Identification of Dlk1, Ptpru and Klhl1 as novel Nurr1 target genes in meso-diencephalic dopamine neurons

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The orphan nuclear receptor Nurr1 is essential for the development of meso-diencephalic dopamine (mdDA) neurons and is required, together with the homeobox transcription factor Pitx3, for the expression of genes involved in dopamine metabolism. In order to elucidate the molecular mechanisms that underlie the neuronal deficits in Nurr1–/– mice, we performed combined gene expression microarrays and ChiP-on-chip analysis and thereby identified Dlk1, Ptpru and Klhl1 as novel Nurr1 target genes in vivo. In line with the previously described cooperativity between Nurr1 and Pitx3, we show that the expression of Ptpru and Klhl1 in mdDA neurons is also dependent on Pitx3. Furthermore, we demonstrate that Nurr1 interacts with the Ptpru promoter directly and requires Pitx3 for full expression of Ptpru in mdDA neurons. By contrast, the expression of Dlk1 is maintained in Pitx3–/– embryos and is even expanded into the rostral part of the mdDA area, suggesting a unique position of Dlk1 in the Nurr1 and Pitx3 transcriptional cascades. Expression analysis in Dlk1–/– embryos reveals that Dlk1 is required to prevent premature expression of Dat in mdDA neuronal precursors as part of the multifaceted process of mdDA neuronal differentiation driven by Nurr1 and Pitx3. Taken together, the involvement of Nurr1 and Pitx3 in the expression of novel target genes involved in important neuronal processes such as neuronal patterning, axon outgrowth and terminal differentiation, opens up new avenues to study the properties of mdDA neurons during development and in neuronal pathology as observed in Parkinson’s disease.

KEY WORDS: Developmental pathways, Dopamine, mdDA, Midbrain, Terminal differentiation, Mouse

INTRODUCTION

The orphan nuclear receptor Nurr1 (Nr4a2) was first discovered by Law et al. (Law et al., 1992) and was found to be expressed in the meso-diencephalic dopaminergic (mdDA) neurons of the substantia nigra (SNc) and ventral tegmental area (VTA) during developmental and adult stages (Zetterström et al., 1996). The first proof of a critical role for Nurr1 in the development of mdDA neurons was provided through analysis of Nurr1–null mouse embryos, which completely lack expression of tyrosine hydroxylase (Th) during development (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998). Subsequent studies in Nurr1-null embryos added Dat, SLC6a3, Vmat2, Slc18a2, Aude, Ddc and Ahd2 (Adil1a1) to the list of genes that are dependent on Nurr1 for developmental expression in mdDA neurons (Wallen et al., 1999; Smits et al., 2003; Jacobs et al., 2009). Interestingly, other important developmental regulators in mdDA neurons, such as the engrailed genes En1/2, Foxa2, Lmx1b and Pitx3, were still expressed in Nurr1-null embryos, pointing to the existence of multiple parallel pathways that act together to build a healthy mdDA population (Saucedo-Cardenas et al., 1998; Smidt et al., 2000; Wallen et al., 1999; Smits et al., 2003).

Despite the observed gene expression deficits, the generation and distribution of mdDA neuronal progenitors to their ventral positions within the mdDA area are largely unaffected in Nurr1–/– embryos (Castillo et al., 1998; Witta et al., 2000; Simon et al., 2003; Smits et al., 2003). However, defects in neuronal migration, patterning and axonal outgrowth of mdDA neurons in Nurr1–/– embryos have been reported (Wallen et al., 1999; Tornqvist et al., 2002). The most dramatic aspect of the Nurr1 phenotype is the progressive loss of mdDA neurons during late developmental stages of Nurr1–/– embryos. Apart from a small, scattered population in the medial part of the midbrain, the vast majority of mdDA neurons are lost in newborn mice (Saucedo-Cardenas et al., 1998; Castillo et al., 1998; Wallen et al., 1999). The mechanistic basis for the observed cellular defects in Nurr1–null embryos remains largely unknown. Interestingly, mice deficient for any of the known Nurr1 targets do not exhibit developmental defects in axonal outgrowth, neuronal migration or neuronal survival during development, similar to what was observed for Nurr1–null mice (known Nurr1 targets: Vmat2 (Mosslehner et al., 2001; Colebrooke et al., 2006); Dat (Giros et al., 1996); D2R (Dr2) (Baik et al., 1995); Th (Zhou and Palmiter, 1995); c-Ret (Jain et al., 2006; Li et al., 2006; Kramer et al., 2007)). This suggests that Nurr1 is required for the expression of other, yet to be identified factors that could be elementary to the multifaceted role of Nurr1 in mdDA neurons. We aimed to identify novel target genes of Nurr1 during terminal differentiation of mdDA neurons. Through microarray analysis of Nurr1–/– mouse embryos combined with in vivo ChiP-on-chip analysis and gene expression microarrays on Nurr1-overexpressing MN9D cells, we identified three novel Nurr1 target genes, Dlk1, Ptpru and Klhl1, that fail to be expressed in the mdDA area of Nurr1–/– embryos. Our results show that in addition to Nurr1, Pitx3 also regulates their expression, underlining the extensive cooperation between Nurr1 and Pitx3 in mdDA neurons. Intriguingly, we demonstrate that embryos deficient for Dlk1 display deficits in the expression of Dat, suggesting a role for Dlk1 in mdDA neuronal differentiation. Taken together, by linking the neuronal pathology in Nurr1- and Pitx3-deficient embryos to genes involved in neuronal processes such as axon formation, neuronal patterning and terminal differentiation, we have opened up new avenues to study the complex developmental mechanism that underlies the generation of a healthy mdDA neuronal population.
MATERIALS AND METHODS

Cell culture
MN9D-Nurr1+/+ or MN9D (MN9D) cells were cultured as described previously (Castro et al., 2001; Jacobs et al., 2007).

