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Publication date
2016

Document Version
Other version

License
Other

Citation for published version (APA):
Kouwenhoven, W. M. (2016). The rise and fall of mesodiencephalic dopaminergic neurons: Molecular programming by transcription factors Engrailed 1, Pitx3, and Nkx2.9 during the development of mesodiencephalic neurons.
Specification of dopaminergic subsets involves interplay of En1 and Pitx3

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PUBLISHED:
DEVELOPMENT, 140 (16), 3373:384 (2013)
ABSTRACT
Mesodiencephalic dopaminergic (mdDA) neurons control locomotion and emotion and are affected in multiple psychiatric and neurodegenerative diseases, including Parkinson’s disease (PD). The homeodomain transcription factor Pitx3 is pivotal in mdDA neuron development and loss of Pitx3 results in programming deficits in a rostrolateral subpopulation of mdDA neurons destined to form the substantia nigra pars compacta (SNc), reminiscent of the specific cell loss observed in PD. We show here that in adult mice in which the gene encoding a second homeoprotein, engrailed 1 (En1), has been deleted, dramatic loss of mdDA neurons and striatal innervation defects were observed, partially reminiscent of defects observed in Pitx3−/− mice. We then continue to reveal developmental crosstalk between En1 and Pitx3 through genome-wide expression analysis. During development, both En1 and Pitx3 are required to induce expression of mdDA genes in the rostrolateral subset destined to form the SNc. By contrast, Pitx3 and En1 reciprocally regulate a separate gene cluster, which includes Cck, demarcating a caudal mdDA subset in wild-type embryos. Whereas En1 is crucial for induction of this caudal phenotype, Pitx3 antagonizes it rostrolaterally. The combinatorial action of En1 and Pitx3 is potentially realized through at least three levels of molecular interaction: (1) influencing each other’s expression level, (2) releasing histone deacetylase-mediated repression of Nurr1 target genes and (3) modulating En1 activity through Pitx3-driven activation of En1 modulatory proteins. These findings show how two crucial mediators of mdDA neuronal development, En1 and Pitx3, interact in dopaminergic subset specification, the importance of which is exemplified by the specific vulnerability of the SNc found in PD.
INTRODUCTION
The pathological hallmark of Parkinson’s disease (PD) is progressive neurodegeneration of the substantia nigra pars compacta (Barzilai and Melamed, 2003). Since cell replacement therapy was acknowledged as a viable treatment for PD, various neurodevelopmental studies have focused on the generation of a good cell replacement model (Arenas, 2010; Gaillard and Jaber, 2011; Toulouse and Sullivan, 2008; Kriks et al., 2011; Caiazzo et al., 2011). To obtain a protocol for successful differentiation of stem/inducible pluripotent cells into transplantable mesodiencephalic dopaminergic (mdDA) neurons that can functionally replace neurons degenerated in PD, detailed understanding of the transcriptional programs leading to generation of healthy mdDA neurons is necessary. Extensive efforts have been made in the last decade to identify signaling pathways and transcription factors crucial for mdDA development (Smidt and Burbach, 2007). A key factor in mdDA neuron development is the orphan nuclear receptor Nurr1 (Nr4a2). Nurr1 is essential for transcriptional activation of genes essential for dopamine (DA) signaling, such as tyrosine hydroxylase (Th), aromatic-L-amino acid decarboxylase (Aadc; Ddc) (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998), vesicular monoamine transporter 2 (Vmat2; Slc18a2), dopamine receptor D2 (D2R; Drd2) and dopamine transporter (Dat; Slc6a3) (Smits et al., 2003; Jacobs et al., 2009a). Whereas Nurr1 is essential for differentiation and survival of all mdDA neurons, the paired-like homeobox gene Pitx3 is crucial only for the induction of a specific mdDA subpopulation that ultimately forms the SNc (Smidt et al., 2004; Smidt et al., 1997; Jacobs et al., 2007; Jacobs et al., 2011). Pitx3 functions as an essential potentiator of Nurr1 in specification of the DA phenotype. Pitx3 and Nurr1 occupy promoters of DA-related genes in concert and Pitx3 releases histone deacetylase (HDAC)-mediated repression from the Nurr1 transcriptional complex (Jacobs et al., 2009a). We recently identified Aldh1a1 and Cck as novel Pitx3 targets (Jacobs et al., 2007; Jacobs et al., 2011). Both are downregulated in Nurr1-deficient embryos but display differential dependence on Pitx3: whereas Aldh2 is downregulated in Pitx3−/− embryos, Cck is upregulated (Jacobs et al., 2007; Jacobs et al., 2011; Wallén et al., 1999) and its expression expands into the rostrolateral mdDA subpopulation. This expansion is accompanied by rostrolateral upregulation of a second homeoprotein that is highly expressed during mdDA neuron development, engrailed 1 (En1) (Jacobs et al., 2011). In the brain, En1 expression is mainly restricted to the mid-hindbrain border (MHB) and is highly expressed by all mdDA neurons continuously from the moment they differentiate until adulthood (Simon et al., 2001). En1 is involved in mdDA survival and maintenance during development (Simon et al., 2003; Albéri et al., 2004) and
complete deficiency of En1 results in a loss of hindbrain tissue with concomitant loss of most of the cerebellum (Wurst et al., 1994). To date, analyses have focused on the En1/En2 double knockout, and a detailed mechanistic study of mdDA system development in En1 single-null mutants has not been performed.

We aimed to fill this gap by detailed analysis of the role of En1 during terminal differentiation and specification of the mdDA area. We show that En1 induces Nurr1 target genes during development (including Th, Dat and Ahd2) in a rostrolateral subset of mdDA neurons, and that absence of En1 results in similar defects in this subset of neurons to those observed in Pitx3–/– embryos. Genome-wide expression analysis revealed that En1 and Pitx3 co-regulated, mostly in a reciprocal manner, various factors, including Cck, which marks a caudal subset of mdDA neurons. The caudal DA phenotype is induced by En1, but repressed by Pitx3 in the mdDA area position. En1-Pitx3 crosstalk might occur through at least three levels of molecular interaction: (1) influencing each others’ expression, (2) releasing HDAC-mediated repression of Nurr1 target genes and (3) modulating En1 activity through Pitx3-driven activation of En1 modulatory proteins that are expressed in specific subsets of the mdDA expression domain. Our data suggest that a delicate balance between transcriptional activity of the homeoproteins En1 and Pitx3 is crucial for the proper induction of distinct mdDA subsets.

