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

Frank M. J. Jacobs1,*, Jesse V. Veenvliet1,2,*, Wadia H. Almirza1,2, Elisa J. Hoekstra1,2, Lars von OeRthel1,2, Annemarie J. A. van der Linden1, Roel Neijts1, Marian Groot Koerkamp3, Dik van Leenen3, Frank C. P. Holstege3, J. Peter H. Burbach1 and Marten P. Smidt1,2,‡

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...
differentiating mdDA neurons (McCaffrey and Dräger, 1994; Wallen et al., 1999; Jacobs et al., 2007). Moreover, cultured embryonic midbrains selectively activated RAR-α to but not RAR-β/γ 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 dopaminergic genes involved in mdDA function. We found that expression of Snca/Pitx3 gfp/gfp crossed with Nurr1+–/–;Pitx3gfp/gfp animals has been described previously (Saucedo-Cardenas et al., 1998). Retinoic acid treatment of pregnant Pitx3+/– Pitx3gfp/gfp mice For in situ hybridization and immunofluorescence analysis, either Pitx3+/– or Pitx3gfp/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 (Niederreiter 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 Pitx3gfp/– 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-mercaptoethanol 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 Pitx3gfp/– and Pitx3gfp/+ embryos were 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. Pitx3gfp/+ 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 Pitx3gfp/– and Pitx3gfp/+ 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 ImageJ 8.0 (BioDiscovery), print-tip Loess normalization was performed (Yang et al., 2002) on mean spot-intensities. Data were analyzed using ANOVA (R version 2.2.0, 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 Benjamin-Hochberg correction) were considered to be significantly changed. Microarray data have been deposited at GEO with accession number GSE32940.

MATERIALS AND METHODS Animals Pitx3+/– and Pitx3+/+ embryos were obtained as described previously (Smits et al., 2003; Jacobs et al., 2007). Pitx3-deficient Aphaia (Pitx3+/–), C57Bl6-Jico wild-type or heterozygous Aphaia (Pitx3+/–) mice were crossed with Pitx3gfp/gfp mice to obtain Pitx3gfp/– and Pitx3gfp/+ embryos. Alternatively, Pitx3gfp/– mice were intercrossed to obtain Pitx3gfp/–, Pitx3gfp/0 and Pitx3+/– embryos. Pitx3gfp/0 embryos are heterozygous for wild-type Pitx3 and green fluorescent protein (GFP), and have a normal development of the midDA system (Maxwell et al., 2005). Pitx3gfp/0 mice are Pitx3 deficient, Pitx3gfp/0 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+/–;Pitx3gfp/–, Nurr1+/–;Pitx3gfp/0 and Nurr1+/–;Pitx3gfp/0 embryos were obtained by first crossing Pitx3gfp/0 animals with Nurr1+/– animals, intercrossing the offspring heterozygous for both genes (Nurr1+/–;Pitx3gfp/0) and then crossing Nurr1+/–;Pitx3gfp/0 with Nurr1+/–;Pitx3gfp/0 animals. The Nurr1–/– mice have been described previously (Saucedo-Cardenas et al., 1998).
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°C 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≤0.05 is considered significant and is indicated by an asterisk; P≤0.01 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

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°C 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≤0.05 is considered significant and is indicated by an asterisk; P≤0.01 is indicated with double asterisks.
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 (Synca) 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 Pitx330+/+ and Pitx330−/− 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).

**Mдра 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 mDA 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 mDA 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 Pitx330−/− embryos revealed that colocalization of Pitx3 and Dlk1 is strictly limited to the more caudal mDA area in medial sections (Fig. 3C,D). Similarly, colocalization of Dlk1 and Th is also limited to caudal mDA 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,H). Interestingly, this region contains the mDA 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 Pitx330+/+ and Pitx330−/− embryos with Dlk1 expression in Pitx330+/−/H11032 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 Pitx330−/− embryos (Fig. 4B). Strikingly, embryonic RA treatment of Pitx330−/− embryos clearly restricted the expression domain of Dlk1 in the rostral part of the mDA area (Fig. 4D), to a pattern highly similar to wild-type embryos (Fig. 4D). These striking effects were not observed in the caudal area (Fig. 4A-C). To verify whether the Pitx3/RA-mediated regulation of Dlk1 transcription was followed by a change in Dlk1 protein in Pitx3-GFP positive DA neurons, we performed immunofluorescence on sagittal sections of Pitx330+/−, Pitx330+/+ and RA-treated Pitx330+/−/H11032 embryos. We confirmed that in the absence of Pitx3, Dlk1 protein is upregulated in mDA neurons in Pitx330+/−/H11032 embryos and observed a rostral expansion of Pitx3/Dlk1 colocalization in Pitx330+/−/H11032 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 rostral lateral 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′).

**Dat, Vmat2 and Ahd2 are dopaminergic genes that are downregulated in Pitx3-deficient embryos and co-regulated by Nurr1 and Pitx3**

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
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 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 rostral lateral 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 rostral lateral 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
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).
**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 rostral-d lateral 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 expression 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 Nurrl 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 Nurrl-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 Nurrl 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 Nurrl 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.

Acknowledgements
We thank Ger Arkestein for assistance with FAC sorting. The Pitx3-GFP animals were a kind gift from Meng Li.

Funding
This work was supported by a HIPO-grant [IUU to M.P.S.]; by a VICI-grant [865.09.002 to M.P.S.]; and by EUFP7 funding to the mdDAneurodev [222999] consortium, coordinated by M.P.S.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071704/-/DC1


