RESEARCH ARTICLE

Otx2 Requires Lmx1b to Control the Development of Mesodiencephalic Dopaminergic Neurons

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Abstract

Studying the development of mesodiencephalic dopaminergic (mdDA) neurons provides an important basis for better understanding dopamine-associated brain functions and disorders and is critical for establishing cell replacement therapy for Parkinson’s disease. The transcription factors Otx2 and Lmx1b play a key role in the development of mdDA neurons. However, little is known about the genes downstream of Otx2 and Lmx1b in the pathways controlling the formation of mdDA neurons in vivo. Here we report on our investigation of Lmx1b as downstream target of Otx2 in the formation of mdDA neurons. Mouse mutants expressing Otx2 under the control of the En1 promoter (En1+/Otx2) showed increased Otx2 expression in the mid-hindbrain region, resulting in upregulation of Lmx1b and expansion of mdDA neurons there. In contrast, Lmx1b−/− mice showed decreased expression of Otx2 and impairments in several aspects of mdDA neuronal formation. To study the functional interaction between Otx2 and Lmx1b, we generated compound mutants in which Otx2 expression was restored in mice lacking Lmx1b (En1+/Otx2; Lmx1b−/−). In these animals Otx2 was not sufficient to rescue any of the aberrations in the formation of mdDA neurons caused by the loss of Lmx1b, but rescued the loss of ocular motor neurons. Gene expression studies in Lmx1b−/− embryos indicated that in these mutants Wnt1, En1 and Fgf8 expression are induced but subsequently lost in the mdDA precursor domain and the mid-hindbrain organizer in a specific, spatio-temporal manner. In summary, we demonstrate that Otx2 critically depends on Lmx1b for the formation of mdDA neurons, but not for the generation of ocular motor neurons. Moreover, our data suggest that Lmx1b precisely maintains the expression pattern of Wnt1, Fgf8 and En1, which are essential for mid-hindbrain organizer function and the formation of mdDA neurons.
Introduction

Meso-diencephalic dopaminergic (mdDA) neurons modulate essential brain functions including motor control, cognition and reward. Their degeneration and dysfunction has been associated with prevalent and devastating brain disorders such as Parkinson’s disease, schizophrenia and drug abuse[1]. mdDA neurons are organized in three major nuclei: substantia nigra, ventral tegmental area and retrorubral field[1]. Characterizing the molecular mechanisms controlling the development of mdDA neurons has attracted special interest since they provide critical information for the differentiation of stem cells into DA neurons used in cell replacement therapy for Parkinson’s disease[2][3].

mdDA neurons originate from the midbrain floor plate and probably partially also from the diencephalon[4]. The morphogens, Shh, Wnt1 and Fgf8 that provide progenitors with positional information, instructing them with regard to their dopaminergic identity, are secreted from the ventral midbrain and the mid-hindbrain organizer (MHO)[4][5][6][7][8][9]. These morphogenes work in concert with a series of transcription factors that include Otx2, Lmx1b, Lmx1a, En1/2, Foxa1/2, Ngn2, Pitx3 and Nurr1 (Nr4a2– Mouse Genome Informatics). Each is important for progenitor cell responsiveness to morphogens, differentiation and survival, as well as for MHO positioning and activity[4][10][11][12][13][14][15][16][17][18][19]. Thus, while many proteins involved in the development of mDA neurons have been identified, understanding how they interact to ensure proper temporal and spatial establishment of mdDA cells remains a major challenge.

Different lines of evidence indicate that Otx2 is a key regulator of mDA development. By employing mouse mutants in which the caudal Otx2 domain, and concomitantly the MHO, are shifted caudally or rostrally, we initially demonstrated that Otx2 controls the number and location of mdDA neurons by positioning the MHO[10]. Subsequent work demonstrated that Otx2 can regulate the formation of mdDA neurons during later stages of development, independent of the position of the MHO[11]. This central role for Otx2 in the ontogeny of mdDA neurons has gained support by reports demonstrating that Otx2 regulates the neurogenic activity in the midbrain floorplate, as well as proliferation and differentiation of mdDA progenitors [20][21][22]. Finally, overexpressing Otx2 in the mid-hindbrain region leads to a lateral expansion of the mdDA neuronal population, indicating that Otx2 controls the medio-lateral extension of the mdDA precursor domain[21]. Different genes are regulated in Otx2 gain- and loss-of-function experiments, suggesting that each might mediate the effects of Otx2 on mdDA neuron ontogenesis. Thus far, however, functional in vivo studies testing whether these genes indeed mediate Otx2 effects on the development of DA neurons are lacking.

