Adult Raphe-Specific Deletion of Lmx1b Leads to Central Serotonin Deficiency

Ning-Ning Song1*, Jian-Bo Xiu1, Ying Huang1, Jia-Yin Chen1, Lei Zhang1, Lise Gutzknecht2, Klaus Peter Lesch2, He Li3, Yu-Qiang Ding1*

1 Department of Anatomy and Neurobiology, Tongji University School of Medicine, Shanghai, China, 2 Molecular and Clinical Psychobiology, Department of Psychiatry and Psychotherapy, University of Würzburg, Würzburg, Germany, 3 Key Laboratory of Nervous System Diseases, Ministry of Education of China, Division of Histology and Embryology, Department of Anatomy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Abstract

The transcription factor Lmx1b is essential for the differentiation and survival of central serotonergic (5-HTergic) neurons during embryonic development. However, the role of Lmx1b in adult 5-HTergic neurons is unknown. We used an inducible Cre-LoxP system to selectively inactivate Lmx1b expression in the raphe nuclei of adult mice. Pet1-CreERT2 mice were generated and crossed with Lmx1blox/lox mice to obtain Pet1-CreERT2; Lmx1blox/lox mice (which termed as Lmx1b iCKO). After administration of tamoxifen, the level of 5-HT in the brain of Lmx1b iCKO mice was reduced to 60% of that in control mice, and the expression of tryptophan hydroxylase 2 (Tph2), serotonin transporter (Sert) and vesicular monoamine transporter 2 (Vmat2) was greatly down-regulated. On the other hand, the expression of dopamine and norepinephrine as well as aromatic L-amino acid decarboxylase (Aadc) and Pet1 was unchanged. Our results reveal that Lmx1b is required for the biosynthesis of 5-HT in adult mouse brain, and it may be involved in maintaining normal functions of central 5-HTergic neurons by regulating the expression of Tph2, Sert and Vmat2.

Introduction

The neurotransmitter serotonin (5-HT) exerts a wide spectrum of actions in a variety of behaviors, such as pain sensation, locomotion, circadian rhythm, food intake and emotional behaviors [1,2]. Extensive efforts have been made to characterize the molecular pathways that control the specification, differentiation and survival of 5-HTergic neurons during brain development [3], because this line of research is very helpful for understanding the genetic basis of central 5-HTergic neurons during brain development [3], because this line of research is very helpful for understanding the genetic basis of central 5-HTergic neurons during brain development [3]. Sonic hedgegog secreted from the floor plate triggers the expression of Mash1 and Gata2 in progenitor cells in the ventricular zone of hindbrain [6], and both genes are essential for the development of 5-HTergic neurons [7,8]. 5-HTergic neurons are classified into two groups based on their anatomical location: a rostral group located in the pons and a caudal group located in the medulla oblongata. Although Nkx2.2 is expressed in the progenitors of all 5-HTergic neurons in hindbrain, evidence from null mutant mice show that it is only required for the generation of 5-HTergic neurons in the dorsal raphe nucleus, one cluster neurons in pons group [9], and Gata3 is thought to be required for the differentiation of the medulla oblongata group [10]. Both Lmx1b and Pet1 are expressed in postmitotic 5-HTergic neurons and essential for the differentiation and survival of 5-HTergic neurons during embryonic development [4,11,12].

Our previous study has shown that Lmx1b is persistently expressed in central 5-HTergic neurons during postnatal development and throughout adulthood suggesting that Lmx1b may be involved in regulating normal expression of 5-HT in adult brain. To test this hypothesis, we used a tamoxifen-inducible Cre-LoxP system [13] to selectively inactivate Lmx1b expression in central 5-HTergic neurons of adult mice. Our data showed that 5-HT level in Lmx1b iCKO mice was reduced to 60% of control mice probably due to down-regulation of Tph2. In addition, Sert and Vmat2 that are implicated in maintaining normal functions of 5-HTergic neurons were greatly reduced in Lmx1b iCKO mice. Thus, Lmx1b, an essential gene for the development of central 5-HTergic neurons, is also required for the normal biosynthesis of 5-HT in the adult brain and possibly for regulating normal functions of central 5-HTergic neurons.

