Lmx1a Encodes a Rostral Set of Mesodiencephalic Dopaminergic Neurons Marked by the Wnt/B-Catenin Signaling Activator R-spondin 2


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Abstract

Recent developments in molecular programming of mesodiencephalic dopaminergic (mdDA) neurons have led to the identification of many transcription factors playing a role in mdDA specification. LIM homeodomain transcription factor Lmx1a is essential for chick mdDA development, and for the efficient differentiation of ES-cells towards a dopaminergic phenotype. In this study, we aimed towards a more detailed understanding of the subtle phenotype in Lmx1a-deficient (dreher) mice, by means of gene expression profiling. Transcriptome analysis was performed, to elucidate the exact molecular programming underlying the neuronal deficits after loss of Lmx1a. Subsequent expression analysis on brain sections, confirmed that Nurr1 is regulated by Lmx1a, and additional downstream targets were identified, like Pou4f1, Pbx1, Pitx2, C13002120Rik, Calb2 and Rspo2. In line with a specific, rostral-lateral (prosomer 2/3) loss of expression of most of these genes during development, Nurr1 and C13002120Rik were affected in the SNc of the mature mdDA system. Interestingly, this deficit was marked by the complete loss of the Wnt/b-catenin signaling activator Rspo2 in this domain. Subsequent analysis of Rspo2−/− embryos revealed affected mdDA neurons, partially phenocopying the Lmx1a mutant. To conclude, our study revealed that Lmx1a is essential for a rostral-lateral subset of the mdDA neuronal field, where it might serve a critical function in modulating proliferation and differentiation of mdDA progenitors through the regulation of the Wnt activator Rspo2.

Introduction

Mesodiencephalic dopaminergic (mdDA) neurons function in regulating motor control and emotion related behavior, and loss of these neurons can cause Parkinson’s disease, and other neurological disorders as well. In order to understand the molecular mechanisms causing the selective degeneration of these neurons, insight in the pathways and factors involved in the development and maintenance of this subset of dopaminergic neurons is needed.

The onset of mdDA development is induced by several extrinsic factors such as Shh, Fgf8 and Wnt1 [1–3]. In addition to this early signaling from the organizing centers in the developing CNS, several developmental factors are essential for the subdivision into different domains, and for the differentiation of newborn neurons towards mdDA neurons [3]. Among the factors implicated in mdDA neuron development, are Wnt1, Wnt5a, En1/2, Otx2, Foxa1, Foxa2, Ngn2, Nurr1 (Nrlh2), Pitx3, Mnx1, and the LIM homeodomain transcription factors Lmx1a and Lmx1b [4–14].

Several studies suggested a role for Lmx1a in establishing the mdDA neuronal phenotype [5,13]. Gain-and-loss-of-function studies in chick revealed that Lmx1a is needed for the specification of mdDA neurons. Moreover, Lmx1a can induce mouse embryonic stem cells (ESCs) into DA neurons [5]. In addition, a Wnt/Lmx1a auto-regulatory loop was identified when differentiating ES cells into mdDA neurons [15]. Together, these experiments, in chick and ESCs, suggest an essential role for Lmx1a in the determination of mdDA neurons. Contradictory, studies performed in Lmx1a mutant mice, revealed a subtle phenotype only, with mild reduction of Th and Nurr1, and compromised Wnt-expression [13]. Other studies in Lmx1a mutants also revealed a moderate reduction in a number of (VTA) neurons [16,17]. Besides several studies indicating that Lmx1a functions in proliferation and neurogenesis, the precise role of Lmx1a in the mouse mdDA neuronal system is still not fully understood.

Therefore, to understand the Lmx1a phenotype in depth, we studied the loss of function of Lmx1a in the Dreher mouse, which can be considered as a null mutant [17–19]. From this, we
established that *Lmx1a* is essential for a rostral-lateral part of the developing mdDA system. Furthermore, to elucidate the molecular programming causing this phenotype, we performed microarray analysis on dissected brain material of *Lmx1a*-dr/dr embryos. This revealed several *Lmx1a* target genes, such as *Nurr1, C130021L20Rik* and *Rspo2*. The loss of these genes was region specific, and occurred in a rostral group of mdDA neurons. Subsequent analysis in *Rspo2−/−* embryos revealed a subtly affected mdDA neuronal field, partially resembling the *Lmx1a* phenotype. Altogether, *Lmx1a* is essential for the correct programming of a rostral sub-set of developing mdDA neurons, marked by the *Wnt/b-catenin* signaling activator *Rspo2.

**Materials and Methods**

**Animals**

The *Lmx1a* mutant (strain name B6C3Fe a/a-Lmx1a^h3j/) was obtained from Jackson Laboratory (Bar Harbor, ME), and maintained on a C57Bl/6j background (Charles River). Genotyping was done by PCR using primers; forward 5′-GAGGCCCCCTGGGAG and reverse 5′-GCTACAGGGTG-GACGTCG, resulting in a 236 bp fragment containing the drJ mutation. Due to this drJ point mutation, a HpyCH4-restriction site disappears. Wild-type PCR product is restricted into fragments of 52, 88 and 95 bp. *Lmx1a*-dr/dr product results in 140 and 95 bp fragments. For embryo generation, heterozygous *Lmx1a* mice were crossed, and the day of detection of a copulatory plug was considered E0.5. Used animals were euthanized by CO2 asphyxiation, or decapitation. *Rspo2-LacZ* mutant mice were generously provided by Jeong Kyo Yoon and Yong-Ri Jin [20]. Mice were maintained under standard conditions and all efforts were made to minimize suffering. All procedures were approved to and fully approved by the Dutch Ethical Committee for animal experimentation of the University Medical Center Utrecht (DEC UMC-U, The Netherlands), and the University of Amsterdam (UvA, The Netherlands).