MATERIALS AND METHODS

ANIMALS

Adult En1tm1Alj/J+/– mice were backcrossed to the C57BL6/J line and then used in heterozygous breeding, generating En1+/+ and En1–/– progeny. Embryos were collected at embryonic day (E)13.5 or E14.5 (E0.5 defined as day of copulatory plug). Genotyping was performed by PCR analysis using specific primers (Wurst et al., 1994).

Pitx3gfp/gfp and Pitx3 gfp/+ littermate embryos were obtained as described previously (Jacobs et al., 2011; Zhao et al., 2004). Pitx3 gfp/+ embryos are heterozygous for wild-type Pitx3 and green fluorescent protein (GFP), and have normal mdDA system development (Maxwell et al., 2005). Pitx3gfp/gfp mice are Pitx3 deficient. All procedures were according to and fully approved by the Dutch Ethical Committees for animal experimentation (UMC-U and UvA).

MICROARRAY ANALYSIS

RNA was isolated from dissected E13.5 En1–/– and En1+/+ ventral midbrains (VMs). Microarray analysis was performed on four independent samples. Each experimental sample consisted of pooled RNA from three En1–/– VMs, which was
hybridized to a reference pool consisting of RNA derived from ten En1+/+ VMs. Analysis was performed as described (Roepman et al., 2005) using custom arrays containing mouse 70-mer oligonucleotides (Operon, Mouse V2 AROS). Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7) (Wu et al., 2003). Genes with P<0.05 after false discovery rate correction were considered to be significantly changed. Data have been deposited in ArrayExpress under accession number E-TABM-711. Gene ontology (GO) analysis was performed using the BiNGO 2.44 plug-in (Cytoscape 2.8.2; Hypergeometric test; FDR correction; GO_Biological_Process; whole annotation as reference) with all En1-regulated genes as input.

IN SITU HYBRIDIZATION (ISH)
ISH was performed as described (Smits et al., 2003). Digoxigenin-labeled probes for Th, Vmat2, Cck, Dat, Nurr1, Aadc, Ahd2, Pitx3 and Nts have previously been described (Grima et al., 1985; Smits et al., 2003; Jacobs et al., 2011; Smits et al., 2004; Jacobs et al., 2007). Other probes used were: Pbx1, bp 1644-2277 of mouse coding sequence (CDS); Pbx3, bp 751-1803 of mouse CDS; Tle3, bp 1950-2928 of mouse CDS; Tle4, bp 2720-3514 of mouse CDS.

NISSEL STAINING
Sections (16 μm) were post-fixed in 4% paraformaldehyde (PFA) for 30 minutes, rinsed in PBS and then milli-Q water (mQ), and subsequently stained in 0.25% Cresyl Violet (+1% acetic acid) for 20 minutes. Sections were rinsed in mQ and 50% ethanol, differentiated in 70% and 96% ethanol, dehydrated in 100% ethanol, cleared in xylene and mounted with Entellan (Merck).

IMMUNOHISTOCHEMISTRY
Sections (16 μm) were post-fixed in 4% PFA for 30 minutes then DAB (3,3′-diamino-benzidine) staining was performed as described (Smidt et al., 2004). Cell culture and transfections followed by silver staining MN9D cells were cultured and transfected as described previously (Jacobs et al., 2007). Cells were transfected with 0.5 μg of En1-pcDNA3.1(−)-myc-His or an equal molar amount of empty pcDNA3.1(−)-myc-His expression vector after which His-tagged proteins were purified using Ni-NTA magnetic agarose beads (Qiagen) according to the manufacturer’s instructions. Purified proteins were separated by SDS-PAGE and visualized by protein gel silver staining as described previously (Jacobs et al., 2009a). Protein bands of interest were excised and subjected to nanoLC-ESI-MS mass spectrometry analysis (Proteome Factory).
IMMUNOPRECIPITATION (IP) AND WESTERN BLOTTING
MN9D-N13-cells were homogenized in lysis buffer and subjected to IP and western blot analysis as previously described (Jacobs et al., 2009a). Antibodies used were: anti-En1 (M04; abnova; 1 μg/ml); anti-En1 (ab32817; abcam; 1:2000); anti-PSF (Sfpq) (B92; Sigma; 1:2500); anti-His (ab9108; abcam; 1:20,000). Blots were incubated with SuperSignal and, exposed to ECL films (Pierce).

EXPLANT CULTURE
E13.5 En1−/− and En1+/+ VMs were dissected, cultured as previously described (Jacobs et al., 2009a) and treated with 0.6 mM sodium butyrate (Sigma) or mock (ddH2O) for 48 hours, after which midbrains were homogenized and RNA extracted with Trizol (Invitrogen).

FLUORESCENCE-ACTIVATED CELL SORTING (FACS)
Freshly dissected VMs were dissociated using a Papain dissociation system (Worthington). Cells were sorted on a Cytopeia Influx Cell sorter or BD FACS Aria III using previously described settings (Jacobs et al., 2011) and collected in Trizol-LS (Invitrogen).

QUANTITATIVE PCR (QPCR)
Relative expression levels were determined by qPCR real-time PCR (Lightcycler) using the QuantiTect SYBR Green PCR LightCycler Kit (QIAGEN) according to the manufacturer’s instructions. For each reaction, 25 ng (dissected midbrain) or 0.1 ng (FAC-sorted neurons) total RNA was used as input. Primer sequences are listed in supplementary material Table S5.

