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Retinoic acid-dependent and -independent gene-regulatory pathways of Pitx3 in meso-diencephalic dopaminergic neurons

Frank M. J. Jacobs[1,*], Jesse V. Veenvliet[1,2,*], Wadia H. Almirza[1,2], Elisa J. Hoekstra[1,2], Lars von Oerthel[1,2], Annemarie J. A. van der Linden[1], Roel Neijts[1], Marian Groot Koerkamp[3], Dik van Leenen[3], Frank C. P. Holstege[3], J. Peter H. Burbach[1] and Marten P. Smidt[1,2,‡]

SUMMARY

Development of meso-diencephalic dopamine (mdDA) neurons requires the combined actions of the orphan nuclear receptor Nurr1 and the paired-like homeobox transcription factor Pitx3. Whereas all mdDA neurons require Nurr1 for expression of *Th* and survival, dependence on Pitx3 is displayed only by the mdDA subpopulation that will form the substantia nigra (SNc). Previously, we have demonstrated that *Pitx3*$^{-/-}$ embryos lack the expression of the retinoic acid (RA)-generating enzyme Ahd2, which is normally selectively expressed in the Pitx3-dependent DA neurons of the SNc. Restoring RA signaling in *Pitx3*$^{-/-}$ embryos revealed a selective dependence of SNc neurons on the presence of RA for differentiation into Th-positive neurons and maintenance throughout embryonic development. Whereas these data are suggestive of an important developmental role for RA in neurons of the SNc, it remained unclear whether other Nurr1 and Pitx3 target genes depend on RA signaling in a manner similar to *Th*. In the search for genes that were affected in Pitx3-deficient mdDA neurons and restored upon embryonic RA treatment, we provide evidence that *Delta-like 1*, *D2R* (*Drd2*) and *Th* are regulated by Pitx3 and RA signaling, which influences the mdDA terminal differentiated phenotype. Furthermore, we show that regulation of Ahd2-mediated RA signaling represents only one aspect of the Pitx3 downstream cascade, as *Vmat2*, *Dat*, *Ahd2* (*Aldh1a1*), *En1*, *En2* and *Cck* were unaffected by RA treatment and are (subset) specifically modulated by Pitx3. In conclusion, our data reveal several RA-dependent and -independent aspects of the Pitx3-regulated gene cascade, suggesting that Pitx3 acts on multiple levels in the molecular subset-specification of mdDA neurons.

KEY WORDS: Pitx3, Dopamine, Neural development, Retinoic acid, Subset specification, Transcription

INTRODUCTION

Development of the meso-diencephalic dopaminergic (mdDA) neurons is dependent on a number of transcription factors playing roles during different stages of development (Smidt and Burbach, 2007). During late differentiation, immature mdDA neurons acquire the characteristics of a dopamine (DA) neuron, characterized by the expression of *Th*, the rate-limiting enzyme for DA synthesis. Expression of the orphan nuclear receptor Nurr1 (Nr4a2 – Mouse Genome Informatics) and the paired-like homeobox transcription factor Pitx3 is induced during late differentiation of mdDA neurons, preceding the expression of *Th* and other DA-related genes. *Pitx3* is selectively expressed in mdDA neurons in the brain and is essential for the development and survival of DA neurons of the substantia nigra (SNc) (Smidt et al., 2004; Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003). These initial observations pointed to a paradoxical role for Pitx3 in the mdDA neuronal population. Whereas *Pitx3* is expressed in all mdDA neurons during late differentiation, null mutation of *Pitx3* leads to selective loss of the DA neuronal population in the SNc, while the majority of DA neurons in the ventral tegmental area (VTA) is maintained. The differential dependence of distinct mdDA neuronal populations on Pitx3 is already visible during development. In *Pitx3*$^{-/-}$ embryos, a distinct subpopulation of immature mdDA neurons in a rostrolateral position, destined to form the SNc, are halted in their final differentiation as indicated by the lack of *Th* expression (Smidt et al., 2004; Maxwell et al., 2005; Jacobs et al., 2007).

We have previously shown that Pitx3 regulates the expression of the aldehyde dehydrogenase gene *Ahd2* (*Aldh1a1* – Mouse Genome Informatics) (Jacobs et al., 2007), which is an efficient generator of retinoic acid (RA) (McCaffery and Dräger, 1994). *Ahd2* is expressed in a very restrictive pattern in the mdDA system, precisely by the mdDA neuronal population that is dependent on Pitx3 for survival. The functional relevance of the transcriptional control of the RA-generating enzyme *Ahd2* by Pitx3 is underlined by the finding that RA treatment of *Pitx3*$^{-/-}$ embryos could bypass the requirement for Pitx3 and Ahd2, restoring the expression of *Th* in the SNc. These observations suggest an important role for RA signaling as part of the Pitx3 downstream cascade during final differentiation of mdDA neurons. In line with this, previous studies have demonstrated the presence of RA in the ventral midbrain during embryonic development. RA is detected in the midbrain area of E13 embryos (Horton and Maden, 1995), which corresponds to the timepoint when *Ahd2* expression is restricted to

[1]Rudolf Magnus Institute, Department of Neuroscience and Pharmacology, UMC Utrecht, Utrecht, The Netherlands. [2]Swammerdam Institute for Life Sciences, Science Park, University of Amsterdam, Amsterdam, The Netherlands. [3]Microarray Facility, Molecular Cancer Research, UMC Utrecht, The Netherlands.

*These authors contributed equally to this work
‡Author for correspondence (m.p.smidt@uva.nl)

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differentiating mdDA neurons (McCaffery and Dräger, 1994; Wallen et al., 1999; Jacobs et al., 2007). Moreover, cultured embryonic midbrains selectively activated *RAR-lacZ* but not *RXR-lacZ* constructs, indicative of the presence of endogenous *all-trans*-RA (here referred to as RA) (de Urquiza et al., 2000). RA is an essential molecule for embryonic brain development for its involvement in cellular differentiation (Mey et al., 2005; Maden, 2007) and has the ability to induce differentiation of the embryonic ventral midbrain-derived dopaminergic cell line MN9D (Eom et al., 2005; Castro et al., 2001). Furthermore, RA is an essential factor in ES-cell differentiation protocols for the generation of DA neurons (Smidt and Burbach, 2007). RA acts on gene transcription through binding to retinoic acid receptors (RAR) α (a), β (b) and γ (g) (Chambon, 1996; Germain et al., 2006) resulting in transactivation of RA-responsive target genes (Mangelsdorf et al., 1995; Durand et al., 1992; Leid et al., 1992; Smirnov, 2002). Notably, the rescue effect of RA in *Pitx3*$^{-/-}$ embryos was maintained into later stages even 4 days after cessation of RA treatment, indicating that local availability of RA in a crucial timeframe during final differentiation is important for maintenance of SNc neurons (Jacobs et al., 2007). This suggests that in addition to the RA-mediated induction of *Th* expression, RA may also affect other aspects of the complex process underlying mdDA neuronal terminal differentiation. In this study, we aimed to pinpoint the molecular effects of RA on gene transcription in Pitx3-deficient mdDA neurons and provide evidence for a relationship between Pitx3/RA and the expression of *Dlk1*, *Th* and *D2R* (*Drd2*). Interestingly, *Dlk1* was recently identified as a novel Nurr1 target gene (Jacobs et al., 2009b). The reciprocal relationship between Pitx3/RA and the expression of *Dlk1* potentially reflects an important role for endogenous RA signaling in DA neurons of the SNc. Next to the effect of RA treatment on *Th*, *Dlk1* and *D2R* expression, we verified the extent to which RA treatment affected the expression of other Nurr1 and Pitx3-dependent genes (Jacobs et al., 2009a; Jacobs et al., 2009b) involved in mdDA function. We found that expression of *Vmat2* (*Slc18a2* – Mouse Genome Informatics), *Dat* (*Slc6a3* – Mouse Genome Informatics) and *Ahd2*, all downregulated in the absence of Pitx3, cannot be rescued by embryonic supplementation of RA. Finally, we show that dopaminergic genes *En1*, *En2* and *Cck* are upregulated in the absence of Pitx3, and that their expression cannot be suppressed by RA supplementation. Our present data shed light on the molecular mechanisms and functional implications of RA signaling in mdDA neurons and unravel novel RA signaling-dependent and -independent aspects of the Pitx3 downstream cascade.