Expression of Lmx1b, found in both the mdDA progenitor domain and in the MHO, is regulated in chickens by Otx2[23]. This supports the contention that Lmx1b is downstream of Otx2 in the genetic network controlling the generation of mdDA neurons. In Lmx1b−/− mutants, different aspects of mdDA development are altered. For example, caudomedial mdDA neurons, that will predominantly form the ventral tegmental area, exhibit impairments in their terminal differentiation as indicated by a reduced number of NURR1+ cells expressing TH, and likewise fail to express PITX3 [12][24]. Moreover, the lateral mdDA neurons, forming the bulk of the substantia nigra, are missing, as indicated by a loss of Wnt1 and D2R expression[24]. Finally, the red nucleus (RN), located lateral to the mdDA precursor domain, inappropriately extends medially into the mdDA domain, suggesting that Lmx1b is critical to define the medio-lateral borders of the mdDA nuclei[24]. Another non-DA population, the ocular motor neurons (OMNs) are almost completely missing in Lmx1b−/− mutants[24].

Experiments featuring conditional activation and inactivation of Lmx1b, using an En1-Cre driver, demonstrated increased and decreased numbers of TH+ neurons respectively, further
indicating the importance of Lmx1b for the formation of mdDA neurons[9]. The conditional inactivation of Lmx1b using a Shh-Cre driver suggest that Lmx1b is non-cell autonomously required for mdDA neuron development, indicating that the effects of Lmx1b on mdDA ontogeny are mediated by the MHO expression domain[25]. Since both Wnt1 and Fgf8 are the key mediators of MHO activity and required for mdDA development, they are the most likely candidates to mediate the MHO effects of Lmx1b on mdDA development. Lmx1b cooperates with Wnt1 and with miR135a2 to control the generation of mdDA neurons[26][27]. Little is known, however, about the aspects of mdDA neuronal development regulated by the Lmx1b/Wnt1 interaction. A role for Fgf8 in mediating Lmx1b activity has not been established and the data on the regulation of Fgf8 by Lmx1b is inconsistent. Fgf8 has not been detected in Lmx1b−/−, suggesting that Lmx1b is absolutely required for Fgf8 induction[28]. However, these findings are in conflict with the induction of medial mdDA neurons in Lmx1b−/−, since previous work indicates that the Fgf8 expression domain at the MHO is required for mdDA induction[5].

In our study, we found that expression of Otx2 and Lmx1b are reciprocally regulated. We furthermore provide evidence that Otx2 cannot compensate for the loss of Lmx1b in the specification of mdDA neurons, but it can replace Lmx1b in the induction of OMNs. This indicates that Lmx1b is downstream of Otx2 in the genetic pathway controlling mdDA formation but not OMNs generation. Moreover, we performed a detailed expression analysis of Lmx1b−/− embryos and found that Lmx1b is not required for the induction of Wnt1, En1 and Fgf8, but is necessary for maintaining their transcripts in a specific, spatio-temporal manner in the mdDA precursor field and MHO.

Materials and Methods

Animals

The generation of En1+/Otx2 and Lmx1b−/− mice has been reported earlier[29][10][30]. En1+/Otx2 embryos were distinguished from their wild type littermates, by genotyping PCR using primers: forward 5'-GGGCTGAGTCTGACCACTTC –3' and reverse 5' -CAGGAAGCTGGTGACTATA –3', resulting in a 625 bp product. Lmx1b null allele genotyping was done by PCR using primers: forward 5'-GATAGGGCATTCAACCAGGACGAGCAAAGA and reverse 5'-AAA CAGAAGCCACAGAGAGGACAAGAAG, resulting in a 397bp fragment. In order to obtain embryos at a specific stage, mice were bred overnight, and females were checked the following morning for the presence of copulatory plugs. Embryos were collected at embryonic day (E) 9.5 to E12.5.

Mice were kept under standardized conditions (22–24°C temperature; 55%±15% humidity) on a 12 h light / 12 h dark cycle in groups of 3–5 in standard laboratory cages. Food and tap water were provided ad libitum. Mice were sacrificed by isoflurane overdose. All animal studies were performed in accordance with local animal welfare regulations and international guidelines. 42 adult animals and 87 embryos were used for the study. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Ben-Gurion University of the Negev (Permit Number: IL-13-03-2010). All efforts were made to minimize suffering.