STATISTICAL ANALYSIS
Quantified qPCR results represent average values of experiments performed on 3-12 biological samples for each condition. Data indicate means with standard errors (s.e.m.). For array validation (Fig. 3B) and rostral versus caudal analysis (Fig. 1C,D; Fig. 8B), statistical analysis was performed by Student’s t-test (one-way unpaired). Other qPCR experiments required multiple litters (Fig. 8D) or explant culture experiments (Fig. 7B-D) to obtain a sufficient sample size. We therefore stratified available embryos between conditions and corrected for inter-litter or inter-experimental variation using one-way ANCOVA with litter/experimental set as covariate. Measurements that differed by ≥2× standard deviations from the mean were excluded. *P≤0.05; **P≤0.01.
**FIG. 1.** AHD2 AND CCK MARK DISTINCT DOPAMINERGIC SUBSETS DURING MD DA NEURON DEVELOPMENT. (A) Pseudo-overlays of adjacent sections (section planes indicated by red box in mdDA schematics) stained for Ahd2, Cck and Dat show that expression of Cck and Ahd2 is mutually exclusive and mark a caudal and rostral lateral subset, respectively. C, caudal; R, rostral. (B) E14.5 Pitx3gfp/+ midbrains were microdissected and separated into a rostral and caudal part. Neurons were dissociated and subjected to FACS to obtain a purified DA neuron population [GFP+, indicated by black arrow, compare with Bl6-Ref (control wild-type midbrain)]. Caudal mdDA: ±15,000 GFP+ cells; Rostral mdDA: ±2000 GFP+ cells. RNA was then isolated and subjected to qPCR. F, forebrain; H, hindbrain; M, midbrain. (C,D) qPCR confirmed rostral enrichment of Ahd2 (C) and caudal enrichment of Cck (D). 18S expression was unchanged (P=0.98), and served to normalize for amount of input RNA. **P<0.01; n=5. Error bars represent s.e.m.
RESULTS
AHD2 AND CCK MARK DISTINCT SUBSETS DURING MD DA NEURON DEVELOPMENT

Pitx3–/– embryos display mdDA neuron programming deficits in a rostral-lateral subpopulation that ultimately forms the SNc (Smidt et al., 2004; Nunes et al., 2003; Hwang et al., 2003; van den Munckhof et al., 2003). This subset is closely marked by Ahd2 expression, which is lost in the absence of Pitx3 (Jacobs et al., 2007). We recently identified Cck as a novel Pitx3 target. Cck is upregulated in Pitx3–/– embryos (Jacobs et al., 2011) and its expression expands into the rostral-lateral subpopulation. We therefore hypothesized that Ahd2 and Cck mark distinct mdDA subsets during normal mdDA neuron development. Indeed, ISH on adjacent E14.5 wild-type sections revealed that Ahd2 and Cck mark distinct mdDA domains (Fig. 1A). Whereas overlap between Ahd2 and the DA neuron marker Dat was confined to a rostral subpopulation, Cck expression was restricted to caudal mdDA neurons. Interestingly, mediolateral Cck expression overlapped with that of Dat, whereas more medially Dat was strictly limited to the Ahd2+ mdDA domain (Fig. 1A). To confirm that Ahd2 and Cck demarcate a rostral and caudal mdDA subpopulation, respectively, we microdissected VMs from Pitx3 gfp/+ embryos and separated the rostral from the caudal midbrain. Neurons from each area were dissociated, subjected to FAC-sorting and RNA was isolated to obtain mRNA from purified rostral and caudal mdDA neurons (Fig. 1B). qPCR confirmed enrichment of Ahd2 in rostral mdDA neurons (Fig. 1C; P<0.01) and Cck enrichment in caudal mdDA neurons (Fig. 1D; P<0.01).

ARCHITECTURE OF THE MD DA AREA OF ADULT EN1 KNOCKOUT MICE

We previously reported that upregulation and expansion of the Cck expression domain in Pitx3–/– embryos is accompanied by En1 upregulation (Jacobs et al., 2011). In analogy with the recent finding that the homeoprotein Otx2 specifies neuron subtype identity in the ventral tegmental area (VTA) (Di Salvio et al., 2010), we set out to investigate whether the homeoproteins Pitx3 and En1 could act in a combinatorial code during dopaminergic subset specification, which ultimately gives rise to the distinct mdDA subpopulations outlined in Fig. 1.

As the adult Pitx3 knockout phenotype is well described (Smidt et al., 2004; Nunes et al., 2003; Hwang et al., 2003; van den Munckhof et al., 2003), we next determined the adult En1 knockout phenotype. Detailed characterization of the mdDA system in En1 single-null mutant mice is lacking because previous studies have focused on deletion of one En1 allele in a wild-type or En2-null background (Sgadò et al., 2006; Sonnier et al., 2007; Wurst et al., 1994; Alvarez-
Fischer et al., 2011), leaving the role of En1 in mdDA neuron development and subset specification undefined. Analysis of the mdDA area in En1−/− adult mice has been hampered because engineered null mutations lead to perinatal lethality accompanied by cerebellar deletion (Wurst et al., 1994). We circumvented this impediment by transferring the original En1-null allele from the 129/Sv strain to the C57BL/6J background, which suppresses the cerebellar phenotype (Bilovocky et al., 2003). C57BL/6J-En1−/− mice were born alive, smaller than littermates and displayed limb defects that are not suppressed in the C57BL/6J background.

**Fig. 2. The architecture of the mdDA area and striatal projections are affected in En1−/− adult mice.** (A) Th expression in coronal sections of the mdDA neuronal region. Th expression in the substantia nigra pars compacta (SNc) and the ventral tectal area (VTA) is mildly affected in En1+/− and severely affected in En1−/− adult mice (red arrowheads). (B) Nissl staining in adjacent sections revealed decreased DA cell density in both rostral and medial parts of the SNc in En1+/− and En1−/− mice in a dose-dependent manner (indicated by red arrowheads). High magnification images of the boxed areas are shown. (C) Coronal sections of the striatal projection area stained for Th protein show innervation defects (red arrowheads) of caudate putamen (Cpu) and nucleus accumbens (Acb) in En1−/− mice compared with wild type, with the En1+/− displaying intermediate phenotype.
To analyze the DA system in these mice, we compared the distribution of Th+ neurons in the mdDA area of 6-week-old En1+/–, En1+/– and wild-type littermates. A decrease of Th+ DA neurons was observed in En1+/– mice in both SNc and VTA compared with wild type (Fig. 2A), indicating an early onset of the progressive DA cell loss that was previously documented at later stages in En1+/– mice (Sonnier et al., 2007). En1–/– mice displayed a dramatic decrease of Th+ neurons in both SNc and VTA compared with wild-type and En1+/– mice (Fig. 2A). To determine whether mdDA neurons were absent in En1–/– mice or whether they no longer expressed Th, we performed Nissl staining on adjacent sections (Fig. 2B) and found a severe reduction of DA cell density in the VTA of En1-deficient mice. In the SNc, almost all DA neurons in the rostrolateral part were absent, whereas more medially the density was reduced.