**In Situ Hybridization**

Embryos and postnatal brains were frozen on dry ice. Sections (16 um) were cut and collected on SuperFrost+ slides (Menzel-Glaser). In situ hybridization (ISH) with digoxigenin-labeled RNA probes was performed as described previously [21,22]. The following probes were used: *Th*, a 1142 bp fragment of rat cDNA [23]; *Aadc*, fragment containing bp 22–488 of the mouse coding sequence [22]; *Nurr1*, the 3′-region of rat *Nurr1* transcript; *Ald2*, fragment containing bp 568–1392 of the coding sequence; *Easl*, the 5′-region of the transcript; *Lmx1a*, an 1150 bp fragment containing bp 218–1366 of the coding sequence; *Ptx3*, a 285 bp fragment of the 5′-region of rat *Ptx3* transcript [22]. For microarray post ISH-analysis, cDNA from RNA originating from E12.5 mouse midbrains was used for PCR with gene specific primers (Table S1). The PCR fragments were cloned into pGEM-t-easy and sequenced. Probes were generated by means of T7 or SP6 RNA DIG-labeling according to manufacturer’s protocol (Roche).

**Immunohistochemistry**

Embryos were incubated in 4% para-formaldehyde in PBS at 4°C, followed by cryoprotection in 30% sucrose in PBS, before freezing on dry ice. For immunohistochemistry, sections were washed twice for 5 min in TBS, blocked in 4% fetal calf serum (FCS) or 5% normal donkey serum in TBS for 30 min, and were washed again. Slides were incubated with primary antibody in THZT (50 mM Tris-HCl pH 7.6, 0.5 M NaCl, 0.5% Triton) overnight, washed 3x with TBS for 5 min and incubated 1 h with secondary antibody. Slides were washed three times 10 min in PBS and mounted using FluorSave (Calbiochem). Antibodies used: rabbit anti-TH (Pel-Freez, 1:1000), sheep anti-TH (Millipore, 1:1000), rabbit anti-LMX1A (a kind gift of M. German, UCSF, 1:1000), rabbit anti-PITX3 [9] (1:500) and AHD2 (Abcam, 1:100). Secondary antibodies: goat anti-rabbit Alexa-Fluor-488 and -555, donkey anti-sheep Alexa-Fluor-488, all 1:1000 (Invitrogen).

**Combined In Situ Hybridization/Immunohistochemistry**

ISH on fresh frozen sections was performed as described [21,22]. After this, slides were washed in TBS, incubated in 0.3% H2O2 in TBS for 30 min, washed again, blocked with 4% FCS in TBS for 30 min, washed again and incubated overnight with rabbit anti-TH (Pel-Freez, 1:1000) in TBST (0.05 M Tris-HCl pH 7.4, 0.9% NaCl, 0.5% Triton). Next day, sections were washed in TBS, incubated for 1 h with avidin-biotin-peroxidase reagent mix (ABC Elite kit, Vector Laboratories) in TBST. Slides were washed again, and stained with 3,3′-diaminobenzidine (DAB) until TH regions showed a clear staining (with maximum of 10 min staining). The reaction was ended through washing with water, slides were dehydrated with ethanol and mounted using Entellan (Merck).

**qPCR**

Analysis was performed on a LightCycler 480 II (Roche) using a One Step SYBR green kit (Qiagen) according to the manufacturer’s protocol. Dissected midbrain total RNA (10 ng) was used as a template. Water was used as a non-template control. All samples were normalized to 18 s reference. Table S2 lists primer sets used for qPCR.

**Microarray Analysis**

RNA was isolated from E12.5 dissected midbrains of Lmx1a-dr/dr and Lmx1a+/- embryos, using Trizol according to manufacturer’s protocol (Invitrogen). Each experimental sample consisted of RNA derived from 5 midbrains, pooled and purified on a column, according to protocol (Qiagen, RNeasy mini kit). All RNA samples were analyzed using a 2100 BioAnalyzer (Agilent Technologies) to ensure the quality of the RNA. Microarray analysis was performed on 4 experimental samples, hybridized to a reference pool of RNA derived from 20 Lmx1a+/- midbrains. Microarray analysis was performed as described with slight modifications [24]. Agilent Mouse Whole Genome Gene Expression Microarrays V1 (Agilent Technologies, Belgium) sets were used for all hybridization’s, in 4×4 K Lay-out, covering 41174 Mus musculus 60-mer oligonucleotide probes, representing genes and transcripts. Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30° PMT. After data extraction using ImaGene 8.0 (BioDiscovery), print-tip Loess normalization was performed on mean spot intensities [25]. Data were analyzed using ANOVA [26] (R version 2.2.1/MAANOVA version 0.98–7; http://www.r-project.org/). P-values were determined by a permutation F2 test, in which residuals were shuffled 5000 times globally. Genes with *P<0.05* after family-wise error correction (FWER) (or Benjamini-Hochberg correction/False discovery rate control (FDR)) were considered significantly changed. Details of the microarray data can be viewed at http://www.ncbi.nlm.nih.gov/geo (Gene Expression Omnibus accession number GSE45831).
Results

_Lmx1a_ is Only Expressed during Development

Initial studies showed that _Lmx1a_ expression starts around E8.5–E9 and remains expressed during life, although later studies suggested a decrease in _Lmx1a_ expression already at P12 [27].

In order to elucidate the temporal window of _Lmx1a_ activity, we performed in situ hybridization (ISH) experiments for _Lmx1a_ and _Th_ on adjacent mouse brain sections, collected directly after birth (P0), at P7 and at P14 (See Fig. S1). At P0, _Lmx1a_ expression was found at high levels in the posterior hypothalamus (the P3 tegmentum), retromammillary area, subthalamic nucleus, substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). At P7, _Lmx1a_ transcript levels were clearly decreased in these regions, and at P14, _Lmx1a_ expression was almost completely lost, which was in clear contrast to the retained high level of _Th_ present in the mdDA region. When analyzing the expression in more detail in the different expression areas, the posterior hypothalamic area and retromammillary nucleus displayed a significantly lower expression level, although still present. In the VTA, expression was clearly lower and in the SNc only a few cells remained that express _Lmx1a_ (Fig. S1, arrowheads). To conclude, the drastically lowering of _Lmx1a_ transcript levels in mdDA neurons shortly after birth, suggests that _Lmx1a_ is not involved in adult mdDA neuronal functions.