Animals
E14.5 Pits3−/−, Pits3+/+ and Nurr1−/− and Nurr1+/+ mouse embryos were obtained as described previously (Jacobs et al., 2009). Genotypes were determined by PCR as described previously (Saucedo-Cardenas et al., 1998). Heterozygous Pits3+/− and Pits3-deficient Pits3−/− embryos were obtained and genotyped as described previously (Jacobs et al., 2009).

Microarray analysis
RNA was isolated from dissected ventral midbrains of E14.5 Nurr1+/+ and Nurr1−/− embryos or from MN9D cells using Trizol according to the supplied protocol (Invitrogen). Microarray analysis was performed in triplicate and for each experimental sample a dye swap was performed to correct for dye effects. Each experimental sample consisted of pooled RNA derived from three Nurr1+/+ ventral midbrains, which was hybridized to a reference pool consisting of RNA derived from ten Nurr1−/− ventral midbrains. Microarray analysis was performed as described (Roepman et al., 2005). Custom arrays containing mouse 70-mer oligos (Opgen, Mouse V2 AROS) spotted onto CodeLink Activated Slides (Surnodics) were used for the hybridizations (ArrayExpress, A-UMCU-7 spotted according to protocol P-UMCU-34). Hybridized slides were scanned on an Agilent scanner (G2565AA) at 100% laser power, 30% PMT. After data extraction using ImaGene 8.0 (BioDiscovery), print-tip Loess normalization was performed on mean spot intensities. Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7; http://www.r-project.org/) (Wu et al., 2002). In a fixed effect analysis, sample, array and dye effects were modeled. 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 (or Benjamini-Hochberg correction) were considered significantly changed. ArrayExpress accession: E-TABM-711.

Semi-quantitative RT-PCR
RNA was isolated using Trizol according to manufacturer’s protocol (Invitrogen). Semi-quantitative RT-PCR was performed on equal amounts of RNA (0.1 ng) using a one-step RT-PCR Kit (Quagen) according to the supplied protocol. The following primers were used: Ptp4r, 5′-ACCTCTGCGCGCTGTCTAC-3′ and 5′-GCCAGCTCTGACGAGAATG-3′; Klhl1, 5′-AGGAGGTATGAGCAGGATG-3′ and 5′-GGGCTATAGGCCTCCTCCTCTG-3′; Dlk1, 5′-GACATCTGAACGATGGTCG-3′ and 5′-AGACACTGAAGCTCACCCTG-3′; Grb10, 5′-CCATTACAGAAGACGAGATG-3′ and 5′-TCATCGGATTTGGCA ACTATG-3′; Cart, 5′-TGCCTGCTACTGCTACCTTTG-3′ and 5′-CAGGTTCAGGGAATTG-3′; Bnn45, 5′-AGTACACCGAGTCACGCAGCAGG-3′ and 5′-GTCAGTACGAGGATGATTGT-3′; Rab3c, 5′-CTTATCTTGAGCAGAAGTCTGCAATTTG-3′ and 5′-ATAAACCCTGGGCATATCCACCCA-3′; Tbp, 5′-GAGAAATAGAAAGACCGGACG-3′ and 5′-TCATACGACGTCCTCCCCAC-3′. The amount of amplified DNA was determined by densitometry on a FLA5000 multi-imaging system (Fujifilm). Statistical analysis was performed by two-way unpaired Student’s t-test, comparing the relative transcript levels of sodium butyrate-treated and untreated cultures.

Western blotting
Proteins were separated on NuPAGE 4-12% gradient gels (Invitrogen), transferred to Hybond-C extra (Amersham), the membranes blocked overnight in 5% milk powder in PBS at 4°C and then incubated with Nurr1 antibody (Santa Cruz E20) in PBS-T [PBS + 0.05% (v/v) Tween 20]. Blots were incubated with SuperSignal West Dura Extended Duration Substrate (Pierce) and exposed to ECL films (Pierce).

Chip-on-chip analysis
Chromatin immunoprecipitation (ChIP) was performed as described previously (Jacobs et al., 2009) according to the protocol supplied by NimbleGen. Briefly, five ventral midbrains from C57BL6-Jco E14.5 embryos were used for each ChIP using ChIP-grade Nurr1 antibodies (Santa Cruz E20). ChIP DNA was amplified using a Whole Genome Amplification Kit according to the manufacturer’s protocol (Sigma) and used for ChIP-on-chip analysis (NimbleGen), in which Cy5-labeled Pits3 and Nurr1 ChIP samples were hybridized to Cy3-labeled input chromatin on a mouse promoter two-array set (MM8). Microarray data were analyzed using SignalMap software (version 1.9, NimbleGen).

In situ hybridization (ISH)
ISH was performed as described previously (Smits et al., 2003; Smidt et al., 2004). The following digoxigenin (DIG)-labeled probes were used: Tcf7l2, bp 366-716 of the mouse cDNA (NM_009333); Th, 1142 bp of rat cDNA; Vmat2, bp 290-799 of mouse coding sequence (eds) (Smits et al., 2003); Dat, bp 789-1153 of rat cds; Nurr1, 3′ region of rat Nurr1; Enl, 5′ region of mouse transcript; Ahhd2, 586-1392 of mouse cds. Probes for Dlk1, Klhl1, Ptp4r, Grb10, Rab3c, Bnn45 and Cart were synthesized using the primers used for the semi-quantitative RT-PCR (see above).

Tissue culture
Ventral midbrains of Pits3−/− and Pits3+/+ E13.5 embryos were dissected in L15 medium (Gibco) and cultured in Neurobasal Medium (Gibco) supplemented with 2% (v/v) B-27 supplement (Gibco), 18 mM HEPES-KOH (pH 7.5), 0.5 mM l-glutamine, 26 μM B-mercaptoethanol and 100 units/ml penicillin/streptomycin. Tissue cultures were treated with (0.6 mM) or without sodium butyrate (Sigma) for 48 hours.