In the striatal projection area, Th+ fibers were lost in the nucleus accumbens (Acb) and caudate putamen (Cpu) of En1–/– mice, although parts of the Cpu were still innervated. En1+/– mice displayed an intermediate phenotype (Fig. 2C). This phenotype is in striking contrast with the restricted connectivity observed in Pitx3–/– mice, in which Th+ fibers are lost in the Cpu, whereas the Acb is still innervated (Smidt et al., 2004). Our data show that En1 has a dose-dependent effect on the development and survival of mdDA neurons and specific innervation of the striatal projection area.

**Identification of En1-regulated genes in En1-deficient embryos**

In Pitx3–/– mice, malformation of the adult mdDA system is preceded by developmental failure of mdDA neurons (Smidt et al., 2004). To study the role of En1 during mdDA neuron development, we performed genome-wide expression analysis of E13.5 En1–/– versus En1+/+ dissected VMs. Microarray analysis (MAANOVA-FDR) revealed 204 significantly regulated genes (supplementary material Tables S1, S2). Gene ontology analysis (Fig. 3A) revealed the biological processes affected in En1–/– embryos, including neurogenesis, neural development, chromatin organization, axonogenesis, axon guidance, neuron migration, Wnt receptor signaling pathway, apoptosis, retinoic acid metabolic process and midbrain/diencephalon/hindbrain development.

Most importantly, key genes in DA metabolism, Th, Dat and Aadc, were identified as novel transcriptional targets of En1 (downregulated in En1–/– embryos; supplementary material Table S2). For validation, we subjected RNA from a separate set of dissected E13.5 En1+/+ and En1–/– VMs to qPCR and confirmed downregulation of Dat, Th and Cck (P<0.01) (Fig. 3B). Aadc expression
FIG. 3. IN VIVO GENOME-WIDE EXPRESSION ANALYSIS REVEALS CROSSTALK BETWEEN THE HOMEOPROTEINS EN1 AND PITX3. (A) GO analysis identified a key role for En1 in multiple biological processes during mdDA development. (B) qPCR analysis confirmed downregulation of key DA-related genes in En1−/− embryos (wild type, n=3; knockout, n=5; **P≤0.01). 18S expression was unchanged (P=0.89), and served to normalize for amount of input RNA. Red line indicates relative wild-type expression. Error bars represent s.e.m. (C) Venn diagram of MAANOVA-FDR-corrected lists of genes affected in Nurr1-, Pitx3- and En1-deficient embryos revealed genes co-regulated by En1, Pitx3 and Nurr1. (D) Heatmap generated by hierarchical clustering (MeV v4.7.3) of genes that are significantly regulated (MAANOVA-FDR) in both Pitx3−/− and En1−/− embryos. Color scale bar represents relative expression [M-value=2log(fold-change); green, upregulation; red, downregulation]. (E) Whereas Dat (Slc6a3) and Scn3b are downregulated in both Pitx3−/− and En1−/− embryos, most genes downregulated in Pitx3−/− embryos are upregulated in En1−/− embryos. (F) Genes downregulated in En1−/− embryos are upregulated in Pitx3−/− embryos. Red lines in E,F indicate relative wild- type expression.
was not significantly reduced in this dataset, but local downregulation was confirmed in independent ISH and qPCR experiments (Fig. 4C,F; Fig. 7D). Importantly, unaffected transcript levels of the housekeeping gene 18s (Rn18s) and the DA neuron marker gene *Nurr1* (Fig. 3B), in combination with an unaffected *Nurr1* expression domain (Fig. 5C,D) indicate that reduced expression of DA-related genes represents programming deficits rather than cell loss at this stage.

**EN1 AND PITX3 CROSSTALK IN THE REGULATION OF NURR1 TARGET GENES**

Downregulation of the *Nurr1*-target genes *Dat* and *Th* (Saucedo-Cardenas et al., 1998) in En1–/– embryos is reminiscent of the phenotype in Pitx3–/– embryos (Jacobs et al., 2011), providing further evidence for En1-Pitx3 interplay. To identify genes regulated by both homeoproteins, we performed an overlay of transcripts regulated in En1–/– embryos with the MAANOVA-FDR analysis of genes regulated in Pitx3–/– embryos (Jacobs et al., 2011) (supplementary material Tables S3, S4). This analysis identified 19 genes that are regulated by both Pitx3 and En1, six of which were previously identified as *Nurr1* target genes (Jacobs et al., 2009b; Jacobs et al., 2011) (Fig. 3C). Strikingly, only two of these genes were regulated in the same direction in both En1 and Pitx3 knockout embryos (including *Dat*). All other genes were reciprocally regulated: genes downregulated in En1–/– embryos were upregulated in Pitx3–/– embryos and vice versa (Fig. 3D-F).