Materials and Methods

Genetic crossings, genotyping and animal maintenance

Lmx1blox/lox mice [14] and Rosa26-LacZ reporter (Rosa26R) mice [15] were generated and genotyped as previously described. In Lmx1blox/lox mice, exons 4-6 of Lmx1b were flanked by two LoxP sites and can be deleted in the presence of Cre in vivo [14]. To specifically inactivate Lmx1b expression in 5-HTergic neurons in the adult mouse brain, Lmx1blox/lox mice were crossed with Pet1-CreER<sup>TK</sup> mice (see below) and their offspring Pet1-CreER<sup>TK</sup> Lmx1blox/lox iCKO mice. Animal care

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: songnn1982@gmail.com (N-NS); dingyuqiang@gmail.com (Y-QD)

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Funding: This work was supported by grants from the National Natural Science Foundation of China (30430260), the Ministry of Science and Technology of China (2006CB940903, 2007CB512303, 2009ZX09501-030) and the Deutsche Forschungsgemeinschaft (KFO 125, SFB 581, SFB TRR 58). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Citation: Song N-N, Xiu J-B, Huang Y, Chen J-Y, Zhang L, et al. (2011) Adult Raphe-Specific Deletion of Lmx1b Leads to Central Serotonin Deficiency. PLoS ONE 6(1): e15998. doi:10.1371/journal.pone.0015998

Editor: Etienne Joly, Universite de Toulouse, France

Received September 9, 2010; Accepted December 2, 2010; Published January 5, 2011

PLoS ONE | www.plosone.org 1 January 2011 | Volume 6 | Issue 1 | e15998
practices and all experiments were reviewed and approved by the Animal Committee of Tongji University School of Medicine, Shanghai, China (TJmed-010-10).

**Generation of Pet1-CreERT2 mice**

Pet1-CreERT<sup>2</sup> BAC construct was obtained by inserting CreERT<sup>2</sup> coding sequence downstream of the Pet1 start codon within the RP23-165D11 BAC (BACPAC Resources Center at Children’s Hospital Oakland Research Institute) via homologous recombination in E. coli cell line EL250 [16]. Sepharose-4B (Sigma)-purified BAC DNA was then introduced into FVB/N fertilized mouse eggs by pronuclear injection using standard methods. Transgenic mice were genotyped by PCR with primers against Cre (forward: TCG ATG CAA CGA GTG ATG AG; reverse: TCC ATG AGT GAA CGA ACC TG) resulting in a ~400 base-pair product. All progeny carrying this transgene were found to be viable and fertile without any obvious abnormalities.

To determine the spatial pattern of Cre activity, Pet1-CreERT<sup>2</sup> mice were crossed with Rosa26R mice [15] and Cre activity was examined by administrating tamoxifen to Pet1-CreERT<sup>2</sup>; Rosa26R progeny. Tamoxifen (20 mg/ml; Sigma) diluted in corn oil (Sigma) was administrated by oral gavage in once-daily doses of 8 mg/40 g of body weight on the following schedule: days 1, 8, 9, 11 and 12. Mice were sacrificed 2–3 weeks after the last dose and brains were removed and fixed in 4% paraformaldehyde (Sigma) in 0.01 M phosphate buffered saline (PBS; pH 7.4) for 3 hours. After cryoprotection with 30% sucrose in PBS, 40 μm-thick sections were cut on a cryostat (CM1900, Leica) and immediately subjected to X-gal staining as described previously [5].

**in situ hybridization and immunohistochemistry**

*In situ* hybridization probes against Tph2, Sert, Aadc and Dopamine β-hydroxylase (Dbh), were constructed according to the description on the website of Allen Brain Atlas (http://www.brain-map.org). The Lmx1b [17] and Pet1 in situ probes encompassed the complete...
Lmx1b and Pet1 coding sequence, respectively. We also generated an in situ probe against exons 4–6 of Lmx1b only. All probes were cloned into pGEM-T vector (Promega). Eight mice (4 wild-type and 4 Lmx1b iCKO) were used for in situ hybridization. For in situ hybridization, brains were fixed in 4% PFA in PBS for 24 hours, cryoprotected with 30% sucrose in PBS, and 30 μm-thick transverse sections were cut on a cryostat and mounted onto glass slides (Fisher Scientific). RNA probes labeled by digoxigenin-UTP (Roche) were generated by in vitro transcription and hybridization signals were visualized upon nitro blue tetrazolium chloride (Fermentas) and 5-bromo-4-chloro-3-indolyl phosphate (Fermentas) staining.

