YQ (2007) *Lmx1b* is essential for *Fgf8* and *Wnt1* expression in the isthmic organizer during tectum and cerebellum development in mice. *Development* 134:317–325.
- Hynes M, Rosenthal A (1999) Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. *Curr Opin Neurobiol* 9:26–36.
- Hynes M, Poulsen K, Tessier-Lavigne M, Rosenthal A (1995a) Control of neuronal diversity by the floor plate: Contact-mediated induction of midbrain dopaminergic neurons. *Cell* 80:95–101.
- Hynes M, Porter JA, Chiang C, Chang D, Tessier-Lavigne M, Beachy PA, Rosenthal A (1995b) Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* 15:35–44.
- Isaka F, Ishibashi M, Taki W, Hashimoto N, Nakanishi S, Kageyama R (1999) Ectopic expression of the bHLH gene *Math1* disturbs neural development. *Eur J Neurosci* 11:2582–2588.
- Joyner AL, Liu A, Millet S (2000) *Otx2*, *Gbx2* and *Fgf8* interact to position and maintain a mid-hindbrain organizer. *Curr Opin Cell Biol* 12:736–741.
- Kaufman MH (1998) The atlas of mouse development. San Diego: Academic.
- Kele J, Simplicio N, Ferri ALM, Mira H, Guillemot F, Arenas E, Ang SL (2006) Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* 133:495–505.
- Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 18:675–679.
- Liu A, Joyner AL (2001) Early anterior/posterior patterning of the midbrain and cerebellum. *Annu Rev Neurosci* 24:869–896.
- Martinat C, Bacci JJ, Leete T, Kim J, Vanti WB, Newman AH, Cha JH, Gether U, Wang H, Abeliovich A (2006) Cooperative transcription activation by *Nurr1* and *Pitx3* induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. *Proc Natl Acad Sci U S A* 103:2874–2879.

Figure 7. Normal expression of TH and Pitx3 in *Wnt1^{Lmx1b}Lmx1b^{-/-}* at P0. **A–F**, Expression of TH (**A–C**) and Pitx3 (**D–F**) in *Wnt1^{Lmx1b}Lmx1b^{-/-}* (**C, F**) is similar to that of wild-type control (**A, D**), whereas they are lost in *Lmx1b^{-/-}* embryos (**B, E**). **G, H**, Cell counts of TH-positive neurons show no significant difference between wild-type control and *Wnt1^{Lmx1b}Lmx1b^{-/-}* embryos (wild type, $n = 4$; *Wnt1^{Lmx1b}Lmx1b^{-/-}*, $n = 3$; $p > 0.05$, wild type vs *Wnt1^{Lmx1b}Lmx1b^{-/-}*), although they are dramatically reduced in *Lmx1b^{-/-}* mice ($n = 4$) ($*p < 0.01$, wild type vs *Lmx1b^{-/-}*; $*p < 0.01$, *Wnt1^{Lmx1b}Lmx1b^{-/-}* vs *Lmx1b^{-/-}*). R, Red nucleus; SN, substantia nigra; VTA, ventral tegmental area. Scale bar: **A–F**, 200 μ m.

Figure 8. Inductive activity of the isthmic organizer remains intact and normal differentiation of mDA neuron in *Lmx1b^{Nestin}* CKO mice. **A–D**, Sagittal sections of embryos show the absence of *Lmx1b* expression in the ventral midbrain in *Lmx1b^{Nestin}* CKO embryos at E10.5 (arrowhead in **B**) and E12.5 (arrowhead in **D**) compared with wild-type controls (**A**, **C**). **E–H**, Expression of *Lmx1a* at E10.5 and E12.5 in *Lmx1b^{Nestin}* CKO embryos (**F**, **H**) is similar to that in wild-type control (**E**, **G**). **I–L**, Expression of *Fgf8* (arrowheads in **J**) and *Wnt1* (arrowhead in **L**) is normal in *Lmx1b^{Nestin}* CKO embryo compared with wild-type control (**I**, **K**). **M–R**, Expression of TH (**M**, **N**), Pitx3 (**O**, **P**), and Nurr1 (**Q**, **R**) in *Lmx1b^{Nestin}* CKO (**N**, **P**, **R**) is similar to that in wild-type control embryos (**M**, **O**, **Q**). **S**, **T**, No serotonin-expressing neuron is observed in *Lmx1b^{Nestin}* CKO embryo (arrow in **T**), whereas many of them are observed in control embryo (**S**) at E12.5. **U**, **V**, Nissl-stained sagittal sections at P0 show normal appearance of the superior colliculus (SC), inferior colliculus (IC), and cerebellum (Cb) in *Lmx1b^{Nestin}* CKO mice (**V**) compared with wild-type brain (**U**). Scale bars: **A–D**, 200 μ m; **E**, **F**, 60 μ m; **M–T**, 100 μ m; **U**, **V**, 500 μ m.

