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Cell-autonomous FGF signaling regulates anteroposterior patterning and neuronal differentiation in the mesodiencephalic dopaminergic progenitor domain

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SUMMARY

The structure and projection patterns of adult mesodiencephalic dopaminergic (DA) neurons are one of the best characterized systems in the vertebrate brain. However, the early organization and development of these nuclei remain poorly understood. The induction of midbrain DA neurons requires sonic hedgehog (Shh) from the floor plate and fibroblast growth factor 8 (FGF8) from the isthmic organizer, but the way in which FGF8 regulates DA neuron development is unclear. We show that, during early embryogenesis, mesodiencephalic neurons consist of two distinct populations: a diencephalic domain, which is probably independent of isthmic FGFs; and a midbrain domain, which is dependent on FGFs. Within these domains, DA progenitors and precursors use partly different genetic programs. Furthermore, the diencephalic DA domain forms a distinct cell population, which also contains non-DA Pou4f1$^+$ cells. FGF signaling operates in proliferative midbrain DA progenitors, but is absent in postmitotic DA precursors. The loss of FGFR1/2-mediated signaling results in a maturation failure of the midbrain DA neurons and altered patterning of the midbrain floor. In FGFR mutants, the DA domain adopts characteristics that are typical for embryonic diencephalon, including the presence of Pou4f1$^+$ cells among TH$^+$ cells, and downregulation of genes typical of midbrain DA precursors. Finally, analyses of chimeric embryos indicate that FGF signaling regulates the development of the ventral midbrain cell autonomously.

KEY WORDS: Midbrain, Diencephalon, Fibroblast growth factor, Dopaminergic, Neurogenesis, Engrailed 1, Mouse

INTRODUCTION

The ventral midline in the midbrain and caudal diencephalon gives rise to dopaminergic (DA) neurons in neuronal groups A8-A10 – the retrorubral field (RRF), substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) (Bjorklund and Lindvall, 1984; Dahlstrom and Fuxe, 1964; Marín et al., 2005; Ono et al., 2007; Bonilla et al., 2008; Joksimovic et al., 2009; Blaess et al., 2011). Their dysfuntion or death has been implicated in various neurological and psychological disorders, such as schizophrenia, depression, addictive behavior and Parkinson's disease (Damier et al., 1999; Braak and Braak, 2000; Meyer-Lindenberg et al., 2002; McClung, 2007; Yadid and Friedman, 2008). Detailed mapping of these populations has revealed a significant amount of heterogeneity in their projection patterns, as well as neurotransmitter properties (Bjorklund and Dunnett, 2007). However, the developmental mechanisms behind this diversity remain elusive.

Several factors are involved in DA neuron induction, specification, maintenance and suppression of alternate fates (Ang, 2006; Prakash and Wurst, 2006; Smidt and Burbach, 2007; Alavian et al., 2008). Numerous transcription factors contribute to DA identity. Early patterning genes of engrailed (*En*), Pax, Lmx and Otx families give the midbrain neuronal progenitors competence to adopt a DA progenitor identity. These cells begin to express transcription factors such as *Foxa2*, *Lmx1a/b* and *Msx1*, which guide the cells towards the DA fate, and *Ngn2* (*Neurog2* – Mouse Genome Informatics), which regulates the neurogenesis and cell cycle exit (Andersson et al., 2006a; Andersson et al., 2006b; Kele et al., 2006; Ferri et al., 2007; Mavromatakis et al., 2011).

Around E11.5, first postmitotic tyrosine hydroxylase (TH)-positive DA precursors appear in the ventral midbrain. They express transcription factors needed for their maturation and maintenance, such as *Nurr1* (*Nr4a2* – Mouse Genome Informatics), *En1/2* and *Lmx1a/b*. Terminally differentiated midbrain DA neurons are characterized by the expression of transcription factor *Pitx3*, together with other indicators of the DA phenotype, such as dopamine transporter (DAT; *Slc6a3* – Mouse Genome Informatics) and dopa decarboxylase (*Ddc*) (Eaton et al., 1993; Smidt et al., 2004; Maxwell et al., 2005).