RNA in situ hybridization

For section in situ hybridization, embryonic mice were immersion fixed with 4% paraformaldehyde (PFA), paraffin embedded and cut on a microtome in 8μm thick sections. All sections were processed for in situ hybridization according to Brodski et al., 2003 using 35S-labeled riboprobes. Antisense RNA probes were transcribed from plasmids containing fragments of the murine: Otx2, Wnt1, Th, Fgf8, Lmx1a, En1 and Nurr1 as described in Brodski et al. 2003.
Immunohistochemistry and Cell Counting

For fluorescence immunohistochemistry, paraffin sections (5 μm) were deparaffinized through xylene, rehydrated through an ethanol series, washed in PBS. After antigen retrieval in boiling citric acid (pH –6) for 10 minutes, sections were blocked in 4% normal goat serum (NGS) for half an hour. Slides were incubated with primary antibody diluted in PBS overnight, washed 3xPBS for 3 minutes and incubated for 1 hour with secondary antibody. Slides were washed 3xPBS for 5 minutes and mounted with Immuno-Mount (Thermo Scientific). Antibodies used: rabbit anti TH (Millipore, 1:200), mouse anti TH (Millipore 1:200), rabbit anti OTX2 (Abcam, 1:300), mouse anti FOX2A (DSHB, 1:10), rabbit anti NURR1 (Santa Cruz, 1:400), rabbit anti LMX1A (Millipore 1:100), rabbit anti Cleaved CASPASE 3 (Cell Signaling, 1:300), mouse anti POU4F1 (Santa Cruz, 1:400), mouse anti NKX6.1 (DSHB, 1:10), mouse anti NKX6.1 (DSHB, 1:10), rabbit anti PHH3 (Millipore,1:1000), mouse anti LIM (DSHB, 1:50), mouse anti ISLET1 (DSHB, 1:50). Secondary antibodies: goat anti rabbit Cy3, goat anti mouse Cy3, goat anti rabbit Alexa-Fluor –488, goat anti mouse Alexa-Fluor –488, all 1:100 (Jackson Laborotories). In pictures depicting sagittal sections, the rostral part was oriented to the left. For cell counting, coronal sections (5 μm thick) of each genotype were immune stained with the relevant antibody and positive cell bodies were counted on every second section of all slides.

All results are expressed as mean ± SE. IBM® SPSS® Statistics 21 software was used for the statistical analysis. Genotypes were analyzed as factors for two-way multifactorial analysis of variance (ANOVA). Where significant effects were detected, Fisher’s LSD post hoc analysis was used to detect differences between individual groups (containing at least 3 animals).

Results

Overexpressing Otx2 in the hindbrain leads ventrally to a specific expansion of the mdDA neuronal population

Previous studies on En1+/Otx2 mice indicate that a caudal shift of the Otx2 expression border (and concomitantly the MHO), leads to caudal enlargement of the entire dorsal midbrain and a complementary reduction of dorsal hindbrain[29]. In the ventral midbrain of En1+/Otx2 mutants, the caudal shift of Otx2 and the MHO leads to an increase in the number of mdDA neurons, but does not affect the formation of OMNs[10]. In order to study the specificity of the enlargement of the mdDA neuronal population in En1+/Otx2 mutants, we investigated the formation of the RN, since it is the nucleus most closely neighboring mdDA nuclei.

Along the anterior-posterior axis of the midbrain in wild-type (WT) animals the RN, as visualized by POU4F1 expression, was located directly lateral to TH+ and NURR1+ mdDA neurons (Fig 1A and 1B). The RN and adjacent mdDA neurons were both posteriorly-extend- ing up to the caudal border of the midbrain, but did not cross the boundary into the hindbrain (Fig 1F and 1G). In contrast, in En1+/Otx2 mutants, the caudally-enlarged NURR1+ and TH+ cell population were not flanked by POU4F1+ cells laterally, indicating that the extended Otx2 expression domain does not induce caudal extension of the RN (Fig 1N and 1O).