**EN1-DEFICIENT EMBRYOS FAIL TO INDUCE THE DOPAMINERGIC PHENOTYPE IN A ROSTROLATERAL MDDA SUBSET**

Analysis of Pitx3–/– mice identified mdDA subsets that are differentially dependent on Pitx3 (Smits et al., 2006; Jacobs et al., 2007; Jacobs et al., 2011). To determine whether En1 deficiency affects *Nurr1* target gene expression in a subset-specific manner, we performed ISH on E14.5 En1–/– embryos and wild-type littermates. *Th* and *Dat* expression was downregulated in the rostrolateral mdDA system, but relatively unaffected in the mediocaudal part of the midbrain (Fig. 4A,B,F). *Aadc* expression was subtly affected throughout the whole mdDA in area En1-deficient embryos (Fig. 4C,F). *Vmat2* and *D2R* were not identified by microarray analysis as En1 target genes, but their expression is downregulated in *Nurr1* and Pitx3 knockout embryos (Jacobs et al., 2009a; Smits et al., 2003). We therefore analyzed En1–/– embryos and found a reduction of *Vmat2* and *D2R* expression (Fig. 3D-F). Interestingly, all analyzed DA-related genes except *D2R* displayed caudal expansion of their domain in more medial sections, with expression of *Th, Dat, Aadc* and *Vmat2* behind the MHB (Fig. 4A-D,G, black dotted line).
**Fig. 4. Nurr1 and Pitx3 Target Genes Are Downregulated in the mdDA Area, but Ectopically Expressed in the Metencephalic Area in En1−/− Embryos.** (A-E) The dopaminergic markers Th (A) and Dat (B) are downregulated in En1−/− embryos. The affected rostrolateral subset is demarcated by red dotted lines and indicated by red arrowheads. The Nurr1 target gene Aadc is downregulated in both rostrolateral (C; demarcated by red dotted lines, indicated by red arrowhead) and caudal (G; red arrow in front of black dotted line) DA neurons. The Nurr1 and Pitx3 target genes Vmat2 (D) and D2R (E) are downregulated throughout the whole mdDA area in En1−/− embryos (red arrowheads). Th, Dat, Vmat2 and Aadc all display ectopic expression in the metencephalon of En1−/− embryos (A-D), indicated by black arrowheads (MHB indicated by black dotted line). (F) Higher magnifications of the affected rostrolateral area (red box in mdDA schematics; whole mdDA area in green). The rostrolateral population is demarcated by red dotted lines, affected areas indicated by red arrows. (G) Higher magnifications of caudal area (red box in mdDA schematic; whole mdDA area in green) showing expansion of DA-related genes (except D2R) behind the MHB (black dotted line, affected area indicated by black arrows).
The rostrolateral defect observed in En1−/− embryos is highly reminiscent of the phenotype in Pitx3−/− embryos (Jacobs et al., 2011; Jacobs et al., 2007). We therefore analyzed Pitx3 expression in En1−/− embryos and observed a downregulation in the rostrolateral mdDA area (Fig. 5A,F). As this subset is closely marked by the expression of Ahd2, a direct transcriptional target of Pitx3 (Jacobs et al., 2007), we analyzed Ahd2 expression in En1+/+ and En1−/− embryos by ISH and observed complete loss of Ahd2 transcript in En1-deficient embryos (Fig. 5B,F).

The unaffected expression of the early post-mitotic mdDA precursor marker Nurr1 (Smidt and Burbach, 2007) in this population (Fig. 5C,D) indicates that the absence of DA neuron marker expression within this area is not the result of neuronal loss or Nurr1 transcriptional activity, but a direct consequence of En1- or Pitx3-driven programming defects. To strengthen this hypothesis, we analyzed the expression of Nurr1 and the mdDA progenitor marker Lmx1a (Smidt and Burbach, 2007) in En1−/− and En1 +/- at E12.5 and observed no differences in the distribution of either marker (supplementary material Fig. S2A,B), suggesting that the identity of
Interplay of En1 and Pitx3

mdDA progenitors and post-mitotic neurons is not affected in the absence of En1.

Notably, both Nurr1 and Pitx3 were ectopically expressed behind the MHB (Fig. 5A,C,E, black dotted line), suggesting that the metencephalic expansion of the DA phenotype (Fig. 4A-D,G) is caused by ectopic expression of Pitx3 and Nurr1.
CAUDAL SUBSET-RESTRICTED GENES CCK AND NTS ARE DOWNREGULATED IN EN1-DEFICIENT EMBRYOS

Cck marks a caudal subpopulation of the mdDA area that is not affected in Pitx3−/− embryos and is destined to form the VTA (Fig. 1). Pitx3 loss leads to upregulation and rostrolateral expansion of Cck (Jacobs et al., 2011). By contrast, our microarray and qPCR data showed downregulation of Cck in En1−/− embryos (Fig. 3). Indeed, ISH revealed severe downregulation of Cck in the caudal mdDA area of En1−/− embryos (Fig. 6A,C). Nts is also downregulated in the absence of En1 but upregulated in Pitx3−/− embryos (Fig. 3). Like Cck, Nts expression was restricted to the caudal mdDA system in wild type and downregulated within this area in En1−/− embryos (Fig. 6B,D). Thus, in contrast to Th, Dat and Vmat2, expression of which is similarly affected in the rostrolateral mdDA in En1−/− and Pitx3−/− embryos, the caudally restricted factors Cck and Nts are reciprocally regulated by Pitx3 and En1.

EN1 RELEASES HDAC-MEDIATED REPRESSION OF NURR1 TARGET GENES

We recently reported that Pitx3 releases HDAC-mediated repression of Nurr1 target genes and showed that treatment with the non-specific HDAC inhibitor sodium butyrate (NaB) fully restores Th transcript levels in Pitx3-deficient Pitx3gfp/− embryos (Jacobs et al., 2009a).

Given the striking similarity in the rostrolateral phenotype of Pitx3- and En1-deficient embryos, we tested whether HDAC-inhibition in En1-deficient mdDA neurons could re-activate Nurr1 target genes. We treated explant cultures of E13.5 En1+/+ and En1 −/− VMs with NaB for 48 hours then subjected isolated RNA to qPCR (Fig. 7A). Relative expression levels were normalized to Tbp, for which no change was observed (data not shown). In En1+/+ midbrains, the relative amounts (untreated relative to treated) of Th and Cck transcripts were unchanged upon NaB treatment (Fig. 7B,C). In agreement with the in vivo expression analysis (Fig. 3), Aadc, Th and Cck were downregulated in mock-treated En1−/− midbrains (Fig. 7B-D). Strikingly, treatment of En1−/− midbrains with NaB significantly increased Th expression, restoring levels to 100% upon treatment (Fig. 7B; P<0.05). A smaller, but significant, increase of Cck transcript was detected (Fig. 7C; P<0.05). For Aadc, a non-significant decrease in wild-type midbrains upon NaB treatment was observed, reducing transcript levels to En1−/− values (Fig. 7D; P=0.07). These data demonstrate that release of HDAC-mediated repression in En1−/− embryos completely restores Th expression, bypassing the requirement for En1.