_Lmx1a_ is Required for Rostral mdDA Neurons

To resolve the mdDA phenotype of the _Lmx1a_ mutant, we characterized the _Lmx1a-dr/dr_ mouse [10]in detail. Therefore, rostral-caudal and medial-lateral mapping by ISH was performed on E12.5 _Lmx1a-dr/dr_ and wild-type sagittal sectioned tissue. Several mdDA markers, and _Lmx1a_ itself were analyzed (Fig. 1). The defect in _Lmx1a_ expression in the medial midbrain was only modest, marking the most caudal part. In contrast, in lateral sections of _Lmx1a-dr/dr_, a clear defect was observed in the diencephalon (P1, P2 and P3; [3,28], where _Lmx1a_ transcript levels were drastically lower (Fig. 1).

We found a subtle loss of _Th_ expression in the medial midbrain (Fig. 1F–G'), which is in line with other studies [13,16,29,30]. Interestingly, in rostral-lateral _Th_ expression domains, a clear defect was observed. In this region _Th_ transcript was almost absent, while in the control still a small set of _Th_ positive cells was observed (Fig. 1F', arrowheads). Also, for _Pitx3_ and _En1_ a small decrease in expression in the most lateral domains was displayed (Fig. 1K–O' and P–T'), although this was more subtle in comparison to the loss of _Th_ expression. Surprisingly, _Aadc_ displayed clear expansion of its rostral expression domain (P3) (Fig. 1U–Y', arrowheads). Since _Aadc_ is suggested to be a marker for early differentiated mdDA neurons, this indicates that in the absence of _Lmx1a_, a rostral expansion of early, _Aadc_ positive neurons might occur.

In order to visualize which mdDA region co-localizes with _Lmx1a_ at E12.5, we created pseudo-overlays of _Lmx1a_ and _Th_ wild-type ISH images (Fig. 1AA EE'). Medially, most _Th_ expressing cells co-expressed _Lmx1a_ (Fig. 1AA' and BB'). However, more laterally _Lmx1a_ was co-expressed with a small set of rostrally located _Th_ expressing neurons only (Fig. 1DD'EE', arrowheads). In contrast to the overlap of medial _Th_ and _Lmx1a_, the loss of _Lmx1a_ in this domain resulted in a very mild loss of _Th_ expressing neurons. However, in the lateral domains, the rostral subset of _Th_ neurons that co-expressed _Lmx1a_, apparently depend on this transcription factor, since this group was severely affected in the _Lmx1a-dr/dr_ mutant.

Figure 1. Phenotype characterization of E12.5 _Lmx1a-dr/dr_ mice. (A–E') Sagittal expression analysis of _Lmx1a_ transcript in medial to lateral brain sections. Laterally, _Lmx1a_ expression is clearly down-regulated in the _Dreher_ homozygous mutant (D',E', arrowheads). (F–J') _Th_ transcript is rostral-laterally decreased (arrowheads), but also medially. (K–O') _Pitx3_ and (P–T') _En1_ display a subtle decreased expression in _Lmx1a-dr/dr_ tissue. (U–Y') _Aadc_ expression is expanded rostrally in the absence of _Lmx1a_ (arrowheads). (AA'–EE') Pseudo-overlays of wild-type _Lmx1a_ (red; generated from A–E) and _Th_ (green; generated from F–J) expression, showing complete overlap in medial sections and only partial overlap in lateral sections (white arrowheads).

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Requirement of _Lmx1a_ in the Rostral mdDA Neuronal Field is Retained During Terminal Differentiation

In order to determine the significance of the initial E12.5 defect, we performed additional analysis on E14.5 _Lmx1a-dr/dr_ and wild-type embryos, and it was revealed that the loss of rostral-lateral...
Figure 2. The observed rostral-lateral phenotype is retained in E14.5 Lmx1a-dr/dr. (AA–JJ) ISH analysis of several mdDA markers in wild-type and Lmx1a-dr/dr tissue. Small arrows indicate the fasciculus retroflexus, as extra anatomical marker for comparing wild-type and mutant sections. (A–F) Lmx1a transcript levels are lowered in Lmx1a-dr/dr (large arrowheads), while between the heterozygous mutant and wild-type control no differences are observed. (G–L) Aadc expression is laterally down-regulated, except for a clear rostral expansion of a group of cells in the diencephalon (G’K’,L’). Medially, Aadc expression is expanded rostrally and dorsally. (M–R) Pitx3 expression defects are found in the rostral-lateral parts were transcript levels are lower (arrowhead). (S–X) A more pronounced defect is observed for Th, which is clearly reduced in lateral sections (arrowheads). (Y–DD) En1 expression is slightly decreased in most lateral domains. (EE–JJ) For Ahd2, the most lateral expression domains are lost while more medial expression domains are unchanged. (KK–KK) IHC analysis of E12.5 sagittal Lmx1a wild-type and knock-out tissue. The initial TH fiber outgrowth (green) appears unaffected in the mutant, since growth patterns towards LMX1A+ cells (red) are almost identical, despite a smaller number of TH+ cells. (LL–OO) Medial to lateral analysis of TH fiber outgrowth and innervation within the striatum, in wild-type and Lmx1a-dr/dr tissue at E14.5, displays slightly fewer TH+ fibers in the knock-out. However, no clear phenotype is observed in direction or organization of the fibers, and they arrive at the expected site in the striatum (OO,OO’, green arrowheads).

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expression was retained in later developmental stages (Fig. 2A–DD).