The phenotypic identity and location of the RN are controlled by the transcription factor NKX6.1[31]. In WTs directly adjacent to mdDA neurons, NKX6.1 was expressed in two domains, one in the ventricular zone above the mdDA neurons and one in the mantle zone (Fig 1C). In the hindbrain, the ventricular expression domain adjacent to the mantle zone expression area was missing (Fig 1H), in accordance with previously reported differences between the mid- and hindbrain NKX6.1 expression domain[32]. In En1+/Otx2 mutants, there was no NKX6.1 expression in the ventricular zone above the caudally-extended mdDA neurons.
This further supports our contention that the posteriorly-extended mdDA neurons in En1+/Otx2 mutants are actually located in the hindbrain. To further characterize the differential effect of Otx2 on the dorsal versus ventral midbrain formation, we visualized POU4F1 expression in the tectal midbrain of En1+/Otx2 mutants. It was previously shown that Otx2 specifies tectal fate independent of the MHO[33]. POU4F1 also plays a critical role in the formation of tectal neurons and their projections[34]. Nevertheless, the interaction of these two genes in tectal formation has been barely investigated. In contrast to the ventral midbrain, in which an increase in Otx2 expression did not affect the number of POU4F1+ neurons, we found that POU4F1 was significantly increased in the dorsal midbrain of mutants (Fig 1D and 1L). In addition, mutants exhibited an upregulation of the mitosis marker, PHOSPHO-HISTONE H3 in this region (Fig 1E and 1M). Taken together, we conclude that an early caudal expansion of Otx2 under the endogenous En1 promoter leads to a specific caudal extension of mdDA neuronal populations. These mutants, therefore, represent a useful tool to study the specific effects of Otx2 on mdDA neuron development. In addition, our data suggest that by regulating the expression of Pou4F1, Otx2 also affects tectal formation.

To further assess how Otx2 and signals generated by the MHO interact in the formation of mdDA neurons, we investigated Lmx1b expression in En1+/Otx2 mutants. We found that the expression domain of Lmx1b at E10.5 in En1+/Otx2 mutants was caudally-extended to the same degree as the mdDA neuronal population, suggesting that Otx2 might well control the formation of mdDA neurons via Lmx1b (Fig 1Q–1T).

Otx2 requires Lmx1b for the development of mdDA neurons

Interaction between Otx2 and Lmx1b in the formation of mdDA neurons was assessed by following the expression of OTX2 in Lmx1b+/− mutants. At E12.5 a significant reduction of OTX2 was recorded in the ventral midbrain of Lmx1b+/− mutants (Fig 2A and 2E), suggesting that a downregulation of OTX2 could mediate the loss of mdDA neurons in Lmx1b+/−. At E12.5 a significant increase (51.3 ± 2.1%) in TH positive cells was noted in En1+/Otx2 mutants compared to WTs (Lmx1b+/−; En1+/Otx2; Lmx1b+/−) and Lmx1b+/− embryos (Fisher’s LSD post hoc p = 0.047; Fisher’s LSD post hoc p = 0.423) (Fig 11). Moreover,
the lateral population of mdDA neurons was missing in En1+/Otx2;Lmx1b−/−, as in Lmx1b−/− (Fig 1E, 1F, 1G and 1H). The remaining medial mdDA neuronal populations exhibited alterations in their terminal differentiation as indicated by a significant reduction (84.0 ± 2.9%) in the number of NURR1+ cells expressing TH (Lmx1bF1,8 = 131.122, p < 0.001; Fisher’s LSD post hoc p < 0.001) (Fig 2F, 2H and 2J). However, there was no significant difference in the number of NURR1+ cells expressing TH between Lmx1b−/− and the compound mutants (Fisher’s LSD post hoc p = 0.464). In WT and En1+/Otx2−/− animals there was a sharp lateral border of the mdDA nuclei to the POU4F1 positive RN and LIM1/2 positive territory (K-N). In Lmx1b−/− the mdDA precursor domain intermingled with laterally-adjacent POU4F1 and LIM1/2 positive cells, which was not reversed by overexpression of Otx2 in compound mutants (Fig 2O–2R). Taken together, we conclude that Otx2 cannot compensate for the loss of Lmx1b in the development of mdDA neurons, suggesting that Otx2 requires Lmx1b for the control of the formation of mdDA neurons.

Otx2 does not require Lmx1b for the induction of OMNs

To study whether expression of Otx2 is sufficient to induce other ventral midbrain populations in the absence of Lmx1b, we studied the formation of OMNs as visualized by ISLET1 expression in En1+/Otx2;Lmx1b−/− mutants.

In Lmx1b−/− animals the number of ISLET1+ cells was reduced to 4.3 ± 1.2% of WTs (Lmx1bF1,21 = 83.716, p < 0.001; Fisher’s LSD post hoc p < 0.001). In compound mutants Otx2 lead to a significant increase of ISLET1+ cells compared to Lmx1b−/− (Otx2−/−Lmx1b−/−F1,21 = 6.954, p < 0.015; Fisher’s LSD post hoc p = 0.001) and to a recovery of 61.2 ± 2.7% of ISLET1+ neurons compared to WTs. The number of ISLET1+ positive cells did not differ between En1+/Otx2 and WTs (Fisher’s LSD post hoc p = 0.691) (Fig 3A–3D).