Release of HDAC-mediated repression and subsequent activation of Nurr1
Interplay of En1 and Pitx3 target genes by Pitx3 might be mediated by recruitment to the Nurr1–PSF transcriptional complex, given the direct interaction of the transcriptional co-repressor PSF with both Nurr1 and Pitx3 (Jacobs et al., 2009a). We therefore tested whether En1, like Pitx3, could physically interact with the Nurr1–PSF complex (Fig. 7E). We affinity purified En1-interacting proteins by using an En1-His fusion protein as bait on a His-column purification set-up. En1-His protein or His protein alone (control) was overexpressed in DA MN9D cells, total lysates were subjected to nickel bead columns and the eluate was isolated for analysis (Fig. 7F,G). SDS-PAGE and subsequent silver staining revealed a specific differential band in the

**FIG. 7. INTERFERENCE WITH HDAC-MEDIATED REPRESSION IN EN1-DEFICIENT MIDBRAINS RESTORES TH AND CCK EXPRESSION.** (A) Microdissected littermate En1+/+ and En1−/− midbrains (area between black lines) were treated with the HDAC inhibitor (HDACi) sodium butyrate (NaB) or ddH20 (mock control) for 48 hours. mRNA was then isolated and subjected to qPCR (n=5-7). F, forebrain; H, hindbrain; M, midbrain. (B) Strikingly, Th expression, decreased in En1−/− embryos, was completely restored by NaB (P=0.045), whereas expression in En1+/+ embryos was not affected by treatment (P=0.789). (C) Likewise, Cck expression was upregulated by HDACi treatment in En1−/− (P=0.046) but not in wild-type embryos (P=0.447). NaB treatment did not restore Aadc expression (P=0.779), but a non-significant decrease of expression by HDACi was observed in wild type (P=0.074) (D). *P<0.05; #P=0.07. (E) Jacobs et al. (Jacobs et al., 2009a) proposed that Pitx3 releases HDAC-mediated repression of Nurr1 target genes by recruitment to the Nurr1–PSF transcriptional complex. Re-activation of Nurr1 target genes upon HDAC inhibition in En1−/− embryos suggests that En1 mediates transcription of Nurr1 target genes in a similar manner. (F-H) His-tag based affinity purification [experimental set-up in F, successful pull-down of En1-His confirmed by immunoblotting (G)] with subsequent silver-staining (H) revealed a 95 kDA protein band (black arrowhead) that specifically interacts with En1-His and was identified as PSF by mass-spectrometry (MASCOT). (I) Specific En1-PSF interaction was confirmed by IP of En1 and subsequent detection of PSF. Ctrl, pre-immune serum control. Error bars represent s.e.m.
En1-His purified sample positioned just above the 95 kDa marker that was identified by mass spectrometry as PSF (Fig. 7H). For validation, we overexpressed En1 or empty vector (control) in MN9D cells, immunoprecipitated with an En1 antibody and confirmed that PSF physically interacts with En1 (Fig. 7I), suggesting that both En1 and Pitx3 release HDAC-mediated repression of Nurr1 target genes by recruitment of the Nurr1–PSF transcriptional complex (Jacobs et al., 2009a).

**Pitx3 regulates En1 modulatory proteins**

As a third possible mechanism of En1-Pitx3 interplay, we hypothesized that Pitx3 regulates the transcription of genes encoding proteins that modulate En1 transcriptional activity. Candidates are Groucho (Grg/Tle) and Pbx family members that bind Engrailed Homology (Eh) domains and modulate En1 transcriptional activity (Serrano and Maschat, 1998; Peltenburg and Murre, 1996; Dasen et al., 2001). ISH analysis in E14.5 wild-type embryos revealed clear *Pbx1*, *Pbx3* and *Tle3* expression in the En1 and Pitx3 mdDA expression domain, whereas *Tle4* expression was restricted to the most rostral tip of the Pitx3 expression domain (Fig. 8A). To verify subset-specific expression of *Pbx1*, *Pbx3*, *Tle3* and/or *Tle4*, we subjected rostral and caudal FAC-sorted mdDA neurons to qPCR and found clear enrichment of *Pbx1* and *Tle3* expression within caudal mdDA neurons (Fig. 8B; *P*<0.01). For *Tle3*, this caudal restriction was clearly visible in, and confined to, medial sections (Fig. 8A). Transcript levels of both *Pbx3* and *Tle4* were higher in rostral compared with caudal mdDA neurons (*P*=0.02 for *Tle4*; *P*=0.04 for *Pbx3*). Rostral enrichment of *Pbx3* was relatively small, but was clearly observed in medial sections (Fig. 8A). We continued to analyze whether *Pbx1*, *Pbx3*, *Tle3* and *Tle4* were regulated by Pitx3. To test this, we microdissected E14.5 Pitx3 gfp/+ (heterozygous) and Pitx3gfp/gfp (knockout) VMs, sorted GFP-positive cells by FACS, and subjected isolated RNA to qPCR (Fig. 8C). *Pbx1* (*P*=0.01), *Pbx3* (*P*=0.03) and *Tle3* (*P*=0.01) transcript levels were significantly reduced, whereas expression of *Tle4* and the housekeeper gene 18s appeared unchanged in Pitx3 gfp/gfp embryos (Fig. 8D). To determine in which mdDA domains expression of *Pbx1/3* and *Tle3* was affected in Pitx3 gfp/gfp embryos, we analyzed wild-type, Pitx3 gfp/+ and Pitx3 gfp/gfp embryos at E14.5 by ISH. *Pbx1* expression was decreased throughout the mdDA area, in particular dorsomedially and rostrolaterally (supplementary material Fig. S3A). Notably, in lateral sections *Pbx1* expression appeared lower in Pitx3 gfp/+ compared with Pitx3+/+ embryos (supplementary material Fig. S3A). For *Tle3*, similar defects were observed, as expression appeared to be abolished in both the dorsomedial and rostrolateral mdDA domains of Pitx3 gfp/gfp embryos (supplementary material Fig. S3C). In agreement with the smaller effect size
observed by qPCR, Pbx3 expression was less clearly affected in Pitx3 gfp/gfp embryos, although small defects in the distribution of Pbx3 could be observed in lateral sections (supplementary material Fig. S3B). Altogether, these data suggest that Pitx3 activates Pbxi, Pbx3 and Tle3 and might thereby modulate En1 activity.
DISCUSSION
Despite extensive efforts to identify signaling pathways and transcription factors that are crucial for mdDA development (Smidt and Burbach, 2007; Van den Heuvel and Pasterkamp, 2008), our understanding of transcriptional programs leading to healthy mdDA neuron generation is still in its infancy. Of particular interest are the transcriptional cascades underlying differential programming of the VTA and SNc, because insight into molecular differences might improve our understanding of the specific vulnerability of the SNc to neurodegeneration as observed in PD. Here, we show how combinatorial action of Pitx3 and En1 might induce dopaminergic subset specification.