MATERIALS AND METHODS

Animals

Pitx3$^{-/-}$ and *Pitx3*$^{+/+}$ embryos were obtained as described previously (Smits et al., 2003; Jacobs et al., 2007). *Pitx3*-deficient *Aphakia* (*Pitx3*$^{-/-}$), C57Bl6-Jico wild-type or heterozygous *Aphakia* (*Pitx3*$^{+/-}$) mice were crossed with *Pitx3*$^{gfp/gfp}$ mice to obtain *Pitx3*$^{gfp/+}$ and *Pitx3*$^{gfp/-}$ embryos. Alternatively, *Pitx3*$^{gfp/+}$ mice were intercrossed to obtain *Pitx3*$^{gfp/+}$, *Pitx3*$^{gfp/gfp}$ and *Pitx3*$^{+/+}$ embryos. *Pitx3*$^{gfp/+}$ embryos are heterozygous for wild-type Pitx3 and green fluorescent protein (GFP), and have a normal development of the mdDA system (Maxwell et al., 2005). *Pitx3*$^{gfp/gfp}$ mice are *Pitx3* deficient. *Pitx3*$^{gfp/-}$ embryos contain both the classical *Ak* allele and an allele in which GFP is knocked into the *Pitx3* locus (Zhao et al., 2004; Maxwell et al., 2005) and are therefore *Pitx3* deficient. *Nurr1*$^{+/+}$;*Pitx3*$^{gfp/+}$, *Nurr1*$^{+/-}$;*Pitx3*$^{gfp/+}$ and *Nurr1*$^{-/-}$;*Pitx3*$^{gfp/+}$ embryos were obtained by first crossing *Pitx3*$^{gfp/+}$ animals with *Nurr1*$^{+/-}$ animals, intercrossing the offspring heterozygous for both genes (*Nurr1*$^{+/-}$;*Pitx3*$^{gfp/+}$) and then crossing *Nurr1*$^{+/-}$;*Pitx3*$^{+/+}$ with *Nurr1*$^{+/-}$;*Pitx3*$^{gfp/gfp}$ animals. The Nurr1$^{-/-}$ mice have been described previously (Saucedo-Cardenas et al., 1998).

Retinoic acid treatment of pregnant *Pitx3*$^{+/-}$ *Pitx3*$^{gfp/+}$ mice

For in situ hybridization and immunofluorescence analysis, either *Pitx3*$^{+/-}$ or *Pitx3*$^{gfp/+}$ mice were intercrossed. Pregnant mice were supplemented twice daily with 0.25 mg/g food of all-trans (at) retinoic acid (Sigma) from E10.75 to E13.75 as described previously (Niederreither et al., 2002; Mic et al., 2003; Jacobs et al., 2007). Embryos were isolated at E14.5.

Ex vivo ventral midbrain culture

Ventral midbrains of Pitx3-deficient *Pitx3*$^{gfp/-}$ embryos at stage E11.5 or E13.5 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 β-mercapto-ethanol and 100 units/ml penicillin/streptomycin. Midbrains were treated with 1 μM at-RA for 48 hours.

Genotyping

Genotypes of *Pitx3*$^{-/-}$ and *Pitx3*$^{+/+}$ embryos were determined by PCR analysis as described previously (Saucedo-Cardenas et al., 1998; Jacobs et al., 2007). Genotypes of *Pitx3*$^{gfp/+}$ and *Pitx3*$^{gfp/-}$ embryos was determined by analyzing the shape of the lens. *Pitx3*-deficient embryos exhibit a clear malformation of the lens body, which can easily be distinguished from heterozygous embryos. *Pitx3*$^{gfp/+}$ embryos were distinguished from *Pitx3*$^{+/+}$ embryos by PCR analysis for GFP.

FAC sorting

Dissected or cultured ventral midbrains were dissociated using a Papain dissociation system (Worthington) and cells were sorted on a Cytopeia Influx Cell sorter. Sort gates were set on forward scatter versus side scatter (life 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 (98% purity) using a 100 μm nozzle at a pressure of 15 PSI with an average speed of 7000 cells/second and collected in Trizol reagent (Invitrogen).

qPCR

Total RNA was purified by applying Trizol reagent (Invitrogen) to whole midbrain tissue or FAC-sorted *Pitx3*$^{gfp/+}$ and *Pitx3*$^{gfp/-}$ neurons according to the manufacturer's instructions. qPCR amplification was performed on a 'Roche' light cycler using OneStep qPCR SYBR green kits (Qiagen) according to the manufacturer's protocol. Either 0.1 ng (RNA from FAC-sorted neurons) or 10 ng (whole midbrain tissue) total RNA was used as input. Table S2 (supplementary material) lists primer sets used for qPCR.

Microarray analysis

RNA was isolated from dissected ventral midbrains of E14.5 *Pitx3*$^{-/-}$ and *Pitx3*$^{+/+}$ embryos using Trizol according to the supplied protocol (Invitrogen). RNA from 3 *Pitx3*$^{-/-}$ ventral midbrains was pooled to form one experimental sample that was hybridized to reference RNA derived from 10 Pitx3$^{+/+}$ ventral midbrains. Microarray analysis (n=3 per condition) was performed as described by Roepman et al. (Roepman et al., 2005). Custom arrays containing mouse 70-mer oligos (Operon, Mouse V2 AROS) spotted onto codelink activated slides (Surmodiscs) 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 (Yang et al., 2002) on mean spot-intensities. Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7) (Wu et al., 2003). 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 to be significantly changed. Microarray data have been deposited at GEO with accession number GSE32940.

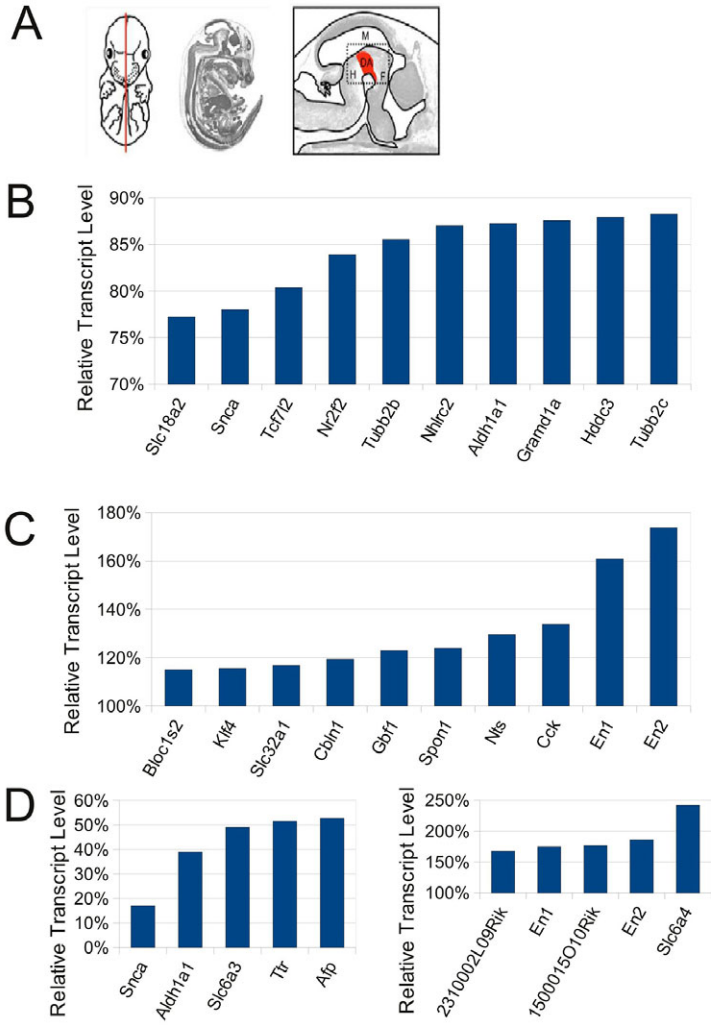

Fig. 1. Genes activated and repressed by Pitx3. Results of microarray analysis. (**A**) microdissected ventral midbrains of E14.5 *Pitx3*$^{-/-}$ and *Pitx3*$^{+/+}$ littermate control embryos were used. (**B**) Relative transcript levels of the 10 genes showing the highest downregulation in *Pitx3*$^{-/-}$ embryos compared with wild type (non-background corrected, $P<0.05$, MAANOVA). This toplist includes the previously described *Pitx3* targets *Vmat2* (*Slc18a2*) and *Ahd2* (*Aldh1a1*) (Jacobs et al., 2009a). (**C**) Relative transcript levels of the 10 genes that show the highest upregulation in *Pitx3*$^{-/-}$ embryos compared with wild type (non-background corrected, $P<0.05$, MAANOVA). This toplist includes the previously described dopaminergic genes *En1*, *En2* and *Cck*. (**D**) Relative transcript levels of genes found to be downregulated (left panel) or upregulated (right panel) after background-corrected MAANOVA analysis ($P<0.05$). This analysis identified the previously described (Jacobs et al., 2009a) dopaminergic gene *Dat* (*Slc6a3*) as an additional *Pitx3* target.

In situ hybridization

In situ hybridization was performed as described previously (Smits et al., 2003; Smidt et al., 2004). The following digoxigenin-labeled probes were used: Th, 1142 bp fragment of the rat Th cDNA (Grima et al., 1985); Vmat2, bp 290-799 of mouse coding sequence (CDS) (Smits et al., 2003); En1, 5′ region of mouse transcript; En2, 5′ region of the mouse transcript; Cck, bp 290-658 of the mouse CDS and 3′UTR; Dat, bp 789-1153 of rat CDS; Nurr1, 3′ region of rat Nurr1; D2R, bp 345-1263 of mouse CDS; Dlk1, bp 366-852 of the mouse CDS.