FACS sorting
Cultured ventral midbrains were dissociated using a Papain Dissociation System (Worthington) and cells were sorted on a Cytopeia Influx cell sorter. Sorted gates were set on forward scatter versus side scatter (live cell gate), on forward scatter versus pulse width (elimination of clumps) and on forward scatter versus fluorescence channel 1 (528/38 filter, GFP fluorescence). Cells were sorted using a 100-μm nozzle at a pressure of 15 PSI with an average speed of 7000 cells/second.

RESULTS

Identification of Nurr1-regulated genes by Nurr1 loss-of-function analysis
In order to identify novel target genes of Nurr1 in mdDA neurons we performed in vivo expression analysis on E14.5 Nurr1−/− mouse embryos. Based on the detection of truncated Nurr1 transcripts in Nurr1−/− embryos, mdDA progenitors adopted their ventral position in the ventral midbrain mantle layer normally, whereas minor alterations in the rostrolateral population of mdDA neurons were observed (Fig. 1A). This confirms that the vast majority of mdDA progenitors were properly generated and distributed to build the mdDA neuronal field. Microarray analysis (Nurr1−/− against Nurr1+/+) revealed that a total of 116 genes were significantly downregulated in Nurr1−/− embryonic midbrains (see Table S1 in the supplementary material). Several known Nurr1 target genes involved in dopamine (DA) metabolism, including Dat, Ahhd2, Th, Vmat2 and Aadc, were found amongst the 15 most-downregulated genes (Table 1 and Fig. 1B). As expected, the transcript levels of Enl1/2 and Foxa2 were unchanged (Fig. 1C). In Nurr1−/− embryos there was an at least 25% reduction in the transcript levels of Lhx2, Ptp4r, Tcf7l2, Kitl, Ptpor, Klhl1, Tmed8 and Dlk1 (Fig. 1C). Furthermore, the microarray analysis revealed a total of 139 upregulated genes in Nurr1−/− embryos (see Table S2 in the supplementary material). The greatest change was observed for a developmentally regulated transcript named Bnn45, followed by an additional 11 transcripts that were upregulated by at least 25% in Nurr1−/− embryos (Fig. 1D). Most of the differentially expressed transcripts in Nurr1−/− embryos are encoded by genes with no known association with Nurr1 or with mdDA neurons. For further analysis, additional lines of evidence would be beneficial to aid the selection of candidate genes that are likely to represent true Nurr1 target genes.
Novel target genes of Nurr1 and Pitx3

In vivo ChIP-on-chip analysis reveals binding of Nurr1 to promoters of Nurr1-regulated genes

The identification of genes that are potentially under direct transcriptional control of Nurr1 is important in order to obtain a more detailed view of the hierarchical composition of the Nurr1 downstream cascade. We performed in vivo ChIP-on-chip analysis, combining chromatin immunoprecipitation (ChIP) with microarray analysis designed to identify transcription factor-bound promoters in a relatively unbiased manner (Jacobs et al., 2006). ChIP for endogenous Nurr1 protein-DNA complexes was performed on dissected tissue harboring the mdDA neurons from E14.5 C57BL6 embryos (Fig. 2A), and linearly amplified ChIP DNA fragments were hybridized to control input DNA (Fig. 2B) on an MM8 two-set promoter array. Nurr1 specifically enriched 208 promoters with a false discovery rate (FDR) of less than 0.01 (see Table S3 in the supplementary material) as described previously (Jacobs et al., 2009). Of special interest were those genes identified as Nurr1-regulated genes in Nurr1+/− mdDA neurons by the in vivo microarray analysis (Fig. 2C and Table 2). Specific enrichment by ChIP for Nurr1 was observed for the promoters of 13 genes that were significantly up- or downregulated in Nurr1+/− embryos, including the previously reported Nurr1 target gene Vmat2 (Jacobs et al., 2009) (Fig. 2D). Of special interest was the enrichment for the promoters of Ptpnu and Tcfl2 because they were in the top ten most-downregulated genes in vivo expression array. The ChIP-on-chip data indicate that a number of Nurr1-regulated genes are bound by Nurr1 at their promoter region in vivo, which is suggestive of direct transcriptional regulation by Nurr1.

Nurr1 gain-of-function analysis in MN9D cells

A second approach might provide additional evidence for the selection of genes that can be considered truly Nurr1 regulated in DA neurons. We analyzed the effects of Nurr1 overexpression in DA MN9D-Nurr1tet on13N cells, which are derived from embryonic ventral midbrain tissue and modified to overexpress Nurr1 upon treatment with doxycyclin (Fig. 3A). In agreement with previous studies (Castro et al., 2001), microarray analysis revealed that Vmat2 was significantly upregulated in MN9D cells by Nurr1 overexpression. Out of the set of 255 genes that showed significant changes in the in vivo expression analysis of Nurr1+/− embryos, 33 transcripts were also differentially expressed upon Nurr1 overexpression in MN9D cells (Table 3). Interestingly, the transcript levels of Ptpnu, Dlk1 and Tcfl2, which were all within the top 15 most-downregulated genes in vivo, were upregulated by Nurr1 overexpression in MN9D cells (Fig. 3B). Similarly, Gfr10, which was upregulated in Nurr1+/− embryos, was downregulated by Nurr1 overexpression in MN9D cells. The