Next we assessed the integrity of the ventral midbrain in mutants by visualizing the expression of patterning genes. We found that NKX2.2 (Fig 3E–3H) and NKX6.1 (Fig 3I–3L) were unaltered in all investigated genotypes. Moreover, we did not find any changes in mitosis as visualized by PHOSPHO-HISTONE H3 in the mdDA precursor domain (Fig 3M–3P) or apoptosis as visualized by activated CASPASE–3 (Fig 3Q–3T) in all genotypes at E12.5.

PHOSPHO-HISTONE H3+ cells in the mdDA progenitor domain were counted in WT, En1+/Otx2−/−, Lmx1b−/− and En1+/Otx2−/−Lmx1b−/− embryos. A two-way ANOVA of the ratio between the PHOSPHO-HISTONE H3+ cells revealed no significant difference between any of the groups, with a trend increase in En1+/Otx2−/− mutants (Lmx1bF1,21 = 0.514, p = 0.497; Otx2F1,21 = 5.206, p = 0.056). Since virtually no CASPASE–3 staining was observed in any of the genotypes a quantification was not performed. The trigeminal ganglion showing strong CASPASE–3 staining was used as a positive control (Fig U-X) [35].

In order to assess whether changes in mitosis or apoptosis are present at earlier developmental time points we also studied PHOSPHO-HISTONE H3 and CASPASE–3 at E11.5. There was a significant increase in the number of PHOSPHO-HISTONE H3 positive cells in
En1+/Otx2 (31.0 ±1.8%) compared to WT animals (Otx2F1,16 = 11.281, p = 0.004; Fisher’s LSD post hoc p = 0.004). Lmx1b-/- embryos and compound mutants did not show significant differences compared to WTs in the number of PHOSPHO-HISTONE H3 positive cells (Lmx1bF1,16 = 1.439, p = 0.248; Fisher’s LSD post hoc p = 0.867 and p = 0.146). As for E12.5, the very few CASPASE–3 cells did not allow quantification. We conclude that in contrast to mdDA neurons, Otx2 does not require Lmx1b for the induction of OMNs.

In Lmx1b-/- embryos NURR1+ neurons express LMX1A and FOXA2 but not EN1

We next studied in each of the different genotypes, the expression of the transcription factors Lmx1a, Foxa2 and En1, which all play central roles in the development of mdDA neurons[4][14][15][36]. We observed unaltered expression of LMX1A (Fig 4A–4D) and FOXA2 (Fig 4I–4L) in the ventral midline of all mutants. Double immune labeling with LMX1A and TH antibodies indicated that the few remaining TH+ cells in Lmx1b-/- and compound mutants were all LMX1A+ (Fig 4C, 4D, 4G and 4H).

En1 is expressed during embryogenesis at the MHO and in developing mdDA neurons. Together with En2, it is essential for the formation of the mature mdDA phenotype and for the survival of these neurons[14][37][38][39]. Interestingly, En1-/- phenocopies important aspects of the mdDA phenotype of Lmx1b-/-, suggesting that Lmx1b and En1 are active in the same pathway directing the development of mdDA neurons[38]. However, the signals inducing and maintaining En1 expression in the mdDA precursors are largely unknown. To address this issue, we followed EN1 expression in the various genotypes. In WTs and in En1+/Otx2 embryos, all NURR1 expressing cells co-expressed EN1 (Fig 4M and 4N). In contrast, in Lmx1b-/- and En1+/Otx2;Lmx1b-/- mutants EN1 expression was lost in NURR1 expressing cells, while EN1 expression was maintained and possibly even augmented in the region adjacent to the NURR1+ mdDA neurons (Fig 4O and 4P). We conclude that in contrast to LMX1A and FOXA2, EN1 expression is dependent on Lmx1b in NURR1+ neurons and cannot be substituted for by Otx2.

Wnt1 and Fgf8 expression is induced but not maintained in Lmx1b-/- embryos

In order to better understand how Lmx1b directs the formation of mdDA neurons via the MHO, we employed Lmx1b-/- embryos to study Fgf8, Wnt1 and Otx2 expression, each of which is critical for MHO positioning, maintenance and activity. Highly sensitive radioactive mRNA in situ hybridization was performed on sagittal paraffin-embedded tissue sections taken from the mesencephalic flexure, parasagittal region of the MHO and from the dorsal (tectal) region of the MHO.