Immunofluorescence

E14.5 embryos were isolated, fixed in 4% PFA overnight, washed in 1×PBS, incubated in 30% sucrose/PBS for 24 hours and frozen on dry ice. For immunostaining, sections were washed three times in 1×TBS and blocked for 30 minutes in TBS with 4% hiFCS or 4% normal donkey serum. Primary antibodies in THZT were applied overnight at 4° and slices were washed three times in 1×TBS. Secondary antibodies were applied in 1×TBS for 1 hour at room temperature. Slices were then washed three times in 1×PBS and mounted using FluorSave Reagent (Merck). Primary antibodies used were chicken anti-GFP (1:750, Abcam), rabbit anti-Dlk1 (1:500, Abcam) and sheep anti-TH (1:1000, Millipore). Secondary antibodies (1:400) used were goat anti-chicken (Alexa 488, Invitrogen), goat anti-rabbit (Alexa 594, Invitrogen), donkey anti-sheep (Alexa 594, Invitrogen) and goat-anti-rabbit (Alexa 488, Invitrogen).

Statistical analysis

The quantified results from the qPCR represent the average values of experiments performed on three to seven biological samples for each condition (exact number per condition is mentioned in the figure legends) and data indicate means with standard errors (s.e.m., $n=3$). Statistical analysis was performed by Student's *t*-test (two-way unpaired). $P\leq0.05$ is considered significant and is indicated by an asterisk; $P\leq0.01$ is indicated with double asterisks.

RESULTS

Molecular effects of RA treatment in Pitx3-deficient mdDA neurons

In order to obtain an elaborate view on the molecular alterations caused by *Pitx3* deficiency, we performed gene expression analysis on dissected ventral midbrains of E14.5 *Pitx3*$^{-/-}$ and *Pitx3*$^{+/+}$ embryos (Fig. 1A). Non-background corrected MAANOVA analysis of microarray data identified 45 transcripts differentially expressed between *Pitx3*$^{+/+}$ and *Pitx3*$^{-/-}$ littermate embryos ($P<0.05$, supplementary material Table S1) of which 24 and 21 were down- and upregulated, respectively (Fig. 1B,C). Background corrected MAANOVA analysis revealed three additional regulated transcripts, two upregulated and one downregulated (Fig. 1D; supplementary material Table S1). The majority of previously described *Pitx3*-regulated genes like *Ahd2* (*Aldh1a1*), *Dat* (*Slc6a3*) and *Vmat2* (*Slc18a2*) were in the downregulated list, confirming the validity of the approach (Fig. 1B,D). Interestingly, overlaying the *Pitx3* expression array with our previously published *Nurr1* (*Nr4a2*) data (Jacobs et al., 2009a; Jacobs et al., 2009b) identified *Dat*, *Vmat2* and *Dlk1* as target of both *Pitx3* and *Nurr1*. Whereas *Dlk1* is downregulated in *Nurr1*-deficient embryos, *Dlk1* was

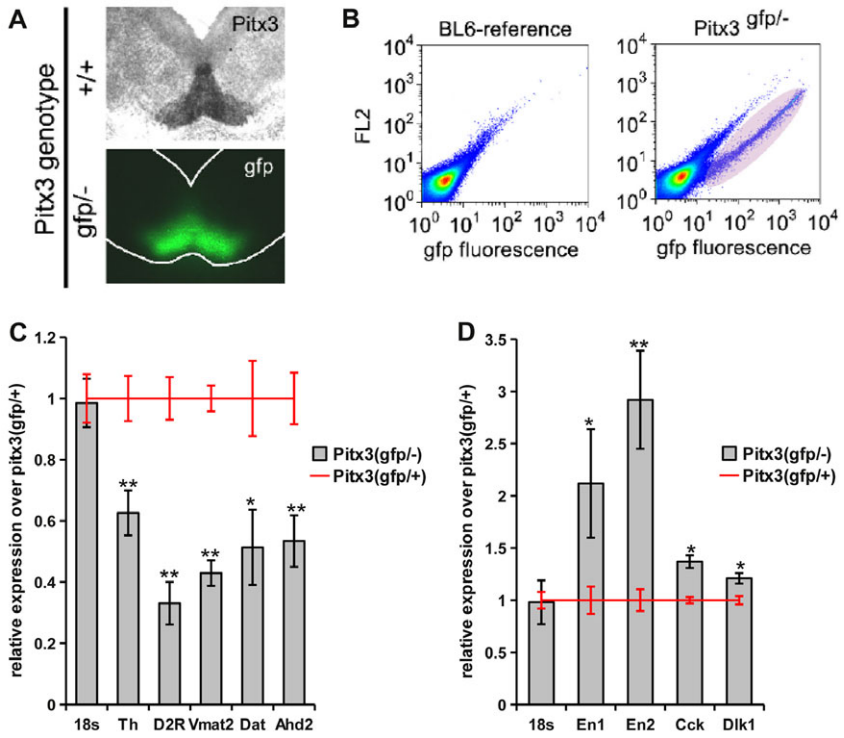

Fig. 2. qPCR validation of Pitx3 target genes in RNA isolated from FAC-sorted mdDA neurons. (**A**) GFP expression in the brain of *Pitx3*$^{gfp/-}$ embryos (lower panel) corresponds to the Pitx3-positive domain in wild-type embryos (upper panel). (**B**) Scatter plots showing distribution of GFP-positive *Pitx3*$^{gfp/-}$ mdDA neurons compared with reference tissue. GFP-positive neurons (purple) were selected by FAC-sorting. (**C**) Significant downregulation of *Ahd2*, *Dat* and *Vmat2* in *Pitx3*-deficient FAC-sorted mdDA neurons was confirmed by qPCR. *Th* and *D2R* (not in the microarray MAANOVA list) were also significantly downregulated. *18s* transcript levels did not differ between wild-type and Pitx3-deficient neurons, and were used to normalize for the amount of input RNA. (**D**) Significant upregulation of *Cck*, *Dlk1*, *En1* and *En2* in *Pitx3*-deficient FAC-sorted mdDA neurons was confirmed by qPCR. *18s* was used for normalization. Mean expression values in wild type (red line) were set at '1' and are indicated with standard errors (s.e.m.). Gray bars indicate mean expression fold relative to wild-type in *Pitx3*$^{gfp/-}$ littermates with s.e.m. Statistical analysis was performed by Student's *t*-test (two-way unpaired). **P*≤0.05 is considered significant; ***P*≤0.01. *n*=3, except for *Cck* and *Dlk1* (*n*=5 for wild type; *n*=7 for *Pitx3*$^{-/-}$).

significantly upregulated in *Pitx3*-deficient embryos, in agreement with what has already been suggested by in situ hybridization (Jacobs et al., 2009b). Other midbrain expressed genes, such as *En1*, *En2* and *Cck* (Simon et al., 2001; Hommer et al., 1985), were in the top ten of most upregulated genes (Fig. 1C).

We note that alpha-synuclein (*Snca*) showed up as the second most downregulated gene. However, this downregulation is due to a chromosomal deletion of the gene in the C57Bl6/6J [C57BL/6S (Harlan)] inbred strain (Specht and Schoepfer, 2001) which we confirmed (data not shown).

To validate our microarray data and to verify whether the genes were in fact expressed in DA neurons, we FAC-sorted *Pitx3*$^{gfp/+}$ and *Pitx3*$^{gfp/-}$ neurons (Fig. 2A,B), isolated RNA and subjected the samples to qPCR analysis. We confirmed downregulation of *Vmat2*, *Dat* and *Ahd2*. Moreover, we analyzed expression of *Th* because it was a previously identified Pitx3/RA target gene (Jacobs et al., 2007) and *D2R* because it was previously described as a target gene of both Nurr1 and Pitx3 (Jacobs et al., 2009a) and known to be regulated by RA in the mouse striatum (Krezel et al., 1998). Here, we show an almost 40% downregulation of *Th* and an almost threefold downregulation of *D2R* transcript levels (Fig. 2C). Furthermore, we confirmed that *Dlk1*, *Cck*, *En1* and *En2* are upregulated in *Pitx3*-deficient dopaminergic neurons (Fig. 2D).

MdDA subset-specific regulation of Dlk1 during development

Dlk1 has recently been identified as downstream target gene of Nurr1 (Jacobs et al., 2009b) and is expressed in mdDA neurons (Christophersen et al., 2007). Although the relationship between Pitx3 and Dlk1 has remained to be elucidated, previous findings suggest an expansion of the expression domain of *Dlk1* in Pitx3-deficient embryos (Jacobs et al., 2009b). These observations prompted us to investigate the role of *Dlk1* in more detail. Detailed in situ hybridization analysis revealed that the *Dlk1*-expression domain largely corresponds to the *Nurr1*-positive domain in the caudal part of the mdDA area (Fig. 3A′,B′). Notably, in rostral sections, *Dlk1* is expressed only in a subset of DA neurons (Fig. 3A,B). Analysis of Dlk1 protein in E14.5 *Pitx3*$^{gfp/+}$ embryos revealed that colocalization of Pitx3 and Dlk1 is strictly limited to the more caudal mdDA area in medial sections (Fig. 3C,D). Similarly, colocalization of Dlk1 and Th is also limited to caudal mdDA neurons (Fig. 3E). To analyze the upregulation of *Dlk1* in *Pitx3*-deficient embryos in more detail, we performed in situ hybridization analysis on sagittal sections of E14.5 *Pitx3*-deficient embryos, showing an enlargement of the *Dlk1* expression domain in a rostral direction (Fig. 3G,G′). Interestingly, this region contains the mdDA neuronal subset that is affected in Pitx3-deficient embryos (Fig. 3F,F′) (Maxwell et al., 2005; Jacobs et al., 2007).