Since the affected Th expression seemed to be restricted to a rostral-lateral subset of mdDA neurons, we used Ahd2 as a marker for this region [31]. Interestingly, and confirming the Th expression data, the affected rostral-lateral mdDA cell-group displayed a specific loss of Ahd2. At E14.5, the paramedian expression domain of Ahd2 was unaffected (Fig. 2F–H), whilst the most rostral-lateral Ahd2 domain was almost completely devoid of transcript (Fig. 2J). These observations suggest a subset-specific loss of Th and Ahd2 in the rostral-lateral mdDA region. Importantly, not all of these affected cells are devoid of transcript (Fig. 2E–J). These observations suggest a subset-specific loss of Th and Ahd2 in the rostral-lateral mdDA region. Importantly, not all of these affected cells are completely lost, since the mdDA markers Pitx3 and En1 are still present in a part of this rostral-lateral sub-population (Fig. 2P–R and Y–DD).

Most prominent defects were consequently observed in the lateral-rostral part of the mdDA neuronal field. Therefore, possible defects in the Th fiber outgrowth were analyzed. TH immunohistochemistry was performed on E14.5 Lmx1a-dr/dr and wild-type tissue to follow fiber outgrowth in the diencephalic region (Fig. 2K–O). The initial guidance direction of TH fibers seemed unchanged between Lmx1a-dr/dr and control (Fig. 2K, KK). To confirm normal striatal innervation, the arrival of TH bundles was analyzed at E14.5, and no obvious defects were observed (Fig. 2O, OO).

Taken together, Lmx1a-dr/dr shows medially a subtle defect, confirming previous reports [13,16,29]. Lateral, a rostral group of mdDA neurons (SNc) is clearly affected, as was observed for Lmx1a, TH, and Ahd2. Likely, the affected neurons are not completely lost at this stage, since Pitx3 and En1 were less affected, and Ahd2 expression was expanded, in this rostral group.

Identification of Downstream Targets of Lmx1a via Loss-of-Function Microarray Analysis in vivo

To elucidate the change in molecular programming as a result of the loss of Lmx1a, we performed microarray analysis on E12.5 mesodiencephalic and retromamillary tegmental (mdDA +RM) material of Lmx1a-dr/dr, compared to control littermates (Fig. 3A). LMX1A Expression is Restricted to the Rostral mdDA Region during Terminal Differentiation

The detailed ISH mapping of Lmx1a-dr/dr suggests a subset-specific requirement for Lmx1a, in the rostral-lateral domains of the mdDA neuronal field. To determine into more detail this rostral-lateral dependency, we analyzed the protein expression patterns of LMX1A and TH, in several mdDA developmental stages.

At E11.5, E12.0 and E12.5, medial expression of LMX1A protein was broad and fully overlapped the mdDA area (Fig. S2). Strikingly, when analyzing the expression pattern in the lateral TH domain, LMX1A protein was co-expressed in a rostral subset of TH neurons only (Fig. S2 D, G, H, I–J, arrowheads). In caudal-lateral mdDA neurons, a set of TH+ neurons did not express LMX1A (Fig. S2 I–J, asterisks), suggesting that there is a group of mdDA neurons that does not depend on LMX1A, during these developmental stages. Moreover, at E14.5 this restricted expression pattern was even more pronounced (Fig. S2 N–O). Medially, LMX1A was co-expressed in a small and select set of rostral TH+ neurons, whilst in the caudal mdDA area, no LMX1A expression was detected. Importantly, in lateral domains at this stage, TH expression largely co-localized with LMX1A protein (Fig. S2 P), suggesting a functional relationship in this area and developmental stage.

Taken together, during early mdDA differentiation, LMX1A is broadly present in TH+ cells. During terminal differentiation, LMX1A expression becomes restricted towards a subset of rostral (P3) and lateral TH+ neurons. Notably, this restricted co-localization underlines the observed phenotype in Lmx1a-dr/dr, where Th, and Ahd2 are clearly affected in similar areas.

Figure 3. Genes regulated by Lmx1a in a microarray analysis of E12.5 Lmx1a-dr/dr embryos. (A) RNA was collected from E12.5 micro-dissected Lmx1a-dr/dr and wt/wt brains. Lmx1a-dr/dr samples were hybridized against wt/wt pooled RNA control. The heatmap represents up- (red) and down- (green) regulated genes based on log2-ratios of four individual microarray samples. Only the top 20 significantly up- and down-regulated genes are shown. (B) Relative expression levels of the 20 most up- and (C) down-regulated genes (microarray FWER ANOVA, p<0.05). With Rspos as most down-regulated gene, and Usf1 as highest up-regulated gene. (D) Venn-diagrams showing a number of genes that are also regulated in Pitx3 and Nurr1 microarrays derived from previous studies. (E) qPCR validation of significant down-regulation of Rspos, Nurr1, Calb2, C130021l20Rik, Lmx1a, Cbn1 and Pitx2, and of significant up-regulation of Pitx1 and Pou4f1, in Lmx1a-deficient embryonic midbrains. 18s was used for normalization. Mean expression values in wt are set at 1 (red line) and are indicated with standard error bars (SEM). Grey bars represent mean expression changes in Lmx1a-dr/dr samples compared to wt samples. Statistical analysis was performed with Student’s t-test. *P<0.05 is considered significant; **P<0.01. N=4 for all analyzed genes and for each phenotype (each experimental sample (n) represents a pool of five micro-dissected midbrains). M, midbrain; MHB, mid-hindbrain border; R, rostral; C, caudal; wt, wild-type.