At E9.5, in the mesencephalic flexure of WTs and Lmx1b-/- mutants, Fgf8 was not expressed (Fig 5A and 5A'). Wnt1 expression did not differ between mutants and WTs (Fig 5B and 5B'). As with immunohistochemistry (Fig 2A and 2E), in the ventral midbrain of Lmx1b-/- embryos, Otx2 transcription was reduced (Fig 5C and 5C). In parasagittal and dorsal sections of the

![Fig 3. Otx2 does not require Lmx1b for the induction of OMNs.](image-url)
MHO, Fgf8 as well as Wnt1 expression was detected in Lmx1b<sup>-/-</sup> embryos (Fig 5D, 5D’, 5E, 5E’, 5G, 5G’, 5H and 5H’). While parasagittal expression levels were reduced, dorsal expression in mutants was similar to WTs. In order to determine if the lack of Lmx1b affects the normal positioning of Fgf8 expression at the MHO, we compared the expression domain of this growth factor to the Otx2 expression area on consecutive sections (Fig 5F, 5F’, 5I and 5I’). As seen in WT and also in sections from Lmx1b<sup>-/-</sup> animals, the Fgf8 expression domain was directly caudal to the Otx2 expression domain, suggesting that the decreased Fgf8 signal is properly positioned along the anterior-posterior axis.

At E10.5, sections from Lmx1b<sup>-/-</sup> embryos exhibited a normal Wnt1 expression at the mesencephalic flexure (Fig 5K and 5K’). A short exposure of the slides to autoradiography films, used for the quantification of in situ hybridization experiments, did not show any differences in signal intensity between WTs and mutants. This suggests that the lack of apparent difference between signal strength in WTs and mutants seen on the slides is unlikely caused by an
Fig 5. Wnt1 and Fgf8 expression is induced but not maintained in Lmx1b−/− embryos. Representative sagittal sections of the mesencephalic flexure (A-C, A′-C′, J-L, J′-L′) and the lateral (D-F, D′-F′, M-O, M′-O′) and dorsal (G-I, G′-I′, P-R, P′-R′) aspect of the MHO of E9.5 (A-I, A′-I′) and E10.5 (J-R, J′-R′) WT (A-R) and Lmx1b−/− (A′-R′) embryos. Genes were visualized by radioactive mRNA in situ hybridization. (B, B′) At E9.5 Wnt1 expression is normally induced in the MF of Lmx1b−/−. (D′-E′, G′-H′) At E9.5, Fgf8 and Wnt1 expression are reduced but present in Lmx1b−/− embryos at the lateral and dorsal aspect of the MHO. (C, C′, F, F′, I, I′) Overlay of adjacent sections indicate that Fgf8 is expressed in Lmx1b−/− as in WT directly posterior to the Otx2 expression domain. (K, K′) At E10.5 Wnt1 expression is maintained at the MF of Lmx1b−/−. (M′-N′, P′-Q′) At E10.5, expression of Fgf8 and Wnt1 are lost in the lateral MHO domain, but still present in the dorsal region of the MHO. (L-R, L′-R′) Overlay of adjacent sections indicate that Fgf8 is expressed in Lmx1b−/− as in WT directly posterior to the Wnt1 expression domain. (Scale bar, 250 μm). doi:10.1371/journal.pone.0139697.g005
overexposure of the slides to the dipping solution which would prevent the reaction to be in the linear range. Fgf8 and Wnt1 expression was not detected at the parasagittal region of the MHO (Fig 5M' and 5N'). In contrast, both Fgf8 and Wnt1 dorsal expression domains were present at this embryonic stage (Fig 5P' and 5Q').

In WTs, Wnt1 and Fgf8 expression at the MHO are reciprocally maintained[40]. Wnt1 is expressed in the caudal midbrain, while Fgf8 expression is found directly adjacent, in the rostral hindbrain[29]. In order to test whether the dependency of these two genes are maintained in Lmx1b-/- mutants, we compared their area of expression. Consecutive sections, hybridized with Wnt1 and with Fgf8 probes, indicate that Fgf8 is expressed in WT as well as in mutants directly caudal to the Wnt1 domain (Fig 5L, 5L', 5O, 5O', 5R and 5R'). While the dorsal Fgf8 domain was no longer detected by E11.5 the dorsal Wnt1 domain was lost by E12.5 (data not shown).

Taken together, Lmx1b is required for the maintenance of Fgf8 and Wnt1 in a ventro-dorsal gradient, but not for the induction of these two morphogens.