Next, we compared the expression of *Dlk1* in E14.5 *Pitx3*$^{+/+}$ and *Pitx3*$^{-/-}$ embryos with *Dlk1* expression in *Pitx3*$^{-/-}$ embryos supplemented with RA from E11.75-E13.75. In agreement to what was observed in sagittal sections of Pitx3-deficient embryos, in situ hybridization on coronal sections revealed a rostral expansion of the *Dlk1* expression domain in control-treated *Pitx3*$^{-/-}$ embryos (Fig. 4B). Strikingly, embryonic RA treatment of *Pitx3*$^{-/-}$ embryos clearly restricted the expression domain of *Dlk1* in the rostral part of the mdDA area (Fig. 4C), to a pattern highly similar to wild-type embryos (Fig. 4A). These striking effects were not observed in the caudal area (Fig. 4A′-C′). To verify whether the Pitx3/RA-mediated regulation of *Dlk1* transcript was followed by a change in Dlk1 protein in Pitx3-GFP positive DA neurons, we performed immunofluorescence on sagittal sections of *Pitx3*$^{gfp/+}$, *Pitx3*$^{gfp/gfp}$ and RA-treated *Pitx3*$^{gfp/gfp}$ embryos. We confirmed that in the absence of Pitx3, Dlk1 protein is upregulated in mdDA neurons in *Pitx3*$^{gfp/gfp}$ embryos and observed a rostral expansion of Pitx3/Dlk1 colocalization in *Pitx3*$^{gfp/gfp}$ embryos (Fig. 4D-G). In agreement with our in situ hybridization data, embryonic RA treatment of *Pitx3*-deficient embryos clearly suppressed the upregulation of Dlk1 to a level similar to that in wild-type embryos (Fig. 4D-G).

Altogether, these data strongly suggest that, in addition to Th, the expression of Dlk1 in mdDA neurons of the SNc is modulated by endogenous Ahd2-mediated RA signaling, and is therefore only indirectly regulated by Pitx3.

Pitx3 causes subset specific repression of *Cck* in a RA-independent manner

In addition to *Dlk1*, one of the upregulated transcripts in *Pitx3*-deficient midbrains is *Cck*. To determine whether *Cck* expression is also dependent on *Nurr1*, we subjected RNA from FAC-sorted Nurr1+/Pitx3(GFP/+) and Nurr1–/Pitx3(GFP/+) neurons to qPCR. We found that *Cck* is massively downregulated in the absence of *Nurr1*, establishing *Cck* as a novel Nurr1 target (Fig. 5A). Moreover, *Cck* transcript level was shown to be increased in FAC-sorted cells of Pitx3$^{(gfp/-)}$ embryos when compared with Pitx3$^{(gfp/+)}$ embryos. Thus, as was observed for a number of other Pitx3 target genes (Jacobs et al., 2009a), *Cck* is regulated through the combinatorial action of Nurr1 and Pitx3 (Fig. 2D). Analysis of the *Cck* expression domain in E13.5 embryos revealed that *Cck* is restricted to the most caudal part of the mdDA neuronal population (Fig. 5B). In *Pitx3*-deficient embryos, we observed an overall increase of *Cck* expression within the DA area, and most remarkably a rostral expansion of the *Cck*-positive domain, creating a complete overlap with the *Th* expression domain (Fig. 5B). Upregulation of *Cck* in *Pitx3*-deficient embryos is most prominent in the more rostrolateral part of the mdDA system (Fig. 5B), where *Cck* is normally not expressed (Fig. 5B). Importantly, this area of ectopic *Cck* expression corresponds to the part of the mdDA system where *Th* expression is lost in *Pitx3*-deficient embryos.

Because the rostral expansion of the *Cck* domain is strikingly similar to the rostral expansion of the *Dlk1* expression domain, we tested whether the upregulation of *Cck* in Pitx3-deficient embryos could be suppressed by RA treatment. In coronal sections of Pitx3-deficient E14.5 embryos the caudal upregulation (supplementary material Fig. S1C-C′) and rostral expansion (Fig. 5B,C,C′) of the *Cck* expression domain is evident. However, in contrast to what was observed for *Dlk1*, this expansion could not be suppressed by RA supplementation (Fig. 5C″). To provide a second line of evidence that RA does not play a role in the regulation of *Cck*, we performed qPCR on dissected ventral midbrains cultured with and without RA for *Cck* and *Th* as a control. This experiment confirmed that *Th* is significantly upregulated by RA treatment (Jacobs et al., 2007) (Fig. 5E), and that *Cck* is not regulated by RA (Fig. 5D) but is kept restricted to the caudal part of the mdDA area by the actions of Pitx3 through an unknown but RA-independent mechanism.

mdDA genes can be suppressed and activated by Pitx3 action

After our finding that Dlk1 is indirectly suppressed through the Pitx3/Ahd2/RA pathway, whereas *Cck* is not, we continued to analyze whether the two highest upregulated genes, *En1* and *En2*, can also be suppressed by RA supplementation. The intense upregulation of the crucial mdDA genes *En1* and *En2* might hint towards a compensatory effect towards the loss of Pitx3, indicating crosstalk between these homeodomain transcription factors. To analyze the upregulation of *En1* and *En2* in more detail and to study the potential role of RA in their regulation, we performed in situ hybridization on E14.5 coronal sections and found that in Pitx3-deficient embryos *En1* and *En2* are heavily upregulated in the rostral part of the mdDA (Fig. 6B,B′,C,C′), whereas upregulation of transcript levels in the caudal region is more subtle (supplementary material Fig. S1A,A′,B,B′). However, RA supplementation did not suppress the *En1/2* upregulation in *Pitx3*-deficient embryos (Fig. 6B″,C″).

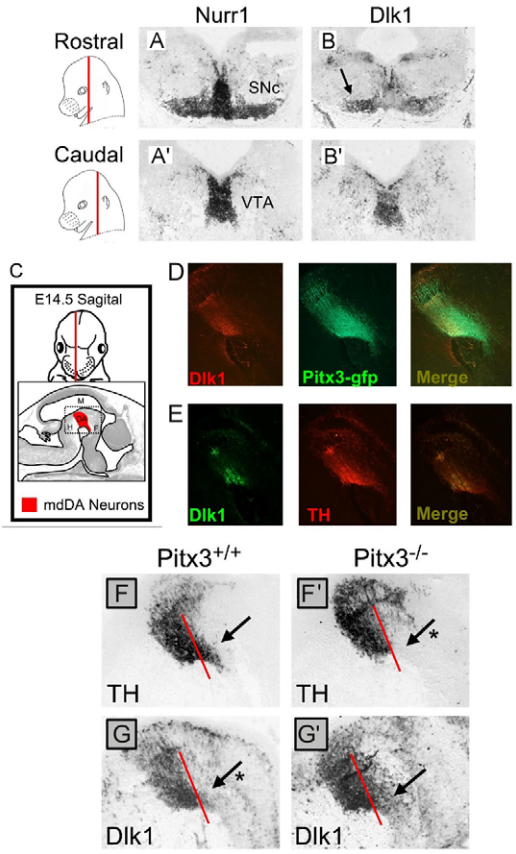

Fig. 3. Dlk1 expression in the wild-type and *Pitx3*-deficient mdDA area. (**A**,**A′**) Expression of *Nurr1* in rostral (A) and caudal (A′) sections of the E14.5 ventral midbrain. (**B**,**B′**) Expression of *Dlk1* in rostral (B) and caudal (B′) sections of the E14.5 ventral midbrain. Whereas the *Dlk1* expression domain highly resembles *Nurr1* expression in caudal sections (compare A′ with B′), *Dlk1* is expressed in only a subset of *Nurr1*-positive DA neurons in the rostral part of the mdDA area (compare A with B), as indicated by the arrow in B. (**C**) Overview of a sagittal section of the mdDA area in E14.5 embryos. (**D**,**E**) Sagittal section showing that colocalization of Dlk1 with Pitx3 (D) and Th (E) is restricted to the caudal area of the mdDA system. (**F-G′**) Sagittal section showing the expression of *Th* (F) and *Dlk1* (G) in the *Pitx3*$^{+/+}$ (F,G) and *Pitx3*$^{-/-}$ (F′,G′) embryos. Red lines indicate the border between the mdDA neuronal subsets that are devoid of *Th* expression in *Pitx3*$^{-/-}$ embryos (right side of line) or that maintain *Th* expression to a normal level (left side of line). The neuronal subset that is devoid of *Th* expression in *Pitx3*$^{-/-}$ embryos (F′) matches the part that ectopically expresses *Dlk1* in Pitx3-deficient embryos (G′), as indicated by the arrows.

