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**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*.

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 ESCs 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 \textit{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 \textit{Lmx1a-dr/dr} embryos. This revealed several \textit{Lmx1a} target genes, such as \textit{Nurr1}, \textit{CI36021120Rik} and \textit{Rspo2}. The loss of these genes was region specific, and occurred in a rostral group of mdDA neurons. Subsequent analysis in \textit{Rspo2}--/-- embryos revealed a subtly affected mdDA neuronal field, partially resembling the \textit{Lmx1a} phenotype. Altogether, \textit{Lmx1a} is essential for the correct programming of a rostral subset of developing mdDA neurons, marked by the \textit{Wnt/b-catenin} signaling activator \textit{Rspo2}.

Materials and Methods

Animals

The \textit{Lmx1a} mutant (strain name B6C3Fe a/a-Lmx1a\textsuperscript{dr/+}) 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'-CACAAGGGCCCGTTGGGAG and reverse 5'-GCCATACGAGTGAGACTTGC, resulting in a 236 bp fragment containing the \text{d}J mutation. Due to this \text{d}J point mutation, a HpyCH4V-restriction site disappears. Wild-type PCR product is restricted into fragments of 52, 88 and 95 bp. \textit{Lmx1a-dr/dr} product results in 140 and 95 bp fragments. For embryo generation, heterozygous \textit{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. \textit{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 by the Dutch Ethical Committee for animal experimentation.

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-Freeze, 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).

Materials and Methods

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In Situ Hybridization

Embryos and postnatal brains were frozen on dry ice. Sections (16 um) were cut and collected on SuperFrost\textsuperscript{+} slides (Menzel-Glaser). In situ hybridization (ISH) with digoxigenin-labeled RNA probes was performed as described previously [21,22]. The following probes were used: \textit{Th}, a 1142 bp fragment of rat cDNA [23]; \textit{Aadc}, fragment containing bp 22–488 of the mouse coding sequence [22]; \textit{Nurr1}, the 3’-region of rat \textit{Nurr1} transcript; \textit{Ahd2}, fragment containing bp 568–1392 of the coding sequence; \textit{En1}, the 5’-region of the transcript; \textit{Lmx1a}, an 1150 bp fragment containing bp 218–1366 of the coding sequence; \textit{Pitx3}, a 285 bp fragment of the 5’-region of rat \textit{Pitx3} transcript [22]. For microarray post ISH-analysis, cDNA from RNA originating from E14.5 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 3 x 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-Freeze, 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).


Microarray Analysis

RNA was isolated from E12.5 dissected midbrains of \textit{Lmx1a-dr/dr} and \textit{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 \textit{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 \textit{P}<0.05 after family-wise error correction (FWER) (or Benjamini-Hochberg correction/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 E9.5–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 [18] 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. 1A–E, arrowheads).

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–J, 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. Lateral, 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) transcript 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 where 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 Abhd2 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 Abhd2. At E14.5, the paramedian expression domain of Abhd2 was unaffected (Fig. 2F–F>H>H’), whilst the most rostral-lateral Abhd2 domain was almost completely devoid of transcript (Fig. 2 EE,,JJ’, arrowheads). These observations suggest a subset-specific loss of Th and Abhd2 in the rostral-lateral mdDA region. Importantly, not all of these affected cells are devoid of transcript (Fig. 2EE–JJ)

whilst the most rostral-lateral expression domain of Ahd2 was unaffected (Fig. 2F–F’), arrowheads). The initial guidance direction of TH fibers seemed unchanged between Lmx1a-dr/dr and control (Fig. 2KK, KK’ and LL–NN’). To confirm normal striatal innervation, the arrival of TH bundles was analyzed at E14.5, and no obvious defects were observed (Fig. 2O0O0’).