**Lmx1b-/-** embryos show a specific spatio-temporal loss of En1 and Wnt1 expression

We next followed the expression pattern of genes associated with the formation of mdDA neurons in Lmx1b-/- embryos at E11.5 and E12.5. We employed radioactive in situ hybridization to visualize gene transcripts on series representing the mesencephalic flexure, along the medial to lateral extension. In Lmx1b-/- at E11.5, En1 expression was reduced in the caudomedial domain and absent in the rostrolateral domain (Fig 6A–6C). At E12.5, En1 was absent from the entire mesencephalic flexure (Fig 6J–6L). In Lmx1b-/- at E11.5, Wnt1 expression was significantly reduced, with only a small region of expression visible in the caudomedial region, and virtually absent at E12.5 (Fig 6D–6F', data not shown). Th expression was reduced in Lmx1b-/- mutants at E12.5 (Fig 6P–6R'), and not detected at around E17.5, as previously reported by Smidt et al. (data not shown). Lmx1b-/- embryos did not exhibit any apparent changes in expression of Lmx1a (Fig 6G–6I') or of Nurr1 (Fig 6M–6O') at either age. Taken together, the inactivation of Lmx1b leads to a specific spatio-temporal loss of En1 and Wnt1 expression.

Discussion

Overexpressing Otx2 in the hindbrain leads ventrally to a specific expansion of the mdDA neuronal population

In dorsal brain stem of En1+/Otx2 mutants ectopic expression of Otx2, induces a general expansion of all midbrain structures at the expense of the rostral hindbrain[29]. In contrast, in the ventral brain stem of En1+/Otx2 mutants, Otx2 expression results in a specific expansion of the mdDA neuronal population. The molecular mechanisms mediating these differential effects of Otx2 on the development in dorsal and ventral parts of the brain stem are unknown. The mutual repression of midbrain Otx2 and hindbrain Gbx2 expression is instrumental in defining midbrain versus hindbrain fate[29][41]. Loss of function experiments for Otx2 as well as Gbx2 indicate however that this antagonizing interaction is less apparent in the ventral brain stem [32][42]. As a consequence, overexpression of Otx2 in the dorsal brainstem could lead to a general expansion of all dorsal midbrain structures at the expense of the rostral dorsal hindbrain.

In the ventral brain stem, Otx2 was previously shown to antagonize Nkx2.2, critically defining the border between the mdDA precursor domain and the adjacent serotonergic precursor domain[6]. Together, these data suggest that the general changes described in the present study for the dorsal hindbrain reflect a mutual repression of Otx2 and Gbx2, whereas the specific changes in the ventral hindbrain seem to be based on the antagonism of Otx2 and Nkx2.2.
**Otx2 requires Lmx1b for the development of mdDA neurons**

Otx2 has been shown to play a critical role at different stages of mdDA neuron development [11][21][22][23][32]. The role of Otx2 in the terminal differentiation of mdDA neurons, however, remains poorly understood. Our data provide evidence that Otx2 requires Lmx1b activity to induce NURR1"TH" neurons from immature NURR1"TH" precursors. The molecular pathway by which Lmx1b regulates the final step is as yet unknown. Similar to Lmx1b\(^{-/-}\) mutants, NURR1"TH" neurons fail to fully differentiate to NURR1"TH" mdDA neurons in Foxa1/2 mutants[15], suggesting that Lmx1b could induce the terminal differentiation of mdDA neurons by regulating Foxa2 expression. However, this hypothesis is not supported by our findings since Foxa2 expression is not altered in Lmx1b\(^{-/-}\) mutants. Since Lmx1b is also normally expressed in Foxa2 mutants, it is clear that Lmx1b is also not downstream of Foxa2 in this cascade.

Otx2 and Lmx1b have both been implicated in defining the lateral border of the mdDA precursor field. Thus, overexpressing Otx2 in the En1 expression domain leads to a lateral expansion of the Lmx1b expression area and concomitant mdDA precursor field[21]. In contrast,
Lmx1b<sup>−/−</sup> embryos are characterized by an invasion of POU4F1 positive RN cells into the mdDA precursor domain[24]. Analysis of Lmx1b<sup>−/−</sup> mutants and embryos ectopically expressing Sim1 in the mdDA precursor domain indicates that an antagonism between Lmx1b and Sim1 is important for defining the lateral border of the mdDA neuronal population to that of the RN[24][43]. An antagonism between Otx2 and Sim1 has not been established in this context. Based on the results of our compound mutants, it appears likely that Otx2 modulates the medio-lateral boundary of mdDA nuclei by regulating Lmx1b, which in turn represses Sim1 and the lateral red nucleus identity.