Dat, *Vmat2* and *Ahd2* are dopaminergic genes that are downregulated in Pitx3-deficient embryos and co-regulated by Nurr1 and Pitx3 (Jacobs et al., 2009a; Jacobs et al., 2009b). In order to test whether these genes were regulated by RA, we performed in situ hybridization analysis on in vivo RA-treated *Pitx3*-deficient mice, control-treated *Pitx3*-deficient mice and their wild-type littermates (Jacobs et al., 2007). Our data confirm that *Dat*, *Vmat2* and *Ahd2* are downregulated in the rostral mdDA area (Fig. 7A) in E14.5 Pitx3-deficient embryos (Fig. 7C′-E′) compared with wild-type embryos (Fig. 7C-E). Interestingly, RA treatment

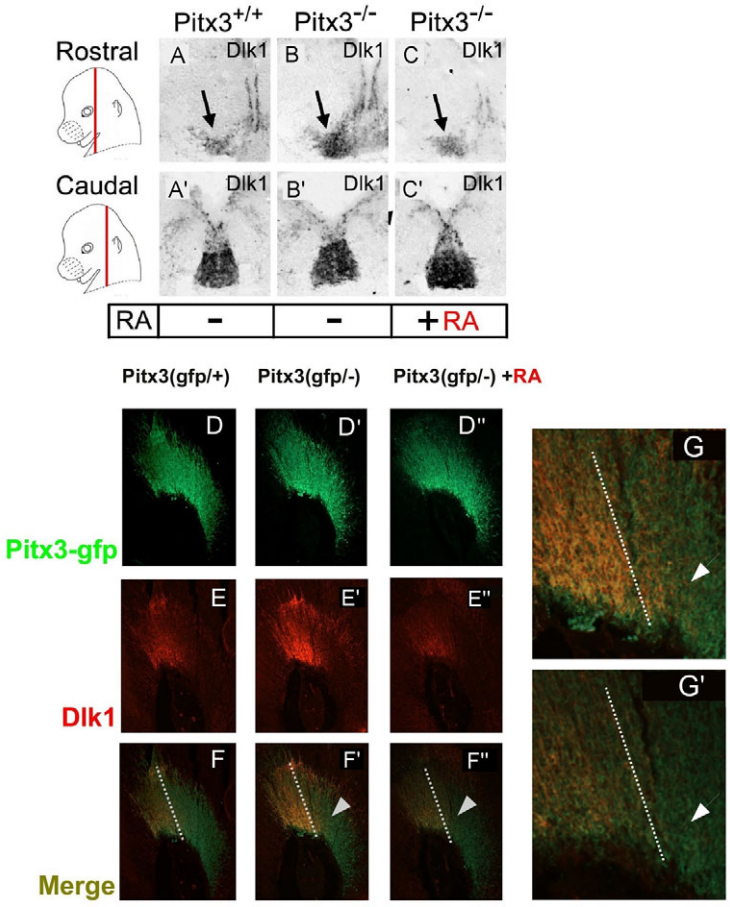

Fig. 4. Dlk1 expression is regulated by both Pitx3 and RA at both transcript and protein levels. (**A-C′**) *Dlk1* expression in the E14.5 *Pitx3*$^{+/+}$ (A), control-treated *Pitx3*$^{-/-}$ (B) and RA-treated *Pitx3*$^{-/-}$ (C) ventral midbrain in rostral (A-C) and caudal (A′-C′) sections. The expression domain of *Dlk1* is clearly enlarged in the rostral part of the mdDA area in *Pitx3*$^{-/-}$ embryos (compare A with B, arrows). Embryonic RA treatment of *Pitx3*$^{-/-}$ embryos restricted *Dlk1* expression domain in rostral sections to a similar pattern of *Dlk1* expression as observed in wild-type embryos (compare B and C with A, arrows). (**D-F″**) Immunofluorescence for Dlk1 (red) and Pitx3-GFP (green) on matching sagittal sections [as shown for Pitx3-gfp (D-D″)] reveals caudal upregulation and rostral expansion of Dlk1 in Pitx3-deficient embryos (compare E with E′) that is repressed by RA treatment of *Pitx3*$^{-/-}$ embryos (E″). Colocalization (F-F″) confirms that upregulation and rostral expansion of Dlk1 in *Pitx3*$^{-/-}$ embryos (F′) and rescue by RA treatment (F″) is within dopaminergic neurons (arrowheads). The broken white lines indicate the border between the mdDA neuronal subsets that are devoid of *Th* expression in *Pitx3*$^{-/-}$ embryos (right side of line) or that maintain *Th* expression (left side of line). RA, retinoic acid-treated embryos. (**G**,**G′**) Higher magnification images of F and F′, respectively, to show the upregulation and ectopic expansion of Dlk1 protein in Pitx3-deficient embryos (G) and the restoration to wild-type expression by RA supplementation (G′) in more detail. Arrowheads indicate the mdDA population that is dependent on RA signaling (right side of white dotted line).

was not able to restore *Dat*, *Vmat2* and *Ahd2* expression (Fig. 7C″-E″), whereas *Th* expression was rescued in RA-treated *Pitx3*$^{-/-}$ embryos compared with age-matched controls (Fig. 7B-B″), consistent with what was observed for Th protein by immunohistochemistry (Jacobs et al., 2007).

D2R expression is partially restored by embryonic RA treatment in Pitx3-deficient embryos

Whereas the expression of *Cck*, *En1*, *En2*, *Dat*, *Ahd2* and *Vmat2* in RA-treated *Pitx3*$^{-/-}$ embryos was almost indistinguishable from control-treated *Pitx3*$^{-/-}$ embryos (Figs 5 and 6; Fig. 7C-E″), the expression of *D2R* was slightly increased in the rostral mdDA area after RA treatment (Fig. 7F-F″). To validate and quantify the increase in *D2R* expression, we micro-dissected E13.5 *Pitx3*$^{gfp/-}$ ventral midbrains, cultured them for 48 hours with or without RA and subjected RNA from FAC-sorted GFP-positive neurons to qPCR. Indeed, these qPCR data validate that the expression of *D2R* expression in *Pitx3*-deficient mdDA neurons is partly restored by RA treatment (Fig. 7G). This positions *D2R* next to *Th* and *Dlk1* as genes that are affected in Pitx3-deficient mdDA neurons and whose expression pattern is partly restored by resupplying RA signaling to compensate effectively for the loss of Pitx3.

Our data suggest that the downstream cascade of Pitx3 can be subdivided in at least two distinct molecular pathways. Pitx3 is required for the expression of *Dat*, *Ahd2* and *Vmat2* in mdDA neurons in a RA-independent manner (Fig. 8). By contrast, the expression of *Th*, *Dlk1* and *D2R* in DA neurons of the SNc display an indirect requirement for Pitx3, and rather depends on endogenous Ahd2-mediated RA signaling (Fig. 8).

DISCUSSION

Loss of *Pitx3* during development leads to a selective deficit of *Th* expression in the rostrolateral mdDA population, harboring the DA neurons committed to form the SNc. The inability of these neurons to express *Th* precedes the highly selective neuronal loss of DA neurons in the SNc by P0 (Smidt et al., 2004; Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003). Strikingly, the RA-generating enzyme Ahd2 closely marks the neuronal subset that is lost in *Pitx3*$^{-/-}$ mice (McCaffery and Dräger, 1994; Jacobs et al., 2007). Previously, we showed that embryonic treatment with RA in *Pitx3*-deficient embryos restored the expression of Th in the rostrolateral mdDA population throughout embryonic development (Jacobs et al., 2007). Here, we investigated the extent of the role for RA as an indirect action of Pitx3 and direct actions of Pitx3 in relation to the differentiation of immature mdDA neurons into Th-positive DA neurons.