Taken together, Lmx1a-dr/dr shows medially a subtle defect, confirming previous reports [13, 16, 29]. Laterally, a rostral group of mdDA neurons (SNc) is clearly affected, as was observed for Lmx1a, Th, and Abhd2. Likely, the affected neurons are not completely lost at this stage, since 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. 2KK–OO’). The initial guidance direction of TH fibers seemed unchanged between Lmx1a-dr/dr and control (Fig. 2KK, KK’ and LL–NN’). To confirm normal striatal innervation, the arrival of TH bundles was analyzed at E14.5, and no obvious defects were observed (Fig. 2O0O0’).

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 retromammillary tegmental (mdDA +RM) material of Lmx1a-dr/dr, compared to control littermates (Fig. 3A).

**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 Rspo2 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 Rspo2, Nurr1, Calb2, C1300210Rik, Lmx1a, Cbln1 and Pitx2, and of significant up-regulation of Pbx1 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 (s.e.m.). Grey bars represent mean expression changes of Lmx1a-dr/dr 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. doi:10.1371/journal.pone.0074049.g003

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, H1–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 Abhd2 are clearly affected in similar areas.
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, Nurr1 (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, Ush1 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 Pou4f1 (Bmi3a) 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; Mga5h; Nurr1; C13002120Rik; Th; Calb2; Pbx1), high fold-change (Rspo2) and migration (Dab1) (Table S3). In addition, Pits2 and Cbhl1 were selected based on their regulation by Nurr1 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, Brunol6, Mga5h and Dab1. Importantly, we confirmed clear down-regulation of Rspo2, Nurr1, Calb2, C13002120Rik, Lmx1a, Cbhl1 and Ptx2. Furthermore, also the up-regulation of Pbx1 and Pou4f1 was confirmed.

**Lmx1a activates Nurr1, 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, Cbhl1, Mga5h and Dab1 (Fig. S3). Mga5h displayed only subtle reduction of expression, in the lateral-caudal midbrain. For Phlda1, Brunol4/6, Dab1 and Cbhl1, 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 Nurr1 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 Nurr1 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 Nurr1 were slightly affected in Lmx1a-dr/dr. Laterally, the anterior segment was severely affected and in the most lateral mdDA domain, Nurr1 expression was almost absent (Fig. 4S–T; Fig. S4). Interestingly, comparable deficits were observed for Pbx2 (Fig. 4Y–BB'), C13002120Rik (Fig. 4L'), 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, Nurr1 expression is strongly down-regulated in rostral-lateral](image)
areas (arrowheads). (Q–T) At E14.5, similar defects are observed; NurrI expression is clearly decreased in the rostral expression domain, specifically in lateral sections (arrowheads). (U–X) Pbx1 expression is slightly decreased in the most rostral-lateral domain. However, expansion of Pbx1 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.

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(Fig. 4C–D), and in the most lateral Rsps2 expression domains, completely lost (Fig. S5).

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 Rsps2. 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-loegmental 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. 5C–E,J–J').

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 Rsps2, Nurr1 and C130021L20Rik Marks the Loss of Rostral-lateral Positioned Adult mdDA Neurons**

TH, Nurr1, Rsps2, 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 medial VTA a dorsal 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 periadequeductal gray, is clearly decreased in the absence of Lmx1a. A subset of TH expressing neurons co-expresses 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; CL, central linear nucleus; fr, fasciculus retroflexus; mtg, mammillotegmental tract; PN, nucleus paranigralis; R, rostral; C, caudal.

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’). Furthermore, in these affected areas, GAL 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. 5). This regulation might mark or even influence the neuronal programming towards a RN phenotype instead of a DA phenotype (Fig. 8). Furthermore, we observed an expansion of Pou4f1 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, C130021120Rik and Rpo2 are drastically down-regulated outside and in the mdDA area, in Lmx1a-dr/dr embryos (Fig. 8). Moreover, a clear deficit of Th, Nurr1, C130021120Rik and Rpo2 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 GABA-ergic 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 C130021120Rik 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 C130021120Rik 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).