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Microarray (ANOVA) analysis resulted in a total of 143 significantly regulated genes, of which 98 genes were down-regulated and 45 were up-regulated. Importantly, Nur1 (Nr4a2) and Th are among the 20 most down-regulated genes (Fig. 3A–B), confirming our phenotypic analysis. Interestingly, also the Lmx1a transcript level was 35% reduced. Furthermore, the most down-regulated gene was Rspo2, which transcript levels were reduced to 43% of wild-type levels. Among the 20 most up-regulated genes, U3f1 was strongest up-regulated to 180% of wild-type levels (Fig. 3C). In addition, Pbx1 was highly up-regulated, and interestingly, the red nucleus (RN) neuronal marker Pwdf1 (Bm3a) was up-regulated as well, suggesting a suppressive effect of Lmx1a on this alternative RN fate. For subsequent analysis, a selection was made among all significantly regulated genes based on expression, and literature related to mdDA neurons (Phlda1; Brunol4/6; Mga5; Nur1; C13002120Rik; Th; Calb2; Pbx1), high fold-change (Rspo2) and migration (Dab1) (Table S3). In addition, Pitx2 and Cbln1 were selected based on their regulation by Nur1 and Ptx3 respectively (Fig. 3D) [32,33]. In order to validate our microarray data first, we subjected the RNA samples to qPCR analysis (Fig. 3E). Despite the previously observed subtle decrease of Th expression in vivo and in the microarray analysis, we could not confirm the down-regulation of this gene with the used qPCR method, and neither of Phlda1, Brunol4, Mga5/6 and Dab1. Importantly, we confirmed clear down-regulation of Rspo2, Nur1, Calb2, C13002120Rik, Lmx1a, Cbln1 and Pitx2. Furthermore, also the up-regulation of Pbx1 and Pwdf1 was confirmed.

**Lmx1a activates Nur1, Rspo2, Calb2 and C13002120RIK**

In order to identify the spatiotemporal regulation of the selected genes by Lmx1a, we performed ISH analysis in Lmx1a-dr/dr and wild-type mice at E12.5.

Small changes in the expression profile as a consequence of the loss of Lmx1a, were displayed for Phlda1, Brunol4/6, Cbln1, Mga5/6 and Dab1 (Fig. 3F). Mga5/6 displayed only subtle reduction of expression, in the lateral-caudal midbrain. For Phlda1, Brunol4/6, Dab1 and Cbln1, expression was slightly diminished, mainly in rostral-lateral expression domains, and the latter two genes also displayed decreased expression in the lateral-caudal midbrain. Importantly, the other selected genes displayed a more striking loss of expression. At E12.5, the anterior midbrain/P1/P2/P3 plus RM area has lost Nur1 expression in Lmx1a-dr/dr mice (Fig. 4M–P’, arrowheads). A large part of this domain was outside the mdDA system, located in the RM hypothalamic area, however, also a rostral part of the mdDA neuronal field was affected. In addition, the remaining Nur1 expression in the mdDA area was reduced as well (Fig. 4N–O’). At E14.5, a similar phenotype was observed (Fig. 4Q–T’). Medially, the overall levels of Nur1 were slightly affected in Lmx1a-dr/dr. Laterally, the anterior segment was severely affected and in the most lateral mdDA domain, Nur1 expression was almost absent (Fig. 4S–T’; Fig. 3F). Interestingly, comparable deficits were observed for Ptx2 (Fig. 4Y–BB’), C13002120Rik (Fig. 4L’–L’’), Calb2 (Fig. 4E–H’’) and Rspo2 (Fig. 4A–D’). C13002120Rik showed a decrease in the anterior-medial brain area. Furthermore, the transcript was almost completely abolished in the RM hypothalamic area (Fig. 4K–L’’). Calb2 expression was widely affected in the Lmx1a-dr/dr. Medially, levels were significantly decreased (Fig. 4E–F’), and laterally almost all Calb2 expression was lost (Fig. 4G–H’’). For Rspo2, a marked decrease in expression was observed. In medial brain areas, the number of Rspo2 expressing cells was significantly lower, mainly in the rostral part (P3) (Fig. 4A–B’). In addition, in the lateral domains, the anterior domain was clearly reduced.

**Figure 4. Validation of several Lmx1a target genes in Lmx1a-dr/dr embryos.** (A–D’) ISH showing that Rspo2 is drastically down-regulated in sagittal E12.5 Lmx1a-dr/dr tissue (arrowheads). (E–H’) Calb2 expression is almost completely lost (arrowheads). (I–L’) C13002120Rik is almost completely lost in lateral sections (arrowheads). (M–P’) Similarly, Nur1 expression is strongly down-regulated in rostral-lateral
areas (arrowheads). (Q–T) At E14.5, similar defects are observed; Nurr1 expression is clearly decreased in the rostral expression domain, specifically in lateral sections (arrowheads). (U–X) Pax6 expression is slightly decreased in the most rostral-lateral domain. However, expansion of Pax6 expression is displayed in the rostral-medial area and dorsal to the medial mdDA region (in the red nucleus area). (Y–BB) Pitx2 expression (blue) level is down-regulated in rostral-medial areas. In the rostral-lateral domain, Pitx2 expression is more clearly affected, as is displayed by a smaller Pitx2 positive cell group. TH protein staining (brown) was taken along as a reference. doi:10.1371/journal.pone.0074049.g004

Taken together, the genes discussed above are all influenced by Lmx1a activity in vivo and the most marked deficits were observed for Nurr1, C130021L20Rik, Calb2 and Rspo2. These findings strongly suggest that these genes are direct or indirect downstream targets of Lmx1a. Moreover, the Lmx1a dependency is region-specific and seems most severe for the rostral-lateral expression domains (P3 and the RM hypothalamic area).

Calb2 Expression is Selectively Affected in the Adult Medial-rostral SNc and Medial-dorsal VTA

During development, Calb2 was severely affected in the absence of Lmx1a, not only in the rostral-lateral areas but also in the medial midbrain domain. To assess whether this loss is maintained in the mature mdDA system, we performed ISH analysis on adult coronal Lmx1a-dr/dr and wild-type material (Fig. 5).