Table 1. The most-downregulated genes in E14.5 Nurr1−/− mouse embryos

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Log2 ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dat</td>
<td>Dopamine transporter (Slc6a3)</td>
<td>−1.77</td>
<td>0.01</td>
</tr>
<tr>
<td>Ahd2</td>
<td>Aldehyde dehydrogenase family 1 member A1 (Aldh1a1)</td>
<td>−1.71</td>
<td>0.02</td>
</tr>
<tr>
<td>Th</td>
<td>Tyrosine hydroxylase</td>
<td>−1.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Vmat2</td>
<td>Vesicular amine transporter 2 (Slc18a2)</td>
<td>−0.98</td>
<td>0.03</td>
</tr>
<tr>
<td>Lhx2</td>
<td>LIM homeobox protein 2</td>
<td>−0.76</td>
<td>0.04</td>
</tr>
<tr>
<td>Aadc</td>
<td>Aromatic-L-amino-acid decarboxylase (Ddc)</td>
<td>−0.70</td>
<td>0.01</td>
</tr>
<tr>
<td>Ptpru</td>
<td>Protein tyrosine phosphatase, receptor type, U isoform 1</td>
<td>−0.70</td>
<td>0.00</td>
</tr>
<tr>
<td>Tcfl2</td>
<td>Transcription factor 7-like 2 (Tcf4)</td>
<td>−0.57</td>
<td>0.01</td>
</tr>
<tr>
<td>Kitl</td>
<td>c-Kit ligand</td>
<td>−0.52</td>
<td>0.02</td>
</tr>
<tr>
<td>Ptprd</td>
<td>Protein-tyrosine phosphatase</td>
<td>−0.49</td>
<td>0.00</td>
</tr>
<tr>
<td>Khli1</td>
<td>Kelch-like protein 1</td>
<td>−0.47</td>
<td>0.00</td>
</tr>
<tr>
<td>Tmed8</td>
<td>Transmembrane emp24 domain containing 8</td>
<td>−0.47</td>
<td>0.02</td>
</tr>
<tr>
<td>Dlk1</td>
<td>Delta-like 1 protein (Pref-1; FA1)</td>
<td>−0.43</td>
<td>0.01</td>
</tr>
<tr>
<td>Avar1a</td>
<td>Vasopressin V1a receptor</td>
<td>−0.40</td>
<td>0.04</td>
</tr>
<tr>
<td>Ccna2</td>
<td>Cyclin A2</td>
<td>−0.38</td>
<td>0.03</td>
</tr>
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</table>
observation that these transcripts were reciprocally regulated upon Nurr1 ablation and overexpression strongly suggests a regulatory effect of Nurr1 on these genes.

Expression of potential Nurr1-regulated genes in embryonic and adult mdDA neurons

Thus far, in vivo expression microarray analysis revealed 255 differentially expressed genes between Nurr1+/+ and Nurr1–/– embryos. Thirty-three transcripts out of this set were also differentially expressed in MN9D cells upon Nurr1 overexpression and displayed a dual dependence on Nurr1 for expression in DA neurons. Furthermore, 13 genes out of the set of deregulated transcripts in Nurr1–/– embryos were identified by ChIP-on-chip analysis as bound by endogenous Nurr1 in their promoter region. Based on these independent datasets, the following genes were selected for further analysis. Ptpru and Tcf7l2 were downregulated in Nurr1–/– embryos, showed significant enrichment by ChIP for Nurr1 and were upregulated in MN9D cells by Nurr1 overexpression. Similarly, Grb10 and Rab3c were upregulated in Nurr1–/– embryos, identified by ChIP-on-chip and were differentially expressed upon Nurr1 overexpression. Dlk1 and Klhl1 were amongst the 15 most-downregulated genes in Nurr1–/– embryos.

Table 2. ChIP-on-chip identifies Nurr1-bound promoters of Nurr1-regulated genes in vivo

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Peak distance to TSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vmat2</td>
<td>Vesicular amine transporter 2 (Slc18a2)</td>
<td>–0.7</td>
</tr>
<tr>
<td>Ptpru</td>
<td>Protein tyrosine phosphatase U</td>
<td>0.5</td>
</tr>
<tr>
<td>Tcf7l2</td>
<td>Transcription factor 7-like 2 (Tcf4)</td>
<td>–1.9</td>
</tr>
<tr>
<td>Sirt2</td>
<td>NAD-dependent deacetylase sirtuin 2</td>
<td>0.4</td>
</tr>
<tr>
<td>Atp1a3</td>
<td>Na(+)/K(+) ATPase alpha-3 subunit</td>
<td>–0.9</td>
</tr>
<tr>
<td>4930546H06Rik</td>
<td>Adult male testis cDNA</td>
<td>–1.7</td>
</tr>
<tr>
<td>Tmem29</td>
<td>Transmembrane protein 29</td>
<td>0.3</td>
</tr>
<tr>
<td>Srrp</td>
<td>Signal recognition particle receptor subunit alpha</td>
<td>–0.8</td>
</tr>
<tr>
<td>Rab3c</td>
<td>Ras-related protein 3C</td>
<td>0.0</td>
</tr>
<tr>
<td>Zfp146</td>
<td>Zinc-finger protein 146</td>
<td>0.4</td>
</tr>
<tr>
<td>Grb10</td>
<td>Growth factor receptor-bound protein 10</td>
<td>–2.1</td>
</tr>
<tr>
<td>Atf5</td>
<td>Activating transcription factor 5</td>
<td>1.3</td>
</tr>
<tr>
<td>Cart</td>
<td>Cocaine and amphetamine-regulated transcript</td>
<td>–2.4</td>
</tr>
</tbody>
</table>

TSS, transcription start site.
embryos, and although no evidence was found for binding of Nurr1 to their promoters, Dlk1 was upregulated by Nurr1 in MN9D cells. Cart (Cartpt) was upregulated in Nurr1−/− embryos and was significantly enriched by ChIP for Nurr1. Finally, whereas Rbm45 was not identified by ChIP for Nurr1, nor was it differentially expressed upon Nurr1 overexpression in MN9D cells, Rbm45 was selected for its high level of upregulation in Nurr1−/− embryos.