RA signaling results in subset specific repression of *Dlk1*

We examined the molecular changes in *Pitx3*-deficient mdDA neurons, as well as the impact of RA treatment of *Pitx3*-deficient embryos during development. We focused on genes that were differentially expressed in *Pitx3*$^{-/-}$ embryos when compared with wild type, and analyzed whether these genes were affected in *Pitx3*-deficient mdDA neurons following maternal RA treatment. Of particular interest was the identification of *Dlk1* as Pitx3/RA dual regulated gene, as we have previously shown that *Dlk1* expression in the mdDA area is highly dependent on Nurr1 (Jacobs et al., 2009b). In *Pitx3*$^{-/-}$ embryos, *Dlk1* mRNA and

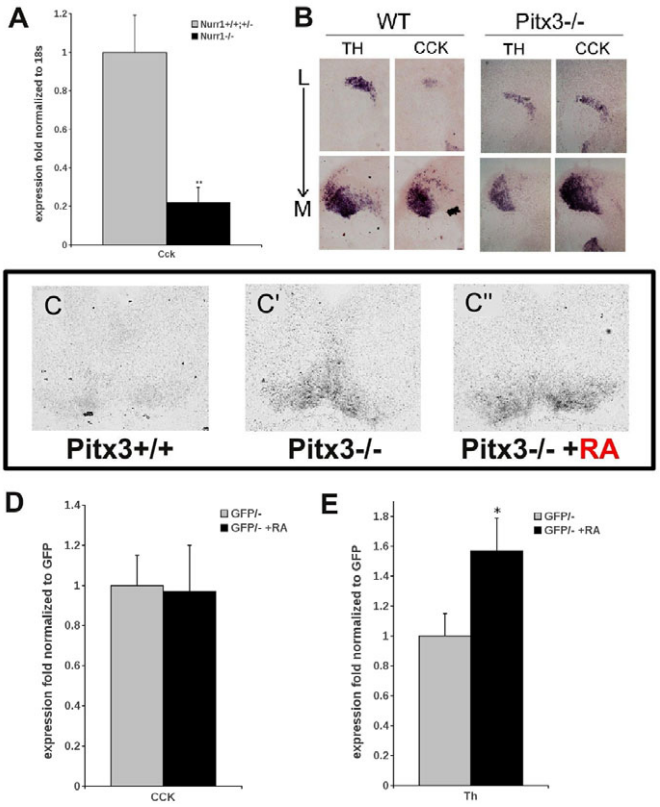

Fig. 5. Pitx3-dependent downregulation of *Cck* in specific rostral mdDA subsets. (**A**) *Cck* is dependent on Nurr1 activity, as measured through qPCR on RNA isolated from FAC-sorted mdDA neurons of *Nurr1*$^{+/+;+/-}$/*Pitx3*$^{gfp/+}$ compared with *Nurr1*$^{-/-}$/*Pitx3*$^{gfp/+}$ animals (**P<0.01). n=3. (**B**) In situ hybridization showing that *Cck* is specifically expressed in the caudal mdDA domain when compared with *Th* expression at E13.5 (wild type) and is expanded in *Pitx3*$^{-/-}$ embryos into the rostral domain, where *Th* expression is lost in *Pitx3*-deficient embryos. (**C-C″**) Rostral expansion of the *Cck* expression domain in *Pitx3*-deficient embryos as shown by in situ hybridization on coronal sections in E14.5 *Pitx3*$^{-/-}$ embryos (C′) and wild-type littermates (C) cannot be suppressed by RA treatment (C″). (**D**) This is confirmed by qPCR on RNA isolated from dissected ventral midbrains (*Pitx3*$^{gfp/-}$) cultured in the absence and presence of RA. (**E**) In the same samples as in D, *Th* is significantly upregulated by RA supplementation (*P=0.05), confirming the validity of this approach. n=4 (*Pitx3*$^{gfp/-}$), n=5 (*Pitx3*$^{gfp/-}$ + RA). Presented data indicate means with standard errors (s.e.m.). Statistical analysis was performed using Student's t-test (two-way unpaired).

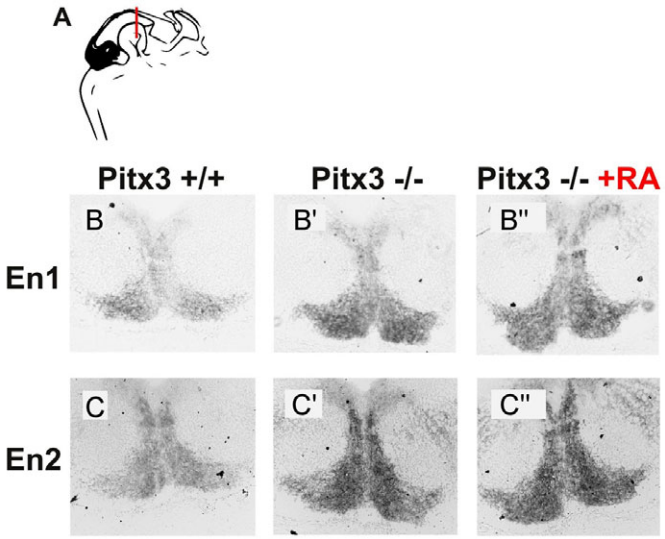

Fig. 6. En1 and En2 are upregulated in the rostral mdDA area in Pitx3-deficient embryos. (**A**) Overview of coronal section area of the mdDA area in E14.5 embryos. (**B-C″**) The expression of *En1* (B) and *En2* (C) in the rostral mdDA area is dramatically increased in *Pitx3*$^{-/-}$ embryos (compare B with B′ for *En1* and C with C′ for *En2*). This increase can not be suppressed by RA treatment of *Pitx3*$^{-/-}$ embryos (compare B′ with B″ for *En1* and C′ and C″ for *En2*).

protein expression was increased throughout the whole mdDA area. Importantly, this led to an expansion of the Dlk1 expression domain into the rostral part of the mdDA area, a region harboring the mdDA neuronal population that had halted in terminal differentiation in the absence of *Pitx3* (Smidt et al., 2004; Jacobs et al., 2007). Noteworthy, this is the area where Ahd2 and Th are colocalized in E14.5 wild-type embryos (Jacobs et al., 2007), indicating that the rostral shift of Dlk1 in *Pitx3*-deficient E14.5 embryos falls into the Ahd2 expressing territory, as found in wild-type animals. Most intriguingly, *Pitx3*-deficient mdDA neurons that have received embryonic treatment with RA showed a decreased level of Dlk1 transcript and protein, reducing Dlk1 expression to wild-type levels and thus removed from the rostral area. We have previously demonstrated that RA treatment selectively affects Th expression in the rostral part of the mdDA area of *Pitx3*-deficient embryos (Jacobs et al., 2007), indicating that both Pitx3 and RA reciprocally affect the expression of Th and Dlk1. The negative effect of RA on the expression of Dlk1 suggests that the increase of Dlk1 expression in *Pitx3*$^{-/-}$ embryos is the consequence of corrupted RA signaling in mdDA neurons in the absence of Pitx3.

Recently, the role of Dlk1 in mdDA neurons has been investigated, leading to contradicting observations. Bauer et al. showed that supplementation of Dlk1-protein to primary cultures promotes the generation of Th-positive neurons (Bauer et al., 2008). By contrast, we recently analyzed the mdDA system in *Dlk1*$^{-/-}$ embryos and did not observe an effect on *Th* expression during multiple developmental stages (Jacobs et al., 2009b). However, we did observe premature expression of *Dat* in migrating young DA neurons and ectopic expression of *Dat* in the caudal-most part of the mdDA area, suggesting a suppressive role for Dlk1 in some aspects of mdDA neuron maturation. These latter observations are in agreement to the role of Dlk1 in a number of peripheral tissues. In adipocytes, osteoblasts and neuroblastoma cells, continued expression of Dlk1 repressed cellular differentiation (Enomoto et al., 2004; Abdallah et al., 2004; Kim, 2010) and downregulation of Dlk1 expression is an important step during differentiation (Hansen et al., 1998; Smas et al., 1999). Although the exact role of Dlk1 in mdDA neurons remains elusive, our present data suggest that one role for Pitx3/Ahd2-mediated local RA-signaling may be to actively downregulate Dlk1 expression in order to allow terminal differentiation of immature neurons into *Th*-positive mdDA neurons. In agreement with this idea, treatment of various doses of RA to neuroblastoma cells or Dlk1 knockdown, can induce differentiation (Kim, 2010). Altogether, our data indicate that Dlk1 is downregulated in rostral differentiating mdDA neurons by RA and is reciprocally correlated with Pitx3-induced mdDA differentiation (Fig. 8).