In the mature midbrain, Calb2 was highly expressed medially in the most rostral domain of the diencephalon, located mostly between the rostral SNc groups, with a slight enrichment of Calb2 expression around the fasciculus retroflexus (fr) and the mammil-logetmental tract (mtg). Interestingly, in Lmx1a-dr/dr sections, Calb2 expression was drastically reduced in this area (Fig. 5A,A',H,H'). Furthermore, in the medial dorsal VTA, a Calb2 positive group clearly being expressed in the wild-type brain, was almost completely lost in the absence of Lmx1a (Fig. 5A–E,I–J'). The loss of Calb2 expression seemed selective since in the more caudal-ventral VTA, close to the nucleus paranigralis (PN), two Calb2 expressing cell-groups were clearly observed in both wild-type and Lmx1a-dr/dr tissue, with no visible change in expression levels (Fig. 5F,F',K,K'). Additionally, more dorsal of the medial mdDA system, located directly ventral of the aqueduct, in the periaqueductal gray, Calb2 positive cells were observed in wild-type tissue. In this same area, a number of TH positive neurons was present as well, and part of these neurons co-expressed Calb2 (Fig. 5L,M,N, arrowheads). Interestingly, in the absence of Lmx1a, a drastic decrease in Calb2 expression was shown, together with a clear decrease in TH positive neurons; in Lmx1a-dr/dr tissue, a clear decrease in Calb2/TH co-expressing neurons was observed (Fig. 5L',M',N', arrowheads).

In conclusion, the domain-specific reduction of Calb2 at selective sites in the adult midbrain, hint towards a region-specific dependency on Lmx1a, during development.

Early Failure of Rspo2, Nurr1 and C130021L20Rik Marks the Loss of Rostral-lateral Positioned Adult mdDA Neurons

Th, Nurr1, Rspo2, and C130021L20Rik were all drastically down-regulated in Lmx1a-dr/dr tissue during development, mainly in the rostral-lateral mdDA neuronal region and in retromammillary areas of the hypothalamus. In order to assess the consequences of this early expression aberration, we analyzed the mdDA neuronal

Figure 5. Calb2 expression is selectively affected in adult Lmx1a-dr/dr. (A–G') Calb2 mRNA expression (blue) combined with TH protein IHC staining (brown). Rostrally, Calb2 expression is drastically lower in the medial midbrain (A,A', arrowheads) and (H–H'). In the medidial VTA a Calb2 positive group is observed in the wild-type, whilst in the...
knock-out, this expression is almost completely lost (C–E’, arrowheads) and (I–J’). In other regions, Calb2 expression appears unaffected. In the medial and caudal VTA, small Calb2 expressing cell groups are identified in wild-type, that appear unchanged in knock-out tissue (F,F’) and (K–K’). (L–N’) Calb2 expression ventrally of the aqueduct, in the periaqueductal gray, is clearly decreased in the absence of Lmx1a. A subset of TH expressing neurons co-express Calb2 and most of these cells are lost in Lmx1a-dr/dr (arrowheads). SNc, Substantia nigra pars compacta; VTA, ventral tegmental area; RRF, retrorubral field; CLI, central linear nucleus; fr, fasciculus retroflexus; mtg, mammilotegmental tract; PN, nucleus paranigralis; R, rostral; C, caudal.

To conclude, Rspo2 expression is specifically localized within mdDA neurons, we performed combined ISH/IHC for Rspo2 transcript and TH protein (Fig. S6). Intriguingly, most cells expressing Rspo2 were observed in the SNc. Importantly, all Rspo2 positive cells in this area co-expressed TH protein (Fig. S6, arrowheads). In addition to some hypothalamic expression in non-dA neurons (data not shown), Rspo2 was in the midbrain uniquely expressed in a subset of mdDA neurons in the VTA and SNc. Interestingly, Rspo2 expression was enriched in the rostral-lateral domains of the SNc (Fig. S6 A’–D’, arrowheads), areas that are clearly affected in Lmx1a mutants.

To conclude, Rspo2 is expressed in a subset of mdDA neurons, which is lost in Lmx1a-dr/dr mutants. The loss of Rspo2 expression in a small number of VTA neurons, and a larger number of SNc neurons, underlines the TH phenotype in these areas, suggesting that in the adult stage these specific mdDA neurons not only lose TH expression, but are lost completely as a consequence of mdDA identity loss.

A Subset of Developing mdDA Neurons is Affected in Embryonic Rspo2 knock-out Mice

To examine if the mdDA phenotype observed in Lmx1a-dr embryos, might be caused by the loss of Rspo2, we analyzed several mdDA markers in E14.5 sagittal midbrain sections of Rspo2-LacZ knock-out mice [20], compared to littermate controls.

TH protein expression analysis revealed a small decrease (65% of wt, data not shown) in TH+ cells in the rostral/lateral mdDA neuronal field (Fig. 7A–G’,V’–V’,Y’–Y’). Furthermore, in these affected areas, BGAL expression was observed, suggesting that the loss of TH expression overlaps with the position of otherwise Rspo2 positive neurons (Fig. 7A’–G’). Similarly, a small but clear decrease of PITX3+ neurons was observed, suggesting that the affected neurons lose their mdDA identity (Fig. 7H–N’,W–W’,Z–Z’). In line with this, AHD2 expression analysis revealed a comparably decreased expression domain (Fig. 7O–U’,X–X’,AA–AA’). This might indicate that two situations occur in the absence of Rspo2; possible loss of neurons as presented by the subtle loss of TH and PITX3 expressing cells, and in addition loss of mdDA coding as shown by affected AHD2 expression in these areas. Remaining BGAL staining and normal Dapi-staining suggest that no massive cell loss occurred in the affected region.

The identification of Nurr1 and Rspo2 as down-stream targets of Lmx1a, made it tempting to hypothesize that one of these factors is maybe regulated by the other. We therefore investigated Rspo2 and Nurr1 expression in E14.5 Nurr1 and Rspo2 knock-out brains, respectively. Rspo2 was normally expressed in the absence of Nurr1.
During development, one of the domains that arises adjacent to the mdDA neuronal field, is the red nucleus (RN). And in several studies, it was observed that Lmx1a can repress Pou4f1, and in the absence of Lmx1a, an up-regulation of Pou4f1 was observed [16,29]. Confirming this, in our microarray analysis Pou4f1 was found in the top of significantly up-regulated genes, and displayed more than two-fold up-regulation with qPCR (Fig. 3). This regulation might mark or even influence the neuronal programing towards a RN phenotype instead of a DA phenotype (Fig. 8). Furthermore, we observed an expansion of Pdx1 towards the mdDA neighboring neuronal fields (Fig. 4), further suggesting changes in neuronal identities in these developing domains due to lack of repression by Lmx1a.