To determine whether the selected genes were expressed in the embryonic mdDA area, we performed ISH analysis on sagittal sections of wild-type (WT) E14.5 embryos (Fig. 4A-I). As discussed above, Nurr1 was detected in the mdDA area and also in the hypothalamic nuclei located anterior to the mdDA neuronal field (Fig. 4A). Dlk1 was expressed in the mdDA area but was clearly restricted to the caudal domain in these medial sections (Fig. 4B). A remarkably similar expression pattern was observed for Ptpru, which was also restricted to the caudal part of the mdDA area (Fig. 4C).

Notably, the expression pattern of Klhl1 closely resembled that of Nurr1 at E14.5 (Fig. 4D), both being expressed in the mdDA area and in anterior neural structures such as the hypothalamus. Tcf7l2 (Fig. 4E), Grb10 (Fig. 4F) and Rbm45 (Fig. 4I) were not detected in the mdDA area by ISH, and expression of Cart was only detected in a region dorsal to the mdDA area (Fig. 4H). Finally, Rab3c was clearly detected in the mdDA area, largely coinciding with the expression domain of Nurr1, and also in the region rostral to the mdDA area (Fig. 4G).

Although the observed expression in the mdDA domain for some of the selected genes is suggestive of expression in mdDA neurons, ISH by itself is no proof of this. Therefore, we analyzed the expression of the selected genes in FACS-sorted mdDA neurons derived from the ventral midbrain of E14.5 Pitx3gfp/+ transgenic mice by RT-PCR (Fig. 4J). In agreement with what was suggested by ISH, a high level of Dlk1, Ptpru, Klhl1 and Rab3c was detected in FACS-sorted Pitx3gfp/+ mdDA neurons. Whereas no clear expression in the ventral midbrain was observed for Tcf7l2, Grb10 and Rbm45 by ISH, their transcripts were detected by RT-PCR, indicating that these genes are expressed in mdDA neurons but with transcript levels too low for detection by ISH. However, in agreement with the ISH data, Cart expression was not detected in Pitx3-positive mdDA neurons.

Next we analyzed the expression of the selected genes in coronal sections of the adult midbrain. Expression of Nurr1 in mdDA neurons of the SNc and VTA was maintained in the adult (Fig. 5A).
Furthermore, Dlk1 was still robustly expressed in the adult mdDA area (Fig. 5C) and its expression pattern was remarkably similar to that of Th (Fig. 5B). Ptpru was only detected in the ventral part of the SNc and its overall expression level was low (Fig. 5D). In agreement with what was observed in the embryonic midbrain, the expression pattern of Klhl1 resembled that of Nurr1 in both the SNc and VTA regions of the adult midbrain (Fig. 5E). A similar pattern was observed for Rab3c (Fig. 5F). A very low signal was detected for Grb10 in the adult mdDA system, and no expression was observed for Tcf7l2, Cart, and Rbm45 (data not shown).

**Expression of Dlk1, Ptpru and Klhl1 is affected in Nurr1<sup>−/−</sup> embryos**

Most of the selected potential Nurr1-regulated genes were expressed in the embryonic mdDA area. These observations made it most interesting to analyze the expression of this set of genes in the mdDA region of Nurr1<sup>−/−</sup> embryos. The expression of truncated Nurr1 transcripts was used to demonstrate the presence of mdDA precursors in the Nurr1-deficient mdDA area (Fig. 6A, J). In agreement with the in vivo microarray analysis on Nurr1<sup>−/−</sup> embryos, the expression of Dlk1 was substantially reduced in the mdDA area in Nurr1<sup>−/−</sup> embryos (Fig. 6B, J) and was only retained in a selective population of neurons along the midline (Fig. 6J). Nurr1 deficiency specifically affected the mdDA neuronal population, as the expression of Dlk1 in other structures in the brain was unaffected. Similarly, Ptpru expression was absent in the mdDA area of Nurr1<sup>−/−</sup> embryos, whereas Ptpru expression outside the mdDA area was unaffected (Fig. 6C, K). Also, the expression of Klhl1 was clearly reduced in the Nurr1-null mdDA area (Fig. 6D, L). Although Klhl1 expression was not completely absent, there was a significant decrease in expression in the area where the Th-positive neurons would normally reside. Importantly, Klhl1 expression in the Nurr1-positive hypothalamic nuclei was unaffected by Nurr1 deficiency. Although the microarray analysis suggested altered transcript levels in Nurr1<sup>−/−</sup> embryos, the expression of Tcf7l2 (Fig. 6E), Cart (Fig. 6F), Rab3c (Fig. 6G) and Grb10 (Fig. 6H) appeared to be unchanged in Nurr1<sup>−/−</sup> versus Nurr1<sup>+/+</sup> embryos. It should be noted that we cannot exclude the possibility that there are small changes in expression that are not detectable by the ISH method used. Altogether, the in vivo expression analysis revealed severe deficits in the expression of Ptpru, Klhl1 and Dlk1 in the mdDA area of Nurr1<sup>−/−</sup> embryos, indicating that they represent novel Nurr1 target genes.