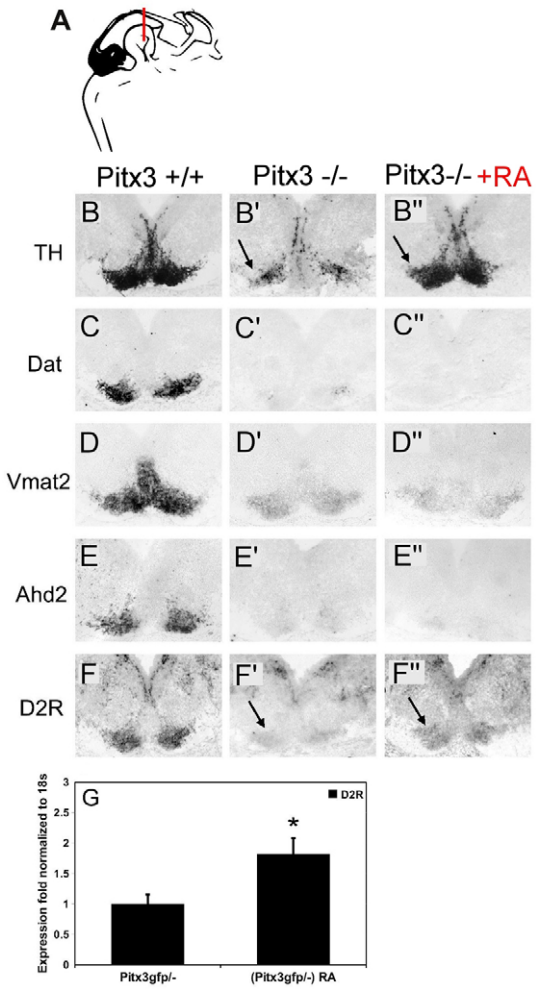

Fig. 7. Selective effects of RA treatment on downregulated mdDA target genes in vivo at E14.5. (**A**) Overview of coronal section area of the mdDA area in E14.5 embryos. (**B-B″**) The expression of *Th* in the mdDA area is dramatically reduced in *Pitx3*$^{-/-}$ embryos (compare B with B′), which is largely restored in *Pitx3*$^{-/-}$ embryos that have received embryonic RA treatment (compare B′ with B″). (**C-E″**) The expression of *Dat* (C), *Ahd2* (D) and *Vmat2* (E) is reduced in *Pitx3*$^{-/-}$ embryos (compare C-E with C′-E′, respectively), but seems unaffected by RA treatment (compare C′-E′ with C″-E″, respectively). (**F-F″**) The expression of *D2R* is reduced in *Pitx3*$^{-/-}$ embryos (compare F with F′), which is partially restored by RA treatment in *Pitx3*$^{-/-}$ embryos that have received embryonic RA treatment (compare F′ with F″). Arrows indicate the mdDA population that is dependent on either Pitx3 or RA signaling. (**G**) RA signaling-dependent upregulation of *D2R* quantified by qPCR for *D2R* in FAC-sorted *Pitx3*$^{gfp/-}$ mdDA neurons cultured from E13.5-E15.5 with and without RA (*P<0.05). Presented data indicate means with standard errors (s.e.m.). Statistical analysis was performed using Student's *t*-test (two-way unpaired).

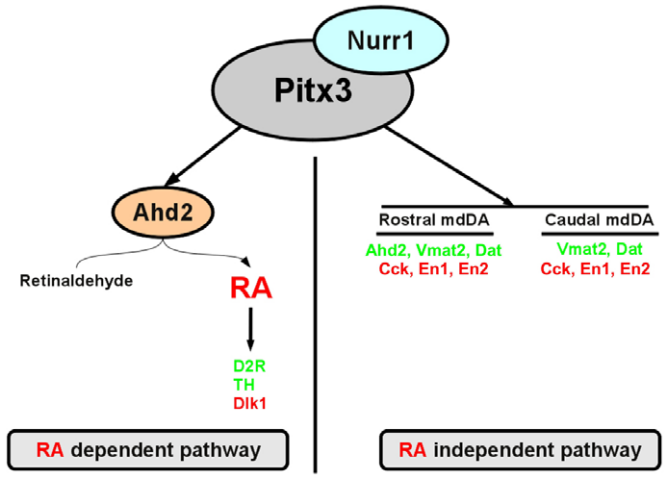

Fig. 8. Model for RA-dependent and -independent aspects of the Pitx3 downstream cascade in the mdDA system. Pitx3 potentiates Nurr1 to drive the expression of Vmat2 both in the caudal and the rostral mdDA region through the previously proposed cooperation of Pitx3 and Nurr1 (Jacobs et al., 2009a). The rostral-selective expression of Ahd2 also requires Pitx3 and Nurr1, presumably through the same mechanism. In the caudal region, Pitx3 is fully dispensable for the expression of Th. However, in the rostral area, Th expression only requires Pitx3 indirectly, for its involvement in the regulation of Ahd2-mediated RA synthesis. Rostral DA neurons demonstrate a selective dependence on RA to induce Th expression. In addition, RA represses Nurr1-mediated expression of Dlk1 in rostral regions, thereby permitting other aspects associated with terminal differentiation. The expression of *D2R*, which at this time point is mainly expressed in the rostral area, may be regulated through a similar mechanism to Th expression. Genes in red are repressed in the presence of Pitx3, whereas genes in green are induced by Pitx3.

RA is not affecting the expression of most Pitx3 target genes, but can partly restore the expression of D2R in Pitx3-deficient embryos

In *Pitx3*$^{-/-}$ embryos, we detected a significant decrease in transcript levels for most of the described *Pitx3*-regulated genes such as *Ahd2*, *Dat*, *Vmat2* and *Th*, and a significant increase in the transcript levels of a number of well-known genes in mdDA neuron development, such as *En1*, *En2* and *Cck*. *Cck* is expressed in the caudal part of the mdDA system. In *Pitx3*$^{-/-}$ animals *Cck* expression is expanded into a rostrolateral direction. Interestingly, in contrast to Dlk1 the expansion of the *Cck* expression domain could not be suppressed by RA treatment. Thus, RA treatment of *Pitx3*-deficient mdDA neurons did not restore the expression of *Ahd2*, *Dat*, *Vmat2*, *Cck*, *En1* and *En2* to wild-type values. However, RA treatment significantly increased *D2R* expression in the *Pitx3*-deficient mdDA area. The apparent involvement of RA in the regulation of *D2R* expression is in agreement to previous studies in the striatum, showing a high dependence of the *D2R* gene on RA signaling (Samad et al., 1997; Krezel et al., 1998; Valdenaire et al., 1998). These data clearly indicate that, whereas RA has the ability to restore part of the DA phenotype of *Pitx3*-deficient SNc neurons, the entire mdDA neuronal population, including DA neurons of the SNc, is still impaired in the expression of other genes involved in DA metabolism.

The observed effect of RA makes it appealing to hypothesize that Nurr1 cooperates with an RA-related factor in this selective subpopulation to drive the expression of *Th*. Although Nurr1 has been shown to heterodimerize with retinoid receptor RXR and RXR ligands promote the survival of DA neurons (Perlmann and Wallen-MacKenzie, 2004), the presence of this complex in the midbrain has so far not been shown. Importantly, cultured embryonic midbrains selectively activate *RAR-lacZ* but not *RXR-lacZ* constructs, indicative of a role of endogenous at-RA in the mdDA area (de Urquiza et al., 2000) and suggesting that the RA signal in the mdDA system is transduced through RAR and/or

RAR-RXR complexes. We were able to mimic the RA-mediated rescue of *Th* transcript in Pitx3-deficient embryos by administration of a pan-RAR-agonist, and RAR transcript levels were upregulated in Pitx3-deficient embryos after treatment of the pregnant mothers with RA (data not shown). In vitro, a direct role for RARs in transcriptional regulation of the Th gene was suggested in SK-N-BE(2)C cells, where RARb bound the promoter of Th and induced Th expression upon activation of RARb by at-RA (Jeong et al., 2006).

Based on the present study, a distinction can be made between RA-dependent and -independent regulatory effects of Pitx3 (Fig. 8). First, Pitx3 directly regulates, together with Nurr1, the expression of *Ahd2*, *Dat* and *Vmat2*, and RA-independently represses *Cck* in a rostrolateral subset of mdDA neurons, although expression levels are also upregulated in the caudal midbrain in the absence of Pitx3, as is also observed for En1/2 (supplementary material Fig. S1). Second, as result of its regulatory effect on the expression of *Ahd2* (Jacobs et al., 2007), Pitx3 is indirectly involved in the generation of RA, thereby affecting the expression of *Th*, *Dlk1* and *D2R*.

We have previously shown that in Pitx3-deficient embryos, SMRT/HDAC complexes are not released from the Nurr1 transcriptional complex, leading to repression of several mdDA target genes. Expression was restored by inhibition of HDACs (Jacobs et al., 2009a). Although it is an interesting possibility that Pitx3-induced RA production releases HDAC/SMRT from Nurr1-containing complexes, this is not likely because: (1) HDAC inhibition rescued not only RA targets *D2R* and *Th*, but also an RA-independent DA target gene, *Vmat2*; and (2) ChIP-on-Chip and co-immunoprecipitation analysis revealed that in wild-type animals release of SMRT/HDAC repression is likely to be mediated by direct interaction of Pitx3 with the Nurr1 transcriptional complex, and thus independent of the Pitx3-mediated induction of RA production.

Our data not only provide novel insights and postulate a new mechanism for Pitx3/Ahd2-mediated subset-specific regulation of RA-dependent target genes, but intriguingly also show that the expression of multiple dopaminergic target genes (*Cck*, *En1* and *En2*), which is upregulated in the absence of Pitx3, is RA independent. Importantly, the transcript level of these genes is not only upregulated in the *Ahd2*-expressing rostral mdDA area, but also in the more caudal mdDA area (supplementary material Fig. S1), strengthening our hypothesis that upregulation of these genes in the absence of Pitx3 is independent of the presence of Ahd2/RA, and mediated by a different mechanism. *Cck*, *En1* and *En2* all did not show up in our in vivo Pitx3 and Nurr1 ChIP-on-Chip analysis (Jacobs et al., 2009a), and might therefore not be regulated by direct binding of Pitx3 to their promoter. Thus, the molecular cascade by which Pitx3 represses *Cck*, *En1* and *En2* is likely to involve multiple, to date unidentified, processes and factors. Interestingly, recent analysis of En1 knockout embryos in our laboratory has provided us with some insight into the crosstalk between Pitx3 and En1. In En1 knockout embryos, *Th* expression is downregulated (data not shown), in line with data published by Simon et al. (Simon et al., 2001) showing the loss of all *Th* expression in *En1/2* double knockout mice (Simon et al., 2001; Wallen and Perlmann 2003; Sonnier et al., 2007). *En1* and *En2* upregulation could therefore partially compensate for the inability of *Pitx3* to induce *Th* expression in the caudal mdDA area, circumventing the need for *Pitx3*. Moreover, we found a downregulation of *Cck* in En1-deficient embryos (data not shown). As we show here that *En1* is upregulated in Pitx3-deficient embryos, the general upregulation and rostrolateral expansion of the *Cck* domain could reflect the upregulation of *En1* (and possibly *En2*).