Importantly, we revealed that the Lmx1a-regulated genes Nurr1, Calb2, C130021L20Rik and Rspo2 are drastically down-regulated outside and in the mdDA area, in Lmx1a-dr/dr embryos (Fig. 8). Moreover, a clear deficit of Th, Nurr1, C130021L20Rik and Rspo2 expression was identified in the adult rostral-lateral SNc, whereas Calb2 expression was affected in medial domains, mostly adjacent to the mdDA system, with only minor defects in TH expression in this area. Interestingly, Calb2 (Calretinin) is a calcium binding protein expressed in GABAergic neurons and it can be considered as a marker for early neuronal differentiation [38,39]. The observed loss of Calb2 expression in Lmx1a-dr/dr, suggests that these cells have a changed calcium homeostasis and therefore lack the proper programming which should normally be present in the Calb2 expressing subset of mdDA neurons. Despite a drastic decrease in Calb2 expression in the Lmx1a-dr/dr embryonic brain, the consequences for the fully developed mdDA system seem only modest, since few TH positive cells are lost, in small and selective domains of the medial mdDA area only.

The Lmx1a-regulated gene C130021L20Rik is a large intervening non-coding RNA molecule (lincRNA). It was discovered recently that many of these lincRNA’s are transcriptionally regulated and moreover, are involved in regulation of gene transcription [40]. Interestingly, the open reading frame of C130021L20Rik is located adjacent to the open reading frame of Lmx1b, and the developmental and adult expression pattern is very similar to the expression pattern of Lmx1b (and Lmx1a).

In the absence of Lmx1a, Nurr1 expression is drastically down-regulated, in the anterior midbrain/P1/P2/P3 plus RM domains. Together with the high fold-change down-regulation of its transcript levels in the microarray study and qPCR analysis, this strongly indicates that this key mdDA factor is a downstream target of Lmx1a. Therefore, the subtle defects found for Th in the developing mdDA system in the absence of Lmx1a, are probably a consequence of affected Nurr1 expression (Fig. 8).

**Lmx1a Regulates Wnt Modulator Rspo2 in a Subset of mdDA Neurons**

Like Nurr1, the gene Rspo2 was severely affected due to the absence of Lmx1a, during development and in adult mdDA neurons. Rspo2 belongs to the group of R-spondins, a family of secreted proteins that activate Wnt/b-catenin signaling [41–43]. In mice, from E8.5 all four known Rspo’s are expressed, displaying a complex expression pattern throughout the whole embryonic body [20]. Rspo2-null mice die immediately after birth because of respiratory failure, and embryos display defects in the limbs (distal limb loss), craniofacial structures (cleft palate and mild facial skeletal defects), lung hypoplasia and pulmonary vascular defects [44–46]. Interestingly, it was shown that all four R-spondin members regulate Wnt signaling at the level of the Fizzled1 and Lrp6 receptors [47,48]. Another study reported that Rspo2 modulates Wnt signaling in mouse mammary epithelial cells, and
that Rspo2 and Wnt1 act synergistically in the b-catenin pathway [49]. Rspo's can stabilize the level of cytosolic b-catenin and synergize with Wnt-ligands in order to promote b-catenin transcriptional activity [47,50,51]. Intriguingly, Wnt1 is important for proliferation and patterning of the mdDA neuronal field [52], and several papers suggested a role of Lmx1a together with Wnt1 in mdDA differentiation. Recently, a novel Wnt1-Lmx1a auto-regulatory loop was identified during mdDA differentiation of mouse ES cells [15]. Moreover, it was described that the reduced number of mdDA progenitors in Lmx1a-dr/dr and Lmx1a/Lmx1b double mutants may be a consequence of proliferation defects and an increase in cell cycle exit of the progenitors [16]. Also, their data indicate that Wnt1 expression was slightly reduced in Lmx1a-dr/dr, and specifically lost in the Lmx1a/Lmx1b double mutant, suggesting that both genes specifically and redundantly regulate Wnt1 expression in the mdDA domain [16]. Since Rspo2 acts in the Wnt1/b-catenin signaling pathway, a reduction in Rspo2 expression might influence the end result of Wnt activity. We speculate that the lack of Rspo2 protein in the affected neurons of the Lmx1a-dr/dr mutant might induce the previously suggested early cell-cycle exit and premature differentiation. In line with this, in the Lmx1a-dr/dr mutant we observed in the current study a clear expansion of Aadc expression, which might represent an expansion of early mdDA neurons. However, it may also display an induction of other monoaminergic neuronal phenotypes in this area.

Additionally, when comparing the loss of mdDA neurons (or a loss of mdDA identity) between the Lmx1a and the Rspo2 mutant, we found that the observed defects are partially identical between both mutants, where the terminal differentiation markers Th and Pitx3 showed the same subtle reduced expression. It is therefore likely that part of the Lmx1a-phenotype is caused by the loss of downstream target Rspo2, affecting a number of mdDA neurons positioned in the VTA and more prominently in the SNc.

Conclusions

We have shown that Lmx1a is essential for a rostral-lateral subset of developing mdDA neurons, and loss of Lmx1a resulted in affected Nurr1 expression, but also in affected expression of Nurr1 transcriptional targets, mainly in rostral-lateral mdDA neurons. Rspo2 is involved in the Wnt1/b-catenin signaling pathways, which is implicated in proliferation and cell-cycle exit. Loss of Lmx1a, resulted in decreased Rspo2 expression and loss of mdDA neuronal markers as Th, Ahd2 and Pitx3. Interestingly, loss of Rspo2 partially phenocopied the defects observed in the Lmx1a-dr/dr mdDA system.