**Pttx3 deficiency affects expression of Dlk1, Ptpru and Klhl1**

Previously, we have shown that Pttx3 potentiates Nurr1 in mdDA neurons, which translates to severe expression deficits of Nurr1 target genes in Pttx3-deficient mdDA neurons (Jacobs et al., 2009). The identification of novel Nurr1 target genes in mdDA neurons prompted us to determine whether Pttx3 deficiency also affects the expression of these genes in the mdDA area. We analyzed the expression of Ptpru, Klhl1 and Dlk1 in Pttx3<sup>−/−</sup> embryos at E14.5. As described previously (Jacobs et al., 2009), the expression of Nurr1 in Pttx3<sup>−/−</sup> embryos was undistinguishable from that in WT and could therefore be used to show the presence of mdDA neurons in Pttx3<sup>−/−</sup> embryos (Fig. 7A). Interestingly, the expression of Ptpru and Klhl1 was dramatically reduced in Pttx3<sup>−/−</sup> embryos (Fig. 7B, C). Similar to what was previously observed for other Nurr1 target genes, Ptpru and Klhl1 expression was still detected in the mdDA area of Pttx3<sup>−/−</sup> embryos, but was significantly reduced compared with WT. However, in contrast to what was observed for Ptpru, Klhl1 and other Nurr1 target genes in Pttx3<sup>−/−</sup> embryos (Jacobs et al., 2009), Dlk1 expression was still abundant in the mdDA area of Pttx3<sup>−/−</sup> embryos (Fig. 7D). Strikingly, whereas Dlk1 expression in WT embryos was confined to the caudal part of the mdDA area, the expression domain of Dlk1 in Pttx3<sup>−/−</sup> embryos was even expanded into anterior positions within the mdDA area (Fig. 7D, insets).
Novel target genes of Nurr1 and Pitx3

We have shown previously that Pitx3 potentiates Nurr1 by inducing the release of the nuclear receptor co-repressor SMRT (Ncor2) from the Nurr1 transcriptional complex, thereby activating Nurr1 target gene expression (Jacobs et al., 2009). The requirement for Pitx3 in driving Nurr1 target gene expression could be bypassed by interfering with the SMRT/HDAC-mediated repression of Nurr1 transcriptional activity. Treatment of Pitx3-deficient cultured ventral midbrains with the HDAC inhibitor sodium butyrate (SB) restored the expression of Nurr1 target genes in the absence of Pitx3 (Fig. 7E). We tested whether the proposed mechanism of Pitx3/Nurr1 cooperativity is also valid for the expression of Ptpru and Klhl1 in mdDA neurons. We cultured ventral midbrains of E13.5 Pitx3<sup>3/3<sup> mutant and Pitx3<sup>3/3<sup> mutant embryos with SB or control diluent to release the SMRT/HDAC-mediated repression of the Nurr1 transcriptional complex. Semi-quantitative RT-PCR on FACs-sorted mdDA neurons revealed that treatment with SB did not affect the expression of Tcf7l2 in heterozygous Pitx3<sup>3/3<sup> and Pitx3<sup>3/3<sup> embryos (Fig. 7F). Furthermore, SB treatment did not affect the level of Ptpru transcripts in FACs-sorted Pitx3<sup>3/3<sup> mdDA neurons, indicating that in the presence of Pitx3, Nurr1-mediated expression of Ptpru is not repressed by SMRT/HDAC complexes (Fig. 7F,G; n=4). In agreement with what was observed for Ptpru expression by ISH, a lower level of Pitx3 transcripts was observed in untreated Pitx3<sup>3/3<sup> mdDA neurons: ~38%, as compared with the level of Ptpru in untreated Pitx3<sup>3/3<sup> mdDA neurons. However, SB treatment resulted in a significant increase in Ptpru transcripts, restoring levels to 73% (n=4; P<0.05), indicating that in the absence of Pitx3, Nurr1-mediated expression of Ptpru is repressed in an HDAC-dependent manner. This further supports a direct transcriptional regulation of Ptpru by Nurr1, which was also suggested by our ChIP-on-chip data. Unexpectedly, SB treatment resulted in a strong decrease in Klhl1 transcript levels in both Pitx3<sup>3/3<sup> and Pitx3<sup>3/3<sup> mdDA neurons (Fig. 7F,H; n=4). Apparently, interference with HDAC-mediated repression negatively affects Klhl1 expression independent of the absence or presence of Pitx3. In summary, these data demonstrate that whereas Dlk1 expression increased in the absence of Pitx3, the expression of Ptpru and Klhl1 dramatically decreased in Pitx3<sup>3/3<sup> embryos. Furthermore, our data indicate that Ptpru is regulated by the combined action of Pitx3 and Nurr1 via the previously proposed mechanism of Pitx3-mediated potentiation of Nurr1 (Fig. 7E).

Expression of Dat is affected in Dlk1<sup>1/1<sup> embryos

A recent study has hinted at a role for Dlk1 in promoting the proliferation and differentiation of Th-positive neurons from in vitro cultured mdDA precursors (Bauer et al., 2008). The contrasting effects of Nurr1 and Pitx3 on the expression of Dlk1 in mdDA neurons have evoked interest concerning the role of Dlk1 during the development of mdDA neurons in vivo. We examined the molecular and morphological consequences of Dlk1 deficiency in Dlk1<sup>1/1<sup> embryos. All the genes tested were still expressed in the Dlk1-deficient mdDA area and, based on the expression pattern of En1 at E14.5, the mdDA field was generated normally (Fig. 8A,E). Furthermore, the expression of Th, Nurr1, Pitx3, Vmat2 and Ahd2 was undistinguishable from that of WT controls (Th, Fig. 8B,F; Pitx3, Fig. 8D,H; data not shown). However, the expression pattern of Dat was strikingly different from that of WT. In WT embryos, Dat was expressed in mdDA neurons during the final stages of differentiation and was restricted to the lateral population in rostral sections (Fig. 8C). Strikingly, in rostral sections of Dlk1<sup>1/1<sup> E14.5 embryos, Dat was also expressed in the medial region of the mdDA area (Fig. 8C'), which is normally completely devoid of Dat expression. This region harbors the young mdDA precursors during their migratory phase (Shults et al., 1990; Kawano et al., 1995). This indicates that in the absence of Dlk1, mdDA neuronal precursors prematurely differentiate into Dat-positive neurons before they have reached their final positions in the mdDA area. Notably, in WT embryos, Dat is not expressed in all
differentiated mdDA neurons, and is predominantly expressed in DA neurons of the SNc (Hurd et al., 1994). Indeed, in E14.5 WT embryos, the caudal (VTA) part of the mdDA area was clearly devoid ofDat expression (Fig. 8G). However, in Dlk1–/– embryos, Dat was highly expressed in this part of the mdDA area (Fig. 8G'), suggesting that Dlk1 might repress Dat in DA neurons of the VTA.