The role of Otx2 and Lmx1b in OMNs formation

In contrast to its effect on mdDA neurons, Otx2 can induce the formation of OMNs independent of Lmx1b. Lmx1b affects the development of OMNs by inducing Phox2a expression and repressing Sim1 transcripts required for formation of the adjacent RN[24]. The conditional inactivation of Otx2 in the En1 domain leads to a hypoplasia of OMNs, indicating that these neurons are dependent upon Otx2[32]. The down regulation of Otx2 in Lmx1b<sup>−/−</sup> embryos and the rescue of OMNs in compound mutants suggest that Lmx1b operates upstream to or independent of Otx2 in the specification of OMNs.

The role of Lmx1b in controlling Fgf8, Wnt1 and En1 expression at the MHO and mdDA progenitor domain

The conditional inactivation of Lmx1b using a Shh-Cre driver results in normal development of mdDA neurons. This indicates that Lmx1b regulates mdDA development not cell autonomously, but via the MHO[25]. Fgf8, Wnt1 and En1 are key elements of the MHO. Moreover, there is a dynamic spatio-temporal requirement of these genes in the development of mdDA neurons. Therefore, it is essential to study their expression in Lmx1b<sup>−/−</sup> embryos in order to understand how Lmx1b directs the formation of mdDA neurons.

Using whole-mount in situ hybridization, it was reported that Fgf8 is not expressed in Lmx1b<sup>−/−</sup> embryos, indicating that Lmx1b is absolutely required for the induction of Fgf8 expression and MHO activity[28]. However, since mdDA neurons are formed in Lmx1b<sup>−/−</sup> mice, their lack of Fgf8 would contradict previous findings that Fgf8 is required for mdDA neuron induction[5]. Our finding that Fgf8 is induced in Lmx1b<sup>−/−</sup> mutants does not support the hypothesis that mdDA neurons can be induced in the absence of Fgf8. It seems possible to us that the discrepancy between our results and previous analysis of Lmx1b<sup>−/−</sup> embryos can be related to the higher sensitivity of radioactive in situ hybridization used in our study.

In Lmx1b<sup>−/−</sup> embryos and in conditional mutants using an En1-Cre driver to inactivate Lmx1b, Wnt1 expression is dramatically reduced at the MHO during early somitogenesis, as well as in the ventral midbrain at E11.5 [24][27][28]. However, it is not known if Lmx1b is required for the induction of Wnt1 at the mdDA precursor domain. Our finding that Wnt1 is expressed normally in the ventral midbrain in Lmx1b<sup>−/−</sup>, indicates that Lmx1b is not necessary for the induction of Wnt1 expression, but rather for its maintenance. Wnt1 has been shown to regulate the expression of Otx2 [6][9]. It is therefore, conceivable that the reduction of OTX2 that we observed at E12.5 in Lmx1b<sup>−/−</sup> is mediated by the loss of Wnt1.

At the MHO of Lmx1b<sup>−/−</sup> mutants, expression of En1 is initially induced but then lost at E9.5 [28]. However, expression of En1 in the mdDA precursor domain in these mutants has not been assessed. Based on the loss of Pitx3 in Lmx1b<sup>−/−</sup>[12] and the fact that Pitx3 represses En1 expression in the rostrolateral domain[44], upregulation of En1 was anticipated in this area in Lmx1b<sup>−/−</sup>. Unexpectedly, we observed downregulation of En1 in the rostrolateral domain of these mutants, suggesting that Lmx1b regulates expression of En1 in a Pitx3 independent
manner. The fact that in Lmx1b−/− mutants NURR1+ neurons, lack En1, in contrast to other genes important for formation of mdDA neurons, including Foxa2 and Lmx1a, suggests that Lmx1b has differential effects on the gene regulator pathways in dopaminergic precursors.

In summary, our study demonstrates that Otx2 is critically dependent on intact Lmx1b gene activity to direct the formation of mdDA neurons, but not for the generation of OMNs. In addition, Lmx1b regulates the spatio-temporal expression of Fgf8, Wnt1 and En1, which are critical for the MHO function and mdDA development.

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Author Contributions
Conceived and designed the experiments: OS LNZ KL CB. Performed the experiments: OS LNZ KL HT VJ KZ. Analyzed the data: OS LNZ KL MMJ CB. Wrote the paper: OS CB.

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