Twelve mice (6 wild-type and 6 Lmx1b iCKO) were used in immunochemistry. Thirty μm-thick brain sections were incubated with primary antibody at 4°C overnight. After washing in PBS, sections were incubated with appropriate secondary antibody for 3 hours at room temperature, washed in PBS, and incubated with Cy3-conjugated streptavidin (1:1000; Jackson ImmunoResearch) for 1 hour. The following primary antibodies were used: goat anti-β-galactosidase (β-gal; 1:1000; AbD Serotec), rabbit anti-Lmx1b (1:2000) [17], rabbit anti-Tph2 (1:4000) [18,19], rabbit anti-Vmat2 (1:1000; Chemicon), mouse anti-Tyrosine hydroxylase (TH; 1:4000; Sigma). For Tph2 and β-gal double staining, sections were incubated with a mix of the anti-Tph2 and anti-β-gal antibodies overnight, then for 3 hours with a mix of Cy3-labeled donkey anti-rabbit (1:400; Jackson ImmunoResearch) and biotinylated horse anti-goat IgG (1:400; Vector Laboratories), and finally with Cy2-conjugated streptavidin (1:1000; Jackson ImmunoResearch) for 1 hour. There were no immunostaining signals when primary antibodies were omitted or replaced with normal IgG. Stained sections were observed and scanned under a fluorescence or confocal microscope.

Figure 2. Pet1-CreERT2 activity is specific to central 5-HTergic neurons. Double immunolabeling of Tph2 and β-gal in the dorsal raphe nucleus (DR; A-B") and raphe magnus nucleus (RMg; C-C") was performed in Pet1-CreERT2; Rosa26R mice. About 85% of Tph2-positive neurons are co-stained with β-gal antibody (arrows), and a few Tph2-labeled neurons are not β-gal positive (arrowhead). Note that all β-gal-expressing neurons are labeled with Tph2 antibody. B-B" show higher magnifications views of A-A", respectively. Scale bars, 100 μm.

doi:10.1371/journal.pone.0015998.g002
Cell count

We counted positive cells in every six sections. Positive cells in the dorsal raphe nucleus (DR) are reduced in Lmx1b iCKO mice (B) relative to that in wild-type controls (A). (C, D) Similar changes in intensities of 5-HT immunofluorescence are also present in the raphe obscurus nucleus (Rob), raphe pallidus nucleus (RPa) and lateral paragigantocellular nucleus (LPGi) of Lmx1b iCKO mice relative to those of wild-type mice (C, D). (E) HPLC analysis showing that level of 5-HT and its metabolite 5-HIAA in the brains of Lmx1b iCKO mice are reduced to about 60% and 30% of that in controls, respectively (* P < 0.001). Scale bar, 100 μm. doi:10.1371/journal.pone.0015998.g003

High performance liquid chromatography (HPLC)

Adult (3 months) tamoxifen-induced wild-type and Lmx1b iCKO mice were used for HPLC (n = 6 for each). Two-three weeks after completion of tamoxifen treatment, whole brains were dissected out immediately after anesthesia with sodium pentobarbital (0.07 mg/g body weight), and HPLC samples were made according to methods described previously [5]. 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), dopamine and its metabolite dihydroxyphenylacetic acid and homovanillic acid, and norepinephrine were measured using HPLC electrochemical detection as described previously [5]. Statistical significance was determined by the Student’s t-test. All data were expressed as mean ± SEM, and error bars represent SEM. P values less than 0.05 were considered statistically significant.

Results

Generation and characterization of Pet1-CreERT² transgenic mice

To delete Lmx1b in the adult mouse brain, we crossed mice carrying two floxed Lmx1b alleles with mice harboring a tamoxifen-inducible form of Cre recombinase (CreERT²) [13] under the control of the Pet1 promoter, to generate Lmx1b iCKO mice (Figure 1A). Genotypes were confirmed by PCR. Pet1 is expressed specifically in central 5-HTergic neurons [4], and the distribution of Cre-recombination activity was determined by crossing to Rosa26R mice [15]. Pet1-CreERT²; Rosa26R mice were administered a
regimen of tamoxifen (see Materials and methods) beginning at P90 and analyzed by X-gal staining 2–3 weeks after completing the induction regimen. The procedure of tamoxifen administration is shown in Figure 1B. The first day of tamoxifen administration was termed as D1, and tamoxifen was administrated in once-daily doses of 8 mg/40 g body weight on D1, D8, D9, D11 and D12. X-gal-positive cells were found in the raphe nuclei and ventrolateral reticular formation of the medulla oblongata (Figure 1C–G), showing that Pet1-driven Cre-recombinase activity can be induced in adulthood. Note that X-gal labeling was not observed in other brain regions outside the raphe nuclei (Figure 1H–J) and no Cre activity was present in negative control-treated and untreated Pet1-CreERT2; Rosa26R mice (data not shown).