AHD2 AND CCK MARK DOPAMINERGIC SUBSETS THAT DISPLAY DIFFERENTIAL DEPENDENCE ON EN1 AND PITX3
In the rostrolateral mdDA area, expression of Nurr1 target genes is abolished in the absence of En1. This phenotype is reminiscent of Pitx3−/− embryos, in which the same neuronal subset, marked by Ahd2 expression and destined to form the SNc, fails to express key DA-related genes (Jacobs et al., 2011). Thus, our data position En1 next to Pitx3 as a crucial inducer of the rostrolateral mdDA phenotype. Although Pitx3 expression is affected in En1−/− embryos, the phenotype of En1−/− and Pitx3−/− embryos is not identical. Aadc is affected in En1−/− but not in Pitx3−/− embryos (Jacobs et al., 2009a), and Pitx3−/− embryos display Cck expansion into the rostrolateral mdDA population (Jacobs et al., 2011), whereas in En1−/− embryos loss of Pitx3 in this subset does not induce ectopic Cck expression. Thus, the relationship between En1 and Pitx3 in terminal mdDA neuron differentiation cannot be explained by a simple model placing Pitx3 under the transcriptional control of En1.

En1 is crucial for correct molecular specification of a second subset, marked by Cck. In this subset, Pitx3 and En1 seem to compensate for each others’ absence partially in the induction of Dat, Th, Vmat2 and Aadc as their expression is affected but not abolished in this area in both En1- and Pitx3-deficient embryos (Fig. 9B). Complete loss of D2R might be explained by its dependence on retinoic acid (RA) (Jacobs et al., 2011), because expression of the RA-synthesizing enzyme Ahd2 is completely absent in En1−/− embryos, suggesting that both D2R and Ahd2 are dependent on En1 activity (Fig. 9A). This could also explain why D2R and Ahd2 are the only DA-related genes not expanded into the En1-deficient metencephalon.
FIG. 9. MODEL FOR INTERPLAY OF EN1 AND PITX3 IN MDPA SUBSET SPECIFICATION. (A) En1 and Pitx3 cooperatively drive expression of the Nurr1 target genes Dat, Th and Ahd2 in a rostralateral subset of mdDA neurons (marked by Ahd2; red in schematic). Aadc is strictly dependent on En1. Local synthesis of RA is abrogated in En1–/– and Pitx3–/– embryos, contributing to rostralateral downregulation of Th and D2R. Pitx3, induced by En1 in this subset, represses genes reciprocally regulated by En1 and Pitx3, such as Cck and Nts, presumably by repression of En1 or by modulating En1 protein activity. (B) A second, caudal mdDA subset is marked by Cck (green in schematic), which is induced by En1. Other DA-related genes, such as Th, are regulated by both En1 and Pitx3, which may compensate for each others’ loss within this subset. (C) In the metencephalon (yellow in schematic), En1 represses the mdDA fate. (D) Generation of mdDA subsets during terminal differentiation. After Nurr1 induction, Pitx3 and En1 cooperate (sequentially, in concert or reciprocally) in mdDA subset specification. Pitx3, initially induced by En1, regulates transcription of genes encoding En1 modulatory proteins (Pbx1, Pbx3, Tle3) and represses En1 rostrolaterally. Once the Ahd2+ rostralateral mdDA subset is generated, En1 induces the caudal (Cck+) DA phenotype. A subpopulation of Cck+ mdDA neurons does not express Dat, possibly explained by Otx2-mediated antagonism (Di Salvio et al., 2010). Green, activation in wild type; red, repression in wild type; dashed black line, modulation in wild type; green arrows with red strike-through, activation inhibited in wild type; dashed red line, possible repressive mechanism.
THE COMBINED ACTION OF EN1 AND PITX3 CAN BE REALIZED THROUGH AT LEAST THREE LEVELS OF MOLECULAR INTERACTION

Our study indicates that En1-Pitx3 crosstalk depends on at least three different mechanisms. First, Pitx3 and En1 influence each others’ transcription, as shown by rostrolateral downregulation of Pitx3 in En1–/– embryos (Fig. 5A,D), and upregulation of En1 in Pitx3–/– embryos (Jacobs et al., 2011). Second, modulation of transcriptional complex composition may be key. Re-activation of Nurr1 target genes in En1–/– embryos by HDAC inhibition is analogous to the previously described upregulation of Th expression upon HDAC inhibition in Pitx3gfp/– embryos (Jacobs et al., 2009a). Pitx3 crucially influences N-CoR2(Smrt)–Sin3–HDAC-mediated repression of mdDA genes by recruitment of the Nurr1–PSF transcriptional complex and release of Smrt–HDAC complexes (Jacobs et al., 2009a; Van Heesbeen et al., 2013). It has been shown that the N-CoR–Sin3–HDAC complex can be recruited to the Engrailed homology 1 domain (Eh1) of HesX1 (Dasen et al., 2001) and our finding that En1 can directly interact with PSF suggests that En1 releases HDAC-mediated repression in a similar manner to that described for Pitx3.

Whereas release of HDAC-mediated repression of Nurr1/En1/Pitx3 target genes seems to be necessary for DA-related gene induction during terminal differentiation, En1-mediated repression might be crucial at earlier developmental stages to repress the mdDA phenotype in the metencephalon (Fig. 9C). During early pituitary development, Hesx1–HDAC repressive complexes define spatial domains in which pituitary organogenesis occurs by restriction and maintenance of the Fgf8 expression domain (Dasen et al., 2001). Fgf8 is crucial for proper positioning of the MHB (Smidt and Burbach, 2007; Joyner et al., 2000) and is regulated by En1 (Shamim et al., 1999), and beads containing recombinant Fgf8 induce ectopic midbrain formation (Martinez et al., 1999). Therefore, absence of En1–HDAC repressive complexes in the metencephalon of En1–/– embryos might result in ectopic induction of the DA phenotype by affecting the Fgf8 expression domain.

