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On p. 3374, a section of the microarray analysis text should read as follows:

Analysis was performed as described (Roepman et al., 2005). 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. Mouse Whole Genome Gene Expression Microarrays V1 (Agilent Technologies, Belgium) containing mouse 60-mer probes were used and data have been deposited in ArrayExpress under accession number E-TABM-1104.

The authors apologise to readers for this mistake.
Specification of dopaminergic subsets involves interplay of En1 and Pitx3

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SUMMARY
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 rostralateral 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 rostralateral 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 rostralateraly. 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.

KEY WORDS: Dopamine, Midbrain, Neurodegeneration, Substantia nigra, Transcription, Mouse

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 Ahd2 (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 Ahd2 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 rostralateral mdDA subpopulation. This expansion is accompanied by rostralateral 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; Alberi 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.

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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 Abd2) in a rostral/lateral subset of mdDA neurons, and that absence of En1 results in similar defects in this subset of neurons to those observed in Ptx3−/− embryos. Genome-wide expression analysis revealed that En1 and Ptx3 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 Ptx3 in the mdDA area position. En1-Ptx3 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 Ptx3-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 Ptx3 is crucial for the proper induction of distinct mdDA subsets.

MATERIALS AND METHODS

Animals

Adult En1tm1Alj/J mice were backcrossed to the C57BL/6J 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).

Ptx3300/00 and Ptx3300/+ littermate embryos were obtained as described previously (Jacobs et al., 2011; Zhao et al., 2004). Ptx3300/− embryos are heterozygous for wild-type Ptx3 and green fluorescent protein (GFP), and have normal mdDA system development (Maxwell et al., 2005). Ptx3300/− mice are Ptx3 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 varied using one-way ANCOVA with litter/experimental set as covariate. 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 into three different groups of E13.5 or E14.5 (E0.5 defined as day of copulatory plug). Genotyping was performed by PCR analysis using specific primers (Wurst et al., 1994). Ptx3300/00 and Ptx3300/+ littermate embryos were obtained as described previously (Jacobs et al., 2011; Zhao et al., 2004). Ptx3300/− embryos are heterozygous for wild-type Ptx3 and green fluorescent protein (GFP), and have normal mdDA system development (Maxwell et al., 2005). Ptx3300/− mice are Ptx3 deficient. All procedures were according to and fully approved by the Dutch Ethical Committees for animal experimentation (UMC-U and UvA).

In situ hybridization (ISH)

ISH was performed as described (Smits et al., 2003). Digoxigenin-labeled probes for Th, Vmat2, Cck, Dat, Nurr1, Aadc, Abd2, Ptx3 and Ns 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); Ptx3, bp 751-1803 of mouse CDS. Tle3, bp 1950-2928 of mouse CDS; Tle4, bp 2720-3514 of mouse CDS.

Cell culture and transfections 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/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 (ab91108; 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 analysis (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 into three different groups of E13.5 or E14.5 (E0.5 defined as day of copulatory plug). Genotyping was performed by PCR analysis using specific primers (Wurst et al., 1994). Ptx3300/00 and Ptx3300/+ littermate embryos were obtained as described previously (Jacobs et al., 2011; Zhao et al., 2004). Ptx3300/− embryos are heterozygous for wild-type Ptx3 and green fluorescent protein (GFP), and have normal mdDA system development (Maxwell et al., 2005). Ptx3300/− mice are Ptx3 deficient. All procedures were according to and fully approved by the Dutch Ethical Committees for animal experimentation (UMC-U and UvA).

RESULTS

Ahd2 and Cck mark distinct subsets during mdDA neuron development

Ptx33−/− 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 Ptx3 (Jacobs et al., 2007). We recently identified Cck as a novel Pitx3 target. Cck is upregulated in Ptx33−/− 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 Pitx3gfp/+ 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 mdDA 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 knocko

![Fig. 1. Ahd2 and Cck mark distinct dopaminergic subsets during mdDA neuron development.](image)

**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, \textit{Th}, \textit{Dat} and \textit{Aadc}, were identified as novel transcriptional targets of En1 (downregulated in \textit{En1}–/– embryos; supplementary material Table S2). For validation, we subjected RNA from a separate set of dissected E13.5 \textit{En1}+/– and \textit{En1}–/– VMs to qPCR and confirmed downregulation of \textit{Dat}, \textit{Th} and \textit{Cck} (\(P<0.01\)) (Fig. 3B). \textit{Aadc} 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 (\textit{Rn18s}) and the DA neuron marker gene \textit{Nurr1} (Fig. 3B), in combination with an unaffected \textit{Nurr1} expression domain (Fig. 5C,D) indicate that reduced expression of DA-related genes represents programming deficits rather than cell loss at this stage.

**Fig. 2.** The architecture of the mdDA area and striatal projections are affected in \textit{En1}–/– adult mice. (A) \textit{Th} expression in coronal sections of the mdDA neuronal region. \textit{Th} expression in the substantia nigra pars compacta (SNC) and the ventral tegmental area (VTA) is mildly affected in \textit{En1}+/– and severely affected in \textit{En1}–/– adult mice (red arrowheads). (B) Nissl staining in adjacent sections revealed decreased DA cell density in both rostralateral and medial parts of the SNC in \textit{En1}+/– and \textit{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 \textit{Th} protein show innervation defects (red arrowheads) of caudate putamen (Cpu) and nucleus accumbens (Acb) in \textit{En1}–/– mice compared with wild type, with the \textit{En1}+/– displaying an intermediate phenotype.