To determine whether Cre-recombinase activity in Pet1-CreERT2; Rosa26R mice was exclusive to 5-HTergic neurons, we performed Tph2/β-gal double immunostaining. Tph2 is the key enzyme responsible for 5-HT synthesis in the brain [21]. All β-gal-positive neurons expressed Tph2, and approximately 85% of

**Figure 5. Decreased expression of 5-HT-specific genes in adult Lmx1b iCKO mice.** (A–L) Tph2 (A–D) and Sert [34] in situ hybridization, and Vmat2 (I–L) immunostaining shows down-regulation of these three genes in rostral and caudal raphe nuclei of Lmx1b iCKO mice (B, D, F, H, J, L) compared with that in wild-type controls (A, C, E, G, I, K). (M–T) Aadc (M–P) and Pet1 (Q–T) expression is unchanged in Lmx1b iCKO raphe nuclei (N, P, R, T) relative to wild-type controls (M, N, Q, S). DR, dorsal raphe nucleus; RMg, raphe magnus nucleus. Scale bar, 100 μm.

doi:10.1371/journal.pone.0015998.g005
Tph2-labeled neurons were β-gal-positive (Figure 2). Thus, Cre activity in Pet1-CreERT2 mice is specific to 5-HTergic neurons, and is capable of inducing recombination in the majority of 5-HTergic neurons in the adult brain.

Deleting Lmx1b in the adult brain leads to 5-HT insufficiency

Previous studies have demonstrated that Lmx1b is required for the differentiation and survival of central 5-HTergic neurons during embryonic development [11,12]. We set out to examine whether Lmx1b also plays a role in the adult central 5-HTergic neurons. Lmx1b iCKO and wild-type control mice were administered with tamoxifen beginning at P90 and examinations were performed 2–3 weeks after completion. We first used Lmx1b antibody [17] to examine whether Lmx1b is deleted in Lmx1b iCKO mice. As shown in Figure S1, similar Lmx1b immunostaining was found in the dorsal raphe nucleus of wild-type mice and Lmx1b iCKO mice showing that the antibody recognizes the truncated Lmx1b protein without exons 4–6 of Lmx1b; in this case the Lmx1b antibody can be used to trace Lmx1b mutant cells. Because the full length of in situ hybridization probe for Lmx1b [17] was unable to distinguish truncated mRNA from normal Lmx1b mRNA either (data not shown), we generated an in situ probe against exons 4–6 of Lmx1b only. However, the sensitivity of this probe was too low to detect Lmx1b mRNA in adult wild-type mice, although it worked in showing Lmx1b mRNA in embryos (data not shown). The floxed Lmx1b alleles were deleted after crossing Lmx1bfl/fl mice with Pet1-Cre or Wnt1-Cre mice [5,14] and Cre activity in Pet1-CreERT2 mice was functional as shown by X-gal staining in 5-HTergic neurons in Pet1-CreERT2; Rosa26R mice (Figures 1, 2), we thus speculate that the exons 4–6 of Lmx1b should be deleted in the majority of 5-HTergic neurons in the adult Lmx1b iCKO mice (also see phenotypes described below).

We next examined 5-HT expression in Lmx1b iCKO mice, and found that intensities of 5-HT immunofluorescence in individual neurons were slightly reduced in the raphe nuclei of Lmx1b iCKO mice compared with wild-type mice (Figure 3A–D). To further investigate whether the content of 5-HT in the brain is altered or not, we used HPLC to measure the levels of 5-HT and its metabolite 5-HIAA in the brain, and found that they were decreased in Lmx1b iCKO mice to about 60% and 30% of control levels, respectively (Figure 3E). We speculate that the discrepancies between no apparent reduction of 5-HT immunofluorescence and 40% reduction of 5-HT revealed by HPLC in Lmx1b iCKO are probably due to the low sensitivity of 5-HT antibody, which is unable to detect this reduction. Taken together, we conclude that Lmx1b is required for normal expression of 5-HT in adult brain.