A third mechanism of Pitx3-En1 crosstalk is Pitx3-mediated regulation of genes encoding En1 modulatory proteins. En1 can act as a transcriptional activator or repressor, depending on its interactors at the Eh domain (Serrano and Maschat, 1998). We found that Pitx3 activates Pbx1, Pbx3 and Tle3, members of the Groucho (Grg/Tle) and Pbx family that bind Eh domains and thereby modulate En1 transcriptional activity (Dasen et al., 2001). In agreement with our data, it was recently shown that Pbx1 and Pbx3 are enriched in Pitx3+ mouse embryonic stem cells compared with Pitx3– cells (Ganat et al., 2012). In flies, Engrailed requires the
Pbx ortholog Exd for activation of specific targets (Serrano and Maschat, 1998), and the murine Eh2 domain interacts with Pbx (Peltenburg and Murre, 1996). A role for Pbx in mdDA subset specification and differential regulation of En1 target genes is supported by the finding that in zebrafish Pbx proteins cooperate with Engrailed proteins to compartmentalize the midbrain and the caudal expansion of diencephalic genes into midbrain territory in Pbx-null and Engrailed-null embryos (Erickson et al., 2007). Moreover, in mice, a role for Pbx1 in mdDA neuron axonal pathfinding has been suggested (Sgadò et al., 2012). Pbx proteins are regulated transcriptionally and post-translationally by RA, which increases mRNA levels and extends protein half-lives, respectively (Qin et al., 2004). Hox and Pbx genes positively reinforce RA signaling by driving Raldh2 (Aldh1a2) expression in the developing hindbrain in a feed-forward mechanism (Vitobello et al., 2011). Local RA synthesis is driven by Ahd2 and is therefore restricted to the rostral lateral mdDA area (Jacobs et al., 2007; Jacobs et al., 2011), suggesting that local RA synthesis reinforces the rostral lateral mdDA phenotype. As Ahd2 expression is Pitx3 dose dependent (Jacobs et al., 2007), this might also explain the decreased lateral expression of Pbx1 in Pitx3gfp/+ and the further downregulation in Pitx3gfp/gfp embryos that we observed.

**How do En1 and Pitx3 interact in dopaminergic subset specification?**

Our data allow us to model how Nurr1, En1 and Pitx3 interplay might generate different mdDA subsets, taking into account that the birth of rostral lateral SNC neurons precedes that of caudal VTA neurons (Bye et al., 2012) (Fig. 9D). Initially, Nurr1 activation generates default DA cells, which can still become caudal Cck+ or rostral lateral Ahd2+ mdDA neurons. Subsequently, crosstalk between Pitx3 and En1 influences mdDA subset specification. Our data suggest that within the rostral lateral mdDA region En1 is initially required to drive Pitx3 expression and induce the DA phenotype, as Pitx3 and all analyzed DA neuron markers (except Nurr1) are downregulated within this domain in En1−/− embryos. Ectopic Cck expression in this subset in Pitx3−/− embryos (Jacobs et al., 2011) indicates that Pitx3 antagonizes the caudal phenotype within this population, either by direct repression of En1 or by regulation of genes encoding proteins that modulate En1 transcriptional activity (Pbx1/3 and Tle3). This complex interplay ultimately generates a rostral lateral mdDA neuron, deprived of Cck, but expressing Ahd2, that is dependent on both En1 (Fig. 5B,D) and Pitx3 (Jacobs et al., 2007a).

After establishing the rostral lateral phenotype, remaining default DA neurons adopt a caudal mdDA subset phenotype (Cck+). Unaffected expression of multiple DA neuron markers in the caudal area of Pitx3−/− embryos indicates that
En1 is crucial for induction of the caudal mdDA phenotype. This fate switch might be initiated by compositional changes in En1-containing complexes (e.g. Pbx or Tle recruitment or release). Notably, within the caudal Cck+ population, we observed a neuronal subset that was Cck+, but Dat–. Possibly, Dat expression is antagonized in this subset by a third homeoprotein, Otx2, as described in adult (Di Salvio et al., 2010). This model explains the mutant mice phenotypes. In Pitx3–/– embryos, the rostromedial phenotype cannot be initiated, but caudal mdDA neuron programming, mainly dependent on En1, can be activated, leading to ectopic Cck expression. These caudal neurons are destined to form the VTA, explaining why the VTA is relatively intact in adult Pitx3–/– mice (Smidt et al., 2004). In En1–/– embryos, by contrast, no active En1 protein is present. Thus, not only is rostromedial mdDA neuron programming affected, but also the Cck+ caudal DA phenotype cannot be induced, because En1 is required for its induction. This ultimately leads to VTA and SNc defects in adult knockout mice.

**Final remarks**

Our genome-wide and in-depth spatial expression analysis revealed multiple genes that are either mutually cooperative or reciprocally regulated by En1 and Pitx3. Which of these are direct targets remains to be assessed, preferably by *in vivo* identification of En1 and Pitx3 binding sites (e.g. by ChIP-Seq analysis). Notwithstanding, we have shown that one crucial difference in the complex coding of different mdDA subsets is the crosstalk between En1 and Pitx3, which has important implications for how we think of subset-specific degeneration (as observed in PD) and reprogramming strategies. In this regard, future studies could focus on the impact of the observed gene expression changes on DA neuron function and physiology. In this aspect, it is interesting it is to note that Cck has mainly been associated with VTA functions such as reward and addiction (Jayaraman et al., 1990; Rotzinger and Vaccarino, 2003) and that Cck can inhibit DA neurotransmission (Lane et al., 1987). Moreover, it has been shown that Cck-B receptor antagonists potentiate locomotor stimulatory effects of L-DOPA in MPTP-lesioned monkeys (Boyce et al., 1990), suggesting that functional blockade of Cck relieves PD symptoms. By contrast, aldehyde dehydrogenase (dys)function, including Ahd2, has mainly been related to PD (Fitzmaurice et al., 2013; Grünblatt et al., 2010), probably for its important role in DA detoxification and/or RA production from vitamin A. In addition, Ahd2/Aldh2 combined knockout mice display significant Th+ neuron loss in the SNc, reduction of dopamine and metabolites in the striatum, and age-dependent deficits in motor performance, that can be alleviated by L-DOPA administration (Wey et al., 2012).
Given these important physiological and functional differences between Ahd2 and Cck function in DA neurons, detailed understanding of En1 and Pitx3 in the differential coding of the Ahd2+ and Cck+ mdDA subsets is of crucial importance, and may hold translational value given the association of polymorphisms in Pitx3 and En1 with sporadic PD (Haubenberger et al., 2011).

ACKNOWLEDGEMENTS
We thank Emmeke Aarts for help with statistical analysis and Robert Post for technical assistance.

AUTHOR CONTRIBUTIONS

SUPPLEMENTARY MATERIAL
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.094565/-/DC1
Chapter 2

REFERENCES


Interplay of En1 and Pitx3