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Figure 8. A model integrating several identified targets of Lmx1a. Pou4f1 (Brn3a) was strongly up-regulated in the Lmx1a−/− expression array, in line with a suggested role of Lmx1a in repressing an alternative, red nucleus, fate during development (dashed line). Lmx1a targets C13002120Rik and Calb2 require Lmx1a directly or indirectly for correct expression in subsets of the mdDA system. The Nurr1 transcriptional pathway induces differentiating mdDA neurons, and loss of Lmx1a resulted in affected Nurr1 expression, but also in affected expression of Nurr1 transcriptional targets, mainly in rostral-lateral mdDA neurons. Rspo2 is involved in the Wnt1/b-catenin signaling pathways, which is implicated in proliferation and cell-cycle exit. Loss of Lmx1a, resulted in decreased Rspo2 expression and loss of mdDA neuronal markers as Th, Ahd2 and Pitx3. Interestingly, loss of Rspo2 partially phenocopied the defects observed in the Lmx1a-dr/dr mdDA system.

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Lmx1a Encodes Dopaminergic Subsets
**Supporting Information**

**Figure S1**  *Lmx1a* expression is down-regulated shortly after birth. Coronal sections of wild-type mouse tissue at P0, P7 and P14. In situ hybridization of *Lmx1a* and *Th* is shown from rostral to caudal. *Th* was taken along to mark the SNc and VTA and as a control for transcript levels. (A–F) A significant loss of *Lmx1a* expression is observed in the SNc at P14, when compared to P7 or P0, and when compared to *Th*. (G–R) Both in caudal SNc, and more caudally, the VTA, the expression of *Lmx1a* is clearly diminished at P14 (arrowheads). *P*, postnatal day; *SnC*, Substantia nigra pars compacta; *VTA*, ventral tegmental area. (TIF)

**Figure S2** LMX1A protein expression is restricted to rostral-lateral *TH* expression at later developmental stages. (A–H) IHC on E11.5 (A–D) and E12.0 (E–H) sagittal wild-type mouse tissue, showing LMX1A protein (red) and *TH* protein (green). Medially, full protein overlap is displayed, whereas in lateral expression domains, only a subset of *TH*+ neurons overlaps with LMX1A. (I–J) Higher magnifications of G and H, showing the group of *TH*+ neurons that co-localize with LMX1A (white arrowheads), and a group of *TH* expressing neurons that do not express LMX1A (green asterisks). (K–M) The observed rostral-lateral overlap is more clear at E12.5, where also in the medial brain, a rostral specificity of LMX1A occurs. (N–P) At E14.5, the lateral LMX1A/*TH* restriction is clearly observed in medial and lateral midbrain areas. (TIF)

**Figure S3** *Lmx1a* regulates *Phlda1*, *Brunol4*, *Brunol6*, *Cbh1*, *Mgat5b* and *Dab1* in selective areas of the Lmx1a-expression domain. Sagittal *Lmx1a* control and *Lmx1a-dr/dr* sections, medial and lateral. On the right, higher magnifications of the boxed areas are shown. (A–E′) *Phlda1* expression is slightly reduced in rostral parts of the brain (arrowheads). (F–O′) *Brunol4* and *Brunol6* both show a slight down-regulation in rostral-lateral domains (G,G′, and arrowheads). (P–T′) *Cbh1* expression is diminished mainly in rostral areas. (U–Y′) *Mgat5b* transcript levels are slightly reduced in the lateral-caudal midbrain (arrowheads). (Z–ZD′) *Dab1* shows loss of expression in medial-rostral areas (Z′,AA′,DD′, arrowheads). Also lateral-caudal, expression is affected (CC′, arrowhead). (TIF)

**Figure S4** *Lmx1a* and *Nurr1* expression throughout the sagittal brain at E14.5. *Lmx1a* expression (left columns) from lateral (L) to medial (M) to lateral brain domains, in wild-type and *Lmx1a-dr/dr* tissue, at E14.5. In lateral domains, *Lmx1a* expression is down-regulated in the *Lmx1a* knock-out, and in all sections throughout the brain, a rostral defect is shown. *Nurr1* expression (right columns) was analyzed in the same set-up. For *Nurr1* a drastic decrease in rostral and lateral expression can be observed (arrowheads). (TIF)

**Figure S5** *Lmx1a* and *Rspo2* expression throughout the sagittal brain at E12.5. *Lmx1a* expression (left columns) from lateral (L) to medial (M) to lateral brain domains, in wild-type and *Lmx1a-dr/dr* tissue, at E12.5. In lateral positions, *Lmx1a* expression is clearly down-regulated in the *Lmx1a* knock-out, and in all sections, a rostral defect can be seen. *Rspo2* expression (right columns) was analyzed in the same set-up. For *Rspo2* an even more drastic decrease in rostral and lateral expression can be observed (arrowheads). (TIF)

**Figure S6** *Rspo2* co-localizes with *TH* in adult mdDA neurons. (A–C) Combined ISH/IHC for *Rspo2* (blue) and *TH* (brown). Most *Rspo2*-positive cells are found in the rostral and lateral mdDA system, in the SNc (A′,B′,C′, arrowheads) and also some cells expressing *Rspo2* are observed in the VTA (A′,B′,C′). All *Rspo2*-positive cells also express *TH*, indicating that *Rspo2* is expressed in a subset of mdDA neurons (D). (TIF)

**Table S1** List of primers used for the generation of in situ hybridization probes. (XLS)

**Table S2** List of primers used for qPCR. (XLS)

**Table S3** A selection of genes regulated in E12.5 *Lmx1a-dr/dr* mouse embryos. (PDF)

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**Author Contributions**

Conceived and designed the experiments: EJH MPS. Performed the experiments: EJH LsO LvIH JvV Wk Bw AjAvL. Analyzed the data: EJH MPS. Wrote the paper: EJH MPS. Carried out microarray analysis: FCPH MJG-K. Contributed Rspo2 mouse material: Y-RJ JKY.

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