Deleteing Lmx1b in the adult brain results in down-regulation of 5-HTergic neuron-associated genes

To explore the mechanisms underlying decreased 5-HT level in Lmx1b iCKO mice, we examined the expression of Tph2, which is a specific enzyme for synthesis of 5-HT in the brain [21]. The number of neurons with intense Tph2 immunofluorescence in the raphe nuclei of Lmx1b iCKO mice was dramatically reduced compared with control mice (Figure 4). Correspondingly, many weakly-labeled neurons were seen in the Lmx1b iCKO raphe nuclei (arrowheads in Figure 4D), whereas they were not observed in wild-type mice. These observations were further confirmed by in situ hybridization for Tph2 (Figure 5A–D). The number of cells with intense in situ signals was significantly decreased in Lmx1b iCKO relative to wild-type mice (Figure 6). Since approximately 15% of 5-HTergic neurons in Pet1-CreERT2 mice did not exhibit Cre activity (Figure 2), the strong Tph2 labeling retained in Lmx1b iCKO may correspond to 5-HTergic neurons in which Lmx1b was not deleted. Nevertheless, these results indicate that deleting Lmx1b in the adult brain impairs Tph2 expression, leading to a deficiency of central 5-HT.

To further investigate the function of Lmx1b, we examined the expression of several genes essential for maintaining the normal function of 5-HTergic neurons. Sert is required for the re-uptake of 5-HT in axonal terminals [22], and its expression was greatly reduced in the raphe nuclei of Lmx1b iCKO mice (Figure 5E–H). Cell counts showed a significant difference in the number of Sert-expressing cells between wild-type and Lmx1b iCKO mice (Figure 6). Vmat2, which is required for packaging 5-HT into synaptic vesicles [23], was also down-regulated in Lmx1b iCKO mice.
mice (Figure 5I–L). These results indicate that expression of the genes associated with the maintaining functions of 5-HTergic neurons is impaired. To test whether deleting Lmx1b decreases the number of 5-HTergic neurons or not, which in turn results in the phenotypes mentioned above, we examined the expression of Aadc and Pet1. We found that the number of Aadc-expressing neurons was unchanged in Lmx1b iCKO mice compared with controls (Figures 5M–P, 6), consistent with the finding that similar Lmx1b immunostaining was present in both Lmx1b iCKO and wild-type mice (Figure S1). The dorsal raphe nucleus contains the most abundant 5-HTergic neurons among the raphe nuclei. In Nissl-stained sections, the Nissl-stained 5-HTergic neurons are larger and more intensely stained relative to non-5-HTergic neurons. Nissl-stained sections from wild-type and Lmx1b iCKO mice showed no obvious difference in cell density and distribution (Figure S2). Furthermore, Pet1 expression in 5-HTergic neurons requires Lmx1b during embryonic development [12], but its expression in the raphe nuclei of Lmx1b iCKO mice showed no difference from wild-type controls (Figure SQ–T). These results suggest that the overall number of 5-HTergic neurons is not affected by deleting Lmx1b in adulthood, and that Pet1 expression in the adult brain is independent of Lmx1b.

Expression of dopamine and norepinephrine is unchanged in Lmx1b iCKO mice

Previous studies have shown that central 5-HT deficiency may affect the expression of other monoamines in the brain [24]. To explore this possibility, we examined the expression of TH, the essential enzyme for the synthesis of both dopamine and norepinephrine, and Dbh, an enzyme that converts dopamine into norepinephrine [25] in Lmx1b iCKO mice. TH immunostaining in the substantia nigra and ventral tegmental area (dopaminergic neurons), and in the locus coeruleus (norepinephrinergic neurons) in Lmx1b iCKO mice was similar to that in control mice (Figure 7A, B, D, E). Dbh in situ hybridization in the locus coeruleus of Lmx1b iCKO mice was also similar to that of wild-type controls (Figure 7C, F). In addition, levels of norepinephrine, dopamine and its metabolites (dihydroxyphenylacetic acid and
homovanillic acid) in Lmx1b iCKO mice were not different from those of controls, as determined by HPLC analysis (Figure 7D). Thus, expression of dopamine and norepinephrine in Lmx1b iCKO mice appeared normal.

Discussion

In the present study, we took advantage of an inducible Cre-LoxP system to inactivate Lmx1b expression in adult 5-HTergic neurons. We found that the level of central 5-HT in Lmx1b iCKO mice is reduced to 60% of controls, and that the expression of 5-HT neuron-associated genes such as Tph2, Sert and Vmat2 are down-regulated in Lmx1b iCKO mice.