Maxwell SL, Ho HY, Kuehner E, Zhao S, Li M (2005) Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development. *Dev Biol* 282:467–479.

Millonig JH, Millen KJ, Hatten ME (2000) The mouse *Dreher* gene *Lmx1a* controls formation of the roof plate in the vertebrate CNS. *Nature* 403:764–769.

Nakamura H, Katahira T, Matsunaga E, Sato T (2005) Isthmus organizer for midbrain and hindbrain development. *Brain Res Rev* 49:120–126.

Ono Y, Nakatani T, Sakamoto Y, Mizuhara E, Minaki Y, Kumai M, Hamaguchi A, Nishimura M, Inoue Y, Hayashi H, Takahashi J, Imai T (2007) Differences in neurogenic potential in floor plate cells along an antero-posterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* 134:3213–3225.

Prakash N, Wurst W (2006) Genetic networks controlling the development of midbrain dopaminergic neurons. *J Physiol* 575:403–410.

Prakash N, Brodski C, Naserke T, Puelles E, Gogoi R, Hall A, Panhuysen M, Echevarria D, Sussel L, Weisenborn DMV, Martinez S, Arenas E, Simeone A, Wurst W (2006) A *Wnt1*-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development* 133:89–98.

Puelles E, Annino A, Tuorto F, Usiello A, Acampora D, Czerny T, Brodski C, Ang SL, Wurst W, Simeone A (2004) *Otx2* regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* 131:2037–2048.

Sakurada K, Ohshima-Sakurada M, Palmer TD, Gage FH (1999) Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* 126:4017–4026.

Figure 9. Normal differentiation of mDA neurons in *Lmx1b*^{Nestin} CKO mice at P0. **A, B**, *In situ* hybridization shows *Lmx1b* expression in the ventral tegmental area (VTA) and substantia nigra (SN) of wild-type (**A**) but not in *Lmx1b*^{Nestin} CKO mice (**B**). **C–J**, mDA neurons are normal in the ventral tegmental area and substantia nigra of *Lmx1b*^{Nestin} CKO mice as shown by expression of TH (**D**), Pitx3 (**E**), Nurr1 (**H**), and Vmat2 (**J**). R, Red nucleus. Scale bar, 200 μ m.

- Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP, Conneely OM (1998) Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc Natl Acad Sci U S A* 95:4013–4018.
- Simeone A (2000) Positioning the isthmic organizer: where *Wnt2* and *Cux2* meet. *Trends Genet* 16:237–240.
- Simeone A (2005) Genetic control of dopaminergic neuron differentiation. *Trends Neurosci* 28:62–65; discussion 65–66.
- Simon HH, Saueressig H, Wurst W, Gillingham JC, O'Leary DDM (2001) Fate of midbrain dopaminergic neurons controlled by the engrailed genes. *J Neurosci* 21:3127–3134.
- Simon HH, Thuret S, Alberch J (2000) Midbrain dopaminergic neurons: control of their cell fate by the engrailed transcription factors. *Cell Tissue Res* 318:53–61.
- Smidt MP, Asbreuk CHJ, Cox JJ, Chen H, Johnson RL, Burbach JP (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires *Lmx1b*. *Nat Neurosci* 3:337–341.
- Smidt MP, Smits SM, Bouwmeester H, Hamers FPT, van der Linden AJA, Hellemons AJCG, Graw J, Burbach JP (2004) Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene *Pitx3*. *Development* 131:1145–1155.
- Urbay B, Koch M, Vaccarino F, Briscoe J, Simeone A, Kageyama R, Ang SL (2005) *Otx2* regulates subtype specification and neurogenesis in the midbrain. *J Neurosci* 25:4856–4867.
- Wallén A, Zetterström RH, Solomin L, Arvidsson M, Olson L, Perlmann T (1999) Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. *Exp Cell Res* 253:737–746.
- Wassarman KM, Lewandoski M, Campbell K, Joyner AL, Rubenstein JL, Martinez S, Martin GR (1997) Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function. *Development* 124:2923–2934.
- Wurst W, Bally-Cuif L (2001) Neural plate patterning: upstream and downstream of isthmic organizer. *Nat Rev Neurosci* 2:99–108.
- Ye W, Shimamura K, Rubenstein JLR, Hynes MA, Rosenthal A (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* 93:755–766.
- Zhuang X, Masson J, Gingrich JA, Rayport S, Hen R (2005) Targeted gene expression in dopamine and serotonin neurons of the mouse brain. *J Neurosci Methods* 143:27–32.