### Discussion

**Novel Targets of Lmx1a Implicated in Neuronal Differentiation and Identity**

Many studies have suggested an essential role for Lmx1a in mdDA neuronal development. This was shown by loss-and-gain-of-function studies, in chick embryos [5], and by analyzing markers in the Lmx1a mutant (Lmx1a-dr/dr) [13, 16]. In addition, the essential role of Lmx1a in dopaminergic differentiation of stem cells was shown [5, 15, 34–37]. In literature, it is suggested that Lmx1a plays a role in proliferation and neurogenesis, but despite the indication that several factors are regulated by Lmx1a, like Nurr1, Msi1 and Wnt1, the precise role of Lmx1a in the mouse is still not clearly identified. In order to unravel the exact molecular programming activated by Lmx1a, we performed an in vivo transcriptome analysis by using Lmx1a-dr/dr embryonic mdDA plus RM brain areas.

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### Lmx1a Regulates Wnt Modulator Rpo2 in a Subset of mdDA Neurons

Like Nurr1, the gene Rpo2 was severely affected due to the absence of Lmx1a, during development and in adult mdDA neurons. Rpo2 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]. Rpo2-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 Fz4/Fzd4 and Lrp6 receptors [47, 48]. Another study reported that Rpo2 modulates Wnt signaling in mouse mammary epithelial cells, and
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 Rsps acts in the Wnt/b-catenin signaling pathway, a reduction in Rsps expression might influence the end result of Wnt activity. We speculate that the lack of Rsps 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 a clear expansion of Ahd2 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 Rsps 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 down-stream target Rsps, 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. Rsps is involved in the Wnt/b-catenin signaling pathways, which is implicated in proliferation and cell-cycle exit. Loss of Lmx1a, resulted in decreased Rsps expression and loss of mdDA neuronal markers as Th, Ahd2 and Pitx3. Interestingly, loss of Rsps partially phenocopied the defects observed in the Lmx1a-dr/dr mdDA system.
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. (A′,C′,E′) Lmx1a expression in the SNc in more detail, showing that only few cells remain that express Lmx1a, in low levels. (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.

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 LMX1A/TH restriction is clearly observed in medial and lateral midbrain areas.

Figure S3 Lmx1a regulates Phidal, Brunol4, Brunol6, Cbhl1, Mgat5b and Dab1 in selective areas of the Lmx1a expression domain. Sagittal Lmx1a control and Lmx1a-dr/d mouse sections, medial and lateral. On the right, higher magnifications of the boxed areas are shown. (A–E′) Phidal 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) Cbhl1 expression is diminished mainly in rostral areas. (U–Y′) Mgat5b transcript levels are slightly reduced in the lateral-caudal midbrain (arrowheads). (Z–DD′) Dab1 shows loss of expression in medial-rostral areas (Z′,AA′,DD′, arrowheads). Also lateral-caudal, expression is affected (CC′, arrowhead).

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/d 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).

Figure S5 Lmx1a and Repo2 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/d 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. Repo2 expression (right columns) was analyzed in the same set-up. For Repo2 an even more drastic decrease in rostral and lateral expression can be observed (arrowheads).

Figure S6 Repo2 co-localizes with TH in adult mdDA neurons. (A–C) Combined ISH/IHC for Repo2 (blue) and TH (brown). Most Repo2-positive cells are found in the rostral and lateral mdDA system, in the SNc (A′,B′,C′, arrowheads) and also some cells expressing Repo2 are observed in the VTA (A′,B′,C′). All Repo2-positive cells also express TH, indicating that Repo2 is expressed in a subset of mdDA neurons (D).

Table S1 List of primers used for the generation of in situ hybridization probes.

Table S2 List of primers used for qPCR.

Table S3 A selection of genes regulated in E12.5 Lmx1a-dr/d mouse embryos.

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Author Contributions
Conceived and designed the experiments: EJH MPS. Performed the experiments: EJH LsO LvdH JWV WK BJ AvdL. Analyzed the data: EJH MPS. Wrote the paper: EJH MPS. Carried out microarray analysis annotations and statistical analysis: FCPH MJG-K. Contributed Rspo2 mouse material: Y–RJ JKY.

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