**Fig. 3.** \textit{In vivo} genome-wide expression analysis reveals crosstalk between the homeoproteins \textit{En1} and \textit{Pitx3}. (A) GO analysis identified a key role for \textit{En1} in multiple biological processes during mdDA development. (B) qPCR analysis confirmed downregulation of key DA-related genes in \textit{En1}–/– embryos (wild type, \(n=3\); knockout, \(n=5\); **\(P\leq0.01\)). 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 \textit{Nurr1}-, \textit{Pitx3}- and \textit{En1}-deficient embryos revealed genes co-regulated by \textit{En1}, \textit{Pitx3} and \textit{Nurr1}. (D) Heatmap generated by hierarchical clustering (MeV v4.7.3) of genes that are significantly regulated (MAANOVA-FDR) in both \textit{Pitx3}–/– and \textit{En1}–/– embryos. Color scale bar represents relative expression \([M\text{-value}= 2\log(\text{fold-change})]\); green, upregulation; red, downregulation). (E) Whereas \textit{Dat} (\textit{Slc6a3}) and \textit{Scn3b} are downregulated in both \textit{Pitx3}–/– and \textit{En1}–/– embryos, most genes downregulated in \textit{Pitx3}–/– embryos are upregulated in \textit{En1}–/– embryos. (F) Genes downregulated in \textit{En1}–/– embryos are upregulated in \textit{Pitx3}–/– embryos.Red lines in E,F indicate relative wild-type expression.
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 rostralateral 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 rostralateral mdDA system, but relatively unaffected in the mediolateral part of the midbrain (Fig. 4A,B,F). Aadc expression was subtly affected throughout the whole mdDA area in En1−/− 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).
expressed in the rostrolateral mdDA area (Fig. 5A,F). As this subset is closely Pitx3 (Jacobs et al., 2007), we analyzed expression of Ahd2, a direct transcriptional target of Pitx3 (Jacobs et al., 2007), which is upregulated in Pitx3−/− embryos (Fig. 3D), is restricted to the caudal mdDA area in wild type (black arrowheads in B), and downregulated in En1−/− embryos (B; higher magnification in D; affected area indicated by black arrows). For both Nts and Cck, no metencephalic expansion was observed behind the MHB (black dotted line in A-D).

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).

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 mdDA progenitors and early 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, which is upregulated in Pitx3−/− embryos (Fig. 3D), is restricted to the caudal mdDA area in wild type (black arrowheads in B), and downregulated in En1−/− embryos (B; higher magnification in D; affected area indicated by black arrows). For both Nts and Cck, no metencephalic expansion was observed behind the MHB (black dotted line in A-D).

Cck expression is restricted to the caudal mdDA area in wild-type embryos and downregulated in the absence of En1 (A; higher magnification in C). Affected area indicated by black arrows; red arrowheads in A mark a small area of Cck+ cells outside the mdDA area. (B) Nts, which is upregulated in Pitx3−/− embryos (Fig. 3D), is restricted to the caudal mdDA area in wild type (black arrowheads in B), and downregulated in En1−/− embryos (B; higher magnification in D; affected area indicated by black arrows). For both Nts and Cck, no metencephalic expansion was observed behind the MHB (black dotted line in A-D).
mdA in En1+/− and Pitx3+/− embryos, the causally restricted factors Cck and Ns 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 Pitx3+/− embryos (Jacobs et al., 2009a).

Given the striking similarity in the rostralateral 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 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 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 domain (Fig. 8A). To verify subset-specific expression, we subjected rostral and caudal FAC-sorted mdDA neurons to qPCR and found clear enrichment of Pbx1 and Tle3 expression within caudal mdDA area (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<0.05 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<sup>300/+</sup> (heterozygous) and Pitx3<sup>300/kp</sup> (knockout) VMs, sorted GFP-positive cells by FACS, and subjected isolated RNA to qPCR (Fig. 8C). qPCR analysis on FAC-sorted rostral and caudal mdDA neurons revealed enriched Pbx1 and Tle3 expression within the caudal mdDA area. Tle4 and Pbx3 expression was decreased throughout the mdDA area, in particular the smaller effect size observed by qPCR, Pbx3 expression was less clearly affected in Pitx3<sup>300/kp</sup> 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 Pbx1, 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.
**Ah2 and Cck mark dopaminergic subsets that display differential dependence on En1 and Pitx3**

In the rostralateral 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 Ah2 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 rostralateral 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 rostralateral 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 Ah2 is completely absent in En1−/− embryos, suggesting that both D2R and Ah2 are dependent on En1 activity (Fig. 9A). This could also explain why D2R and Ah2 are the only DA-related genes not expanded into the En1-deficient metencephalon.

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 rostralateral 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 Pitx3+/0 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 (Sgardò 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 rostralateral mdDA area (Jacobs et al., 2007; Jacobs et al., 2011), suggesting that local RA synthesis reinforces the rostralateral mdDA phenotype. As Ahd2 expression is Pitx3 dose dependent (Jacobs et al., 2007), this might also explain the decreased lateral expression of Pbx1 in Pitx3+/0 and the further downregulation in Pitx3+/0 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 rostralateral 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 rostralateral Ahd2+ mdDA neurons. Subsequently, crosstalk between Pitx3 and En1 influences mdDA subset specification. Our data suggest that within the rostralateral 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 rostralateral 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 rostralateral 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 rostralateral 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 rostralateral 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 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+ mDA 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).

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References