Taken together, our current study provides multiple lines of evidence for a functional subdivision of the Pitx3 downstream cascade into RA-dependent and -independent gene-regulatory pathways. Intriguingly, the RA-dependent aspect of the Pitx3 downstream cascade is directly linked to the RA-independent regulation of the *Ad2* gene. This forms the basis for a simplified model regarding the role for Pitx3 in the mdDA neuronal population in which the RA-dependent aspects of the Pitx3-downstream cascade could be fully attributed to Ahd2 and to its involvement in the generation of RA (Fig. 8). Importantly, this indicates that the developmental defects in *Pitx3*-deficient embryos associated with RA-dependent aspects of the Pitx3-downstream cascade could also be attributed to the loss of Ahd2 and RA signaling. Therefore, the main cause for the selective vulnerability of the rostrolateral (SNc) mdDA neuronal subpopulation in *Pitx3*$^{-/-}$ mice may be directly linked to the restricted expression pattern of Ahd2 and the selective dependence of rostral emerging mdDA neurons (becoming SNc neurons) on RA signaling for repression of Dlk1, expression of *Th* and *D2R*, and possibly other aspects of mdDA differentiation. In addition, RA-independent regulation of Pitx3 target genes (such as *Ahd2*, *Vmat2*, *Dat*, *Cck* and *En1/2*) can affect both the rostrolateral (SNc) and caudal (VTA) mdDA neuronal subpopulations and RA-independent Pitx3-mediated modulation of subset-specific gene expression (as shown here for *Cck*) might add to the molecular distinction of SNc neurons compared with other cells in the mdDA neuronal population. This suggests that additional regulatory mechanisms restrict Pitx3-mediated regulation of some genes to a specific mdDA neuronal subset. These realizations further strengthen the importance of the understanding of subset specification and the role of Ahd2 and local RA signaling for neurons of the SNc. A closer understanding of the distinct molecular programming of different mdDA subsets sheds light on the black box of the complex process of mdDA subset formation and opens new avenues to investigate the selective vulnerability of SNc neurons as observed in PD. Complete understanding of the subset-specific molecular programming of distinct mdDA neuron populations provides us with an important tool to generate a specific subset of mdDA neurons out of stem/pluripotent cells, instead of a more heterogeneous DA population. Grafting of stem cells, differentiated into a specific mdDA neuronal subset fate, is likely to be favorable in conditions where a specific DA subset is affected, as grafting cells that mimic the originally affected cells as closely as possible will probably increase graft survival and minimize possible side-effects.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Table S1. List of in vivo Pitx3-regulated genes

Transcripts downregulated in E14.5 *Pitx3*-deficient mdDA neurons (*Pitx3*$^{-/-}$ versus *Pitx3*$^{+/+}$)

Non background corrected			
Gene symbol	BioSeq description	M-value	*P*-value
Slc18a2	Solute carrier family 18 (vesicular monoamine)	–0.37	0
Snca	Synuclein, alpha	–0.36	0
Tcf7l2	Transcription factor 7-like 2, T-cell specific, HMG-box	–0.32	0.01
Nr2f2	Nuclear receptor subfamily 2, group F, member 2	–0.25	0
Tubb2b	Tubulin, beta 2B	–0.23	0
Nhlrc2	NHL repeat containing 2	–0.2	0
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	–0.2	0
Gramd1a	GRAM domain containing 1A	–0.19	0.03
Hddc3	HD domain containing 3	–0.19	0
Tubb2c	Tubulin, beta 2C	–0.18	0.01
4833420G17Rik	RIKEN cDNA 4833420G17 gene	–0.17	0
Pi4k2a	Phosphatidylinositol 4-kinase type 2 alpha	–0.15	0.01
Lphn2	Latrophilin 2	–0.15	0.03
Crmp1	Collapsin response mediator protein 1	–0.15	0
Tubb2a	Tubulin, beta 2A	–0.15	0.01
Socs2	Suppressor of cytokine signaling 2	–0.14	0.02
AC102575.2		–0.14	0.04
Gsto1	Glutathione S-transferase omega 1	–0.12	0
2700078E11Rik	RIKEN cDNA 2700078E11 gene	–0.12	0
Usmg5	Upregulated during skeletal muscle growth 5	–0.12	0.01
AC142115.1	Putative uncharacterized protein fragment	–0.12	0
Cox15	COX15 homolog, cytochrome c oxidase assembly protein (yeast)	–0.11	0.05
Afp	Alpha fetoprotein	–0.11	0.03
Tmem130	Transmembrane protein 130	–0.1	0.01
Background corrected			
Gene symbol	BioSeq description	M-value	*P*-value
Snca	Synuclein, alpha	–2.55	0
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	–1.36	0
Slc6a3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	–1.03	0
Ttr	Transthyretin	–0.96	0.01
Afp	Alpha fetoprotein	–0.93	0

Transcripts upregulated in E14.5 *Pitx3*-deficient mdDA neurons (*Pitx3*$^{-/-}$ versus *Pitx3*$^{+/+}$

Non background corrected			
Gene symbol	BioSeq description	M-value	*P*-value
En2	Engrailed 2	0.8	0
En1	Engrailed 1	0.69	0
Cck	Cholecystokinin	0.42	0
Nts	Neurotensin	0.37	0
Spon1	Spondin 1, (f-spondin) extracellular matrix protein	0.31	0.03
Gbf1	Golgi-specific brefeldin A-resistance factor 1	0.3	0.05
Cbln1	Cerebellin 1 precursor protein	0.25	0
Slc32a1	Solute carrier family 32 (GABA vesicular transporter), member 1	0.22	0
Klf4	Kruppel-like factor 4 (gut)	0.21	0
Bloc1s2	Biogenesis of lysosome-related organelles complex-1, subunit 2	0.2	0
Olig1	Oligodendrocyte transcription factor 1	0.2	0
Slc6a4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	0.19	0.05
As3mt	Arsenic (+3 oxidation state) methyltransferase	0.18	0
Dlk1	Delta-like 1 homolog (Drosophila)	0.15	0.01
Tph2	Tryptophan hydroxylase 2	0.15	0.01
Glod5	Glyoxalase domain containing 5	0.14	0.01
Ociad1	OCIA domain containing 1	0.14	0.02
Pdgfra	Platelet derived growth factor receptor, alpha polypeptide	0.12	0.02

Nhedc2	Na+/H+ exchanger domain containing 2	0.12	0.01
Wls	Wntless homolog (Drosophila)	0.11	0.04
1500015O10Rik	RIKEN cDNA 1500015O10 gene	0.11	0.04
Background corrected			
Gene symbol	BioSeq description	M-value	*P*-value
Slc6a4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	1.27	0
En2	Engrailed 2	0.9	0
1500015O10Rik	RIKEN cDNA 1500015O10 gene	0.82	0
En1	Engrailed 1	0.81	0.01
2310002L09Rik	RIKEN cDNA 2310002L09 gene	0.75	0.02

The log differential-expression ratio (M-value) of differentially expressed genes in the mdDA area of E14.5 *Pitx3*$^{-/-}$ embryos compared with *Pitx3*$^{+/+}$ littermates with and without background correction (P<0.05, MAANOVA).

Table S2. List of qPCR primers

Primer	Forward (5′ to 3′)	Reverse (5′ to 3′)
q18s	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
qCCK	TAGCGCGATACATCCAGCAGGT	GGTATTCGTAGTCCTCGGCACT
qTH	TGCACACAGTACATCCGTCATGC	GCAAATGTGCGGTACGCCAACA
qeGFP	CGACGGCAACTACAAGAC	TAGTTGTACTCCAGCTTGTGC
qD2R	GATGTGCACAGCAAGCATCT	AGGACAGGACCCAGACAATG
qEn1	GGTCTACTGCACACGCTATTCG	AACTCCGCCTTGAGTCTCTGCA
qEn2	GGTCTACTGCACGCGCTATTCT	AAACTCAGCCTTGAGCCTCTGG
qDlk1	TGGCTGTGTCAATGGAGTCTGC	CCACGCAAGTTCCATTGTTGGC
qDat	GGTGCTGATTGCCTTCTCCAGT	GACAACGAAGCCAGAGGAGAAG
qVmat2	CCTCTTACGACCTTGCTGAAGG	GCTGCCACTTTCGGGAACACAT
qAhd2	GGAATACCGTGGTTGTCAAGCC	CCAGGGACAATGTTTACCACGC