Further analysis revealed that Dat was ectopically expressed in other regions of the Dlk1-deficient embryonic brain. Whereas Dat expression was highly restricted to the mdDA area in WT embryos (Fig. 8L,M), Dlk1–/– embryos showed ectopic expression ofDat in the thalamus (Fig. 8J) and hindbrain (Fig. 8N). Notably, these neuronal tissues were positive for both Nurr1 (Fig. 8K,O) and Dlk1 (Fig. 8L,P) transcripts in WT embryos. These observations indicate that Nurr1-mediated induction of Dlk1 might be necessary to repress Dat expression in neuronal cell types other than DA neurons of the SNc. Although these observations suggest a role for Dlk1 in the timing and fine-tuning of Dat expression in the mdDA area and other neural structures at E14.5, we did not observe differences in Dat expression at E18.5, nor did we detect any molecular or morphological alterations of the mdDA area when compared with WT controls at this stage (data not shown). Altogether, the molecular analysis of the mdDA area in Dlk1–/– embryos suggests an inhibitory role for Dlk1 in the expression of Dat at E14.5, which is reflected in premature expression in precursors of SNc neurons, misexpression of Dat in the caudal (VTA) part of the mdDA area, and ectopic expression of Dat in other Nurr1-positive structures in the absence of Dlk1.

Taken together, we have established Dlk1, Ptprru and Klhl1 as novel targets of the Nurr1 and Pitx3 downstream cascades in the mdDA neuronal population. The involvement of these genes in neuronal processes that are affected in Nurr1–/– embryos by novel clues as to the molecular basis for some aspects of the multifaceted role of Nurr1 in mdDA neurons (Fig. 9).

**DISCUSSION**

**Novel targets of Nurr1 and Pitx3 in mdDA neurons**

Nurr1 is best known for its role in promoting the DA phenotype of mdDA neurons, leading to expression deficits of multiple DA-related genes upon genetic deletion of Nurr1. The involvement of Nurr1 in the DA phenotype only partially reflects the complex role of this protein in mdDA neurons, as other developmental defects in Nurr1–/– embryos have remained unexplained. These include mdDA-specific neuronal loss, defective formation of nerve fiber bundles and defects in neuronal migration and patterning. We hypothesized that Nurr1 might regulate additional target genes or molecular cascades that could further elucidate the role of Nurr1 during mdDA development. In this study, we identified Dlk1, Ptprru and Klhl1 as novel target genes of Nurr1 in vivo. In agreement with a cooperative function of Pitx3 and Nurr1 in mdDA neurons, the expression of Ptprru and Klhl1 was also severely affected in Pitx3–/– mdDA neurons. Whereas the co-regulatory effect of Nurr1 and Pitx3 is observed for most Nurr1 target genes, the regulation of Dlk1 in mdDA neurons appears to be the exception to the rule.

**The role of Klhl1 in mdDA neurons**

Klhl1 is primarily expressed in the brain (Chen et al., 2008), and we showed for the first time that the expression pattern of Klhl1 strongly resembles that of Nurr1 in the mdDA area and other Nurr1-positive structures. Klhl1 is the homolog of the actin-organizing kelch gene in...
Novel target genes of Nurr1 and Pitx3

**Fig. 8. The role of Dlk1 during mdDA neuronal differentiation.** (A–H) Coronal sections of the rostral (A–D) and caudal (E–H) mdDA area of wild-type (WT) (A–H) and Dlk1–/– (A’–H’) E14.5 mouse embryos. There is normal expression of En1 (A,E) and Th (B,F) but abnormal expression of Dat in the medial region of the rostral (C’) and caudal (G’) mdDA area of Dlk1–/– embryos, corresponding to the region where Pitx3 is expressed (D,H). (I–P) Coronal sections of the thalamic (I–L) and hindbrain (M–P) area showing lack of Dat expression in WT embryos (I,M) and ectopic expression of Dat in Dlk1–/– embryos (J,N) at E14.5. Dat is ectopically expressed in tissues positive for Nurr1 (K,O) and Dlk1 (L,P) in WT embryos. Asterisks indicate abnormal Dat expression and arrows indicate expression of Nurr1 and Dlk1.

Drosophila (Nemes et al., 2000) and is described as playing a central role in neurite outgrowth (Seng et al., 2006). In line with this role, Klhl1-null mice exhibit deficits in neurite formation of Purkinje cells (He et al., 2006) and display abnormal gait and progressive loss of motor coordination. Although these Parkinson-like symptoms are most intriguing in view of the association of Klhl1 with mdDA neuronal pathology, they have been mainly attributed to Klhl1-related deficits in Purkinje cells of the cerebellum (He et al., 2006). However, in humans, a direct link between abnormal expansions of trinucleotide repeats within the SCA8 locus, where KLHL1 is located, and cases of Parkinson’s disease have been reported (Worth et al., 2000; Izumi et al., 2003; Wu et al., 2004). The expression of Klhl1 in mdDA neurons of the SNc and VTA and the notion that Klhl1 is linked to mdDA pathology in Nurr1- and Pitx3-deficient embryos provide a new basis for studying the role of Klhl1 in the mdDA system.