We generated Pet1-CreERT2 mice, and X-gal staining data from Pet1-CreERT2, Rosa26R mice treated with tamoxifen in adulthood showed that Cre was functional and restricted to central 5-HTergic neurons. Our previous studies have shown that the flanked Lmx1b allele was deleted in Wnt1-Cre; Lmx1bfllox/ and Pet1-Cre; Lmx1bfllox/ mice [5,14]. Although we failed to provide direct morphological data showing the deletion of Lmx1b, based on the data mentioned above and phenotypes observed in Lmx1b iCKO mice, it is reasonable to speculate that Lmx1b is inactivated in 5-HTergic neurons of Lmx1b iCKO mice. Pet1-CreERT2 mice are very useful in time-controlled deletion of interest genes in central 5-HTergic neurons particularly in adulthood.

The function of Lmx1b in the development of 5-HTergic neurons has been studied extensively [5,11,12,26]. In Lmx1b null mice, postmitotic 5-HTergic neurons fail to express 5-HT and several genes (e.g. Pet1) critical for 5-HT neuron development [11,12]. When Lmx1b is conditionally deleted after 5-HTergic neuron development has initiated (around embryonic day 12.5), 5-HTergic neurons differentiate normally, but end up dying at later embryonic stages [5,26]. Thus, Lmx1b is required for both differentiation and survival of 5-HTergic neurons during embryonic development. As we showed in the present study, the inactivation of Lmx1b in adulthood led to a reduction in central 5-HT levels, probably as a consequence of Tph2 down-regulation. In addition, Sert, the protein responsible for the re-uptake of 5-HT into axonal terminals, and Vmat2, which is involved in packaging 5-HT into synaptic vesicles [22,23], were both greatly reduced in the raphe nuclei of Lmx1b iCKO mice. In contrast, the expression of both Pet1 and Aadt appeared unchanged in Lmx1b iCKO mice relative to control mice, indicating that there was no loss of 5-HTergic neurons in the raphe nuclei. It has been shown that Pet1 is required for terminal differentiation of 5-HTergic neurons during embryonic development [4], and its expression is lost in Lmx1b null mice [12]. Recently, it is reported that Pet1 is required for maintaining the serotonergic neurotransmitter system during adult stages [27]. Loss of Pet1 in the 5-HTergic neurons leads to a decrease of Tph2 expression but no change in Lmx1b expression. In the present study, normal Pet1 expression was found in Lmx1b iCKO mice. It is likely that Lmx1b and Pet1 act in parallel to regulate central 5-HTergic system, the expression of Pet1 in adult brain is independent of Lmx1b, and Pet1 is not involved in alterations of gene expression in Lmx1b iCKO mice. Taken together, these results indicate that Lmx1b is required for 5-HT biosynthesis and expression of several key genes associated with functions of 5-HTergic neurons, but not their survival in adult brain.

Central 5-HT deficiency has been associated with some mental disorders, such as depression and posttraumatic stress disorder [28,29,30]. We previously generated Lmx1b iCKO mice in which Lmx1b is deleted specifically in 5-HTergic neurons at embryonic stage with the help of Pet1-Cre, and found that 5-HT level in brain is less than 10% of that in wild-type mice. Interestingly, these mice showed enhanced contextual fear memory [5]. On the other hand, 5-HT plays important roles in the development of nervous system at embryonic stages and during early postnatal development, such as axonal growth [31], spine formation [32] and barrel formation in the somatosensory cortex [33]. It is likely that abrogating 5-HT biosynthesis or 5-HT neuronal development with traditional genetic ablation techniques might have uncontrolled pleiotropic effects by interfering with the development of other brain systems. The use of Lmx1b iCKO mice circumvents these complications by allowing the brain to develop normally through to adulthood, and they serve as a new mouse model to study mental disorders associated with central 5-HT deficiency.

Supporting Information

Figure S1 The expression of truncated Lmx1b in Lmx1b iCKO mice is comparable to that of full-length Lmx1b in wild-type control. (A) The expression of full-length Lmx1b in dorsal raphe of wild-type control. (B) The expression of truncated Lmx1b in dorsal raphe of Lmx1b iCKO mice. Scale bar, 100 μm. (DOC)

Figure S2 Nissl staining shows no difference in the morphological features of the dorsal raphe nucleus between wild-type and Lmx1b iCKO mice. Scale bar, 100 μm. (DOC)

Author Contributions

Conceived and designed the experiments: NNS HL YQD. Performed the experiments: NNS JBX YH JYC LZ. Analyzed the data: NNS JBX. Contributed reagents/materials/analysis tools: LG KPL. Wrote the paper: NNS YQD.

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