**The involvement of Ptpru in Wnt signaling in mdDA neurons**

Ptpru expression in mdDA neurons requires both Pitx3 and Nurr1 and we provided evidence for a direct transcriptional regulation of Ptpru by their combined action. Receptor-type protein tyrosine phosphatases (PTPRs), like Ptpru, dephosphorylate tyrosine residues on target proteins, thereby counteracting the activity of protein tyrosine kinases (Stoker and Dutta, 1998). Reversible protein phosphorylation is an essential mechanism to regulate intracellular signaling cascades. The central role of PTPRs in determining the intensity and duration of multiple signaling cascades implicates PTPRs in multiple cellular processes in the brain (Paul and Lombroso, 2003). Previously, PTPRs have been implicated in axonal growth and guidance (Stepanek et al., 2005) and Ptpru is directly involved in Wnt signaling through binding and sequestering β-catenin (Yan et al., 2002; Yan et al., 2006; Badde and Schulte, 2008). Intriguingly, the Ptpru ortholog in chick has been implicated in patterning of the ventral midbrain (Badde et al., 2005; Badde and Schulte, 2008). Through a series of elegant studies, the authors showed that Ptpru is intimately linked to Fgf8 and Wnt signaling in the ventral midbrain area. Ptpru exhibited a repressive effect on the expression of Wnt1, which is a crucial factor for proliferation and patterning of the mdDA neuronal field (Megaso and McMahon, 2002). In line with this role, overexpression of Ptpru had an anti-proliferative effect on DA progenitors (Badde and Schulte, 2008). These data indicate an important role for Ptpru in patterning of the chick midbrain. Our analysis revealed that Ptpru continues to be expressed in the mdDA area during terminal differentiation and into adulthood, suggesting that this is not the only role for Ptpru.

**Evidence for a role of Dlk1 in mdDA neuronal differentiation**

Dlk1 was expressed in the embryonic mdDA area and its expression was maintained to a high level in the adult. We showed that Dlk1 expression was largely abolished in the mdDA area in Nurr1–/– embryos. Dlk1 encodes a transmembrane protein containing six EGF repeats and is mainly described for its role in the differentiation of adipocytes and osteoblasts (Moon et al., 2002; Abdallah et al., 2004). Continued expression of Dlk1 in cultured adipocytes is inhibitory to differentiation (Enomoto et al., 2004) and differentiation of adipocytes to mature adipocytes is preceded by downregulation of Dlk1 (Hansen et al., 1998; Smas et al., 1999). Although Dlk1 was previously shown to be expressed in Th-positive neurons of the SNc and VTA, its role in mdDA neurons is largely unknown (Jensen et al., 2001; Christopherson et al., 2007). In vitro studies on ventral midbrain-derived precursors of DA neurons revealed that treatment...
with Dlk1 protein during expansion of the primary culture increased DA precursor proliferation, whereas treatment during DA neuron differentiation did not alter the number of Th-positive neurons. However, interference with Dlk1 expression during differentiation resulted in decreased levels of a subset of mdDA markers, which suggested a permissive role for Dlk1 in the terminal differentiation of mdDA precursors in vitro (Bauer et al., 2008).

To examine the role of Dlk1 in the development of mdDA neurons in vivo, we analyzed the mdDA area in Dlk1−/− mice. Strikingly, whereas Dat expression is restricted to the rostralateral population of differentiated mdDA neurons in WT embryos, Dat was abnormally expressed in the medial region of the rostral mdDA area of Dlk1−/− embryos. This region harbors mdDA precursors that are migrating ventrally to their final positions in the mantle layer (Shults et al., 1990; Kawano et al., 1995). The expression of Dat in these mdDA precursors suggests a premature differentiation into Dat-positive neurons in the absence of Dlk1. Furthermore, in Dlk1−/− embryos, Dat was expressed in the mdDA area harboring the DA neurons of the VTA, whereas this area is largely devoid of Dat expression in WT embryos. This suggests that Dlk1 is important to repress Dat expression in mdDA neurons of the VTA, which might be fundamental to the relatively restricted expression of Dat in mdDA neurons of the SnC in WT embryos. In addition to the aberrant expression of Dat in the mdDA area, Dat was ectopically expressed in Nurr1/Dlk1-positive tissues in the thalamus and hindbrain. It should be noted that in line with previous reports (Moon et al., 2002), we observed a partial penetrance of the phenotype, as not all embryos displayed the aberrant expression pattern of Dat. Altogether, these data indicate an inhibitory role for Dlk1 in Nurr1-mediated expression of Dat, an important intrinsic regulator of the timing and cell type specificity of Dat expression in the brain. Notably, our in vivo data did not support a permissive role for Dlk1 in the differentiation of mdDA neurons in vitro (Bauer et al., 2008). By contrast, our in vivo data point to the premature expression of Dat in mdDA progenitors in the absence of Dlk1, which is in line with the suppressive role of Dlk1 in the differentiation of many non-neuronal tissues (Enomoto et al., 2004; Abdallah et al., 2004; Hansen et al., 1998; Smas et al., 1999). Although the exact mechanism remains to be clarified, our present data provide evidence of an important role for Dlk1 in the development of mdDA neurons in vivo.

In conclusion, the identification of Klhl1, Ptpru and Dlk1 as novel downstream targets of Nurr1 has provided further insight into its multifaceted role in the mdDA neuronal population. We have established Nurr1 as an essential regulator of genes that are involved in neuronal processes such as axonal outgrowth, neuronal patterning and terminal differentiation, processes that are also affected in Nurr1−/− embryos (Fig. 9). Therefore, our data might form the molecular basis for a subdivision of the Nurr1 downstream cascade into several distinctive pathways, each accounting for separate aspects of the complex phenotype of mdDA neurons in Nurr1−/− embryos. The connection of at least one of the newly described Nurr1 target genes with mdDA neuronal differentiation in vivo underlines the importance of identifying novel players and pathways in mdDA neurons in order to obtain a detailed view of the developmental properties of these neurons in health and disease.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/2363/DC1

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