The exact function of each of these transcription factors and their connection with extrinsic signals, such as Wnts, Shh, TGFβ and FGFs, is not fully understood. Both Shh and canonical Wnt pathways regulate *Lmx1a/b*, and the Wnt1-Lmx1a autoregulatory loop controls *Otx2*, *Nurr1* and *Pitx3* expression (Andersson et al., 2006b; Prakash and Wurst, 2006; Chung et al., 2009). TGFβ signaling is required for Shh-mediated DA neuron induction, and with FGF8 and Shh for the survival of DA neurons (Farkas et al., 2003; Roussa et al., 2004). Together with Shh, FGF8 from the isthmic organizer is required for the induction of midbrain DA neurons (Ye et al., 1998). FGF8 maintains the expression of, and also depends on, the transcription factors En1 and En2 (Liu and Joyner, 2001). Both En1 and En2 are cell-autonomously needed for the survival of midbrain DA neurons, as, in their absence, DA precursors undergo apoptosis by E14.5 (Albéri et al., 2004). During embryogenesis, FGF8 is involved in various other functions in the midbrain-hindbrain region, such as patterning, cell survival,

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proliferation and neurogenesis (Crossley et al., 1996; Xu et al., 2000; Chi et al., 2003; Partanen, 2007; Basson et al., 2008; Sato and Joyner, 2009). However, the mechanism by which FGFs regulate these processes and how they are related to DA neuron development remain unclear.

We have previously analyzed the role of FGF signaling in the developing mouse midbrain and anterior hindbrain using conditionally inactivated alleles of FGF receptor 1 (*Fgfr1*) and *Fgfr2*, and a null allele of *Fgfr3* (Trokovic et al., 2003; Trokovic et al., 2005; Jukkola et al., 2006; Saarimaki-Vire et al., 2007; Lahti et al., 2011). In *Fgfr1* conditional mutant embryos ($En1^{Cre};Fgfr1^{cko}$), a rhombomere 1-to-midbrain transformation at the midbrain-hindbrain boundary shifts the DA neuron population posteriorly (Jukkola et al., 2006). Although SNpc and VTA fail to form coherent nuclei, TH^+ neurons are still detected in postnatal animals (Trokovic et al., 2003; Jukkola et al., 2006). In $En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}$ ventral midbrain, DA progenitors show increased cell cycle exit, whereas the cell cycle length remains relatively unaffected (Lahti et al., 2011). Despite the premature neuronal differentiation, post-mitotic TH^+ neurons are initially produced in $En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}$ midbrain (Saarimaki-Vire et al., 2007). However, they fail to display characteristics of mature midbrain DA neurons, such as *Pitx3* and *DAT*, and disappear by birth.

Here, we studied how FGFs regulate DA neuron differentiation. Although the full DA defect in $En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}$ embryos is detected at postmitotic stage, our results suggest that FGF signaling operates mainly in proliferative DA progenitors. Furthermore, our studies reveal a novel anteroposterior (AP) pattern in the early mesodiencephalic DA region. We show that early DA precursors in the caudal diencephalon are intermingled with $Pou4f1^+Lmx1a^+$ non-DA neuronal precursors, and they molecularly differ from their midbrain counterparts. Our data indicate that, in the absence of FGF signaling, full maturation of DA neurons fails, and both proliferative DA progenitors and postmitotic DA precursors in the midbrain adopt many characteristics similar to the embryonic caudal diencephalon.

MATERIALS AND METHODS

Generation and genotyping of mice and embryos

$En1^{Cre}$ (Kimmel et al., 2000), DAT^{Cre} (Ekstrand et al., 2007), Th^{Cre} (Lindeberg et al., 2004), *R26R* (Soriano, 1999), $Fgfr1^{flox}$ (Trokovic et al., 2003), $Fgfr2^{flox}$ (Yu et al., 2003) and $Fgfr1^{IIICn}$ (Partanen et al., 1998) mouse strains have been previously described and were maintained in an ICR outbred genetic background. These strains were crossed to generate $En1^{Cre/+};Fgfr1^{flox/flox};Fgfr2^{flox/flox};R26R^{/+}$ ($En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}$), $DAT^{Cre/+};Fgfr1^{flox/flox};Fgfr2^{flox/flox};R26R^{/+}$ ($DAT^{Cre};Fgfr1^{cko};Fgfr2^{cko}$) and $Th^{Cre/+};Fgfr1^{flox/flox};Fgfr2^{flox/flox};R26R^{/+}$ ($Th^{Cre};Fgfr1^{cko};Fgfr2^{cko}$) embryos. Chimeric embryos were generated by aggregating wild-type (ICR) and $En1^{Cre/+};Fgfr1^{flox/IIICn};Fgfr2^{flox/flox};R26R^{/+}$ morulae, using standard methods. Embryonic day (E) 0.5 was the noon of the day of the vaginal plug or, for the chimeras, the day of the implantation. The embryonic age was determined more precisely by counting the somites. Wild type refers to NMRI or ICR embryos. Control refers to a littermate of the mutant embryos, which either carried non-recombined *Fgfr* floxed alleles, were heterozygous for recombined $Fgfr1^{flox}$ or $Fgfr2^{flox}$ alleles, or were homozygous for recombined $Fgfr2^{flox}$ alleles. Control embryos displayed an identical phenotype to true wild-type embryos. Experiments were approved by the National Committee of Experimental Animal Research in Finland.

Histology

For in situ hybridization (ISH) and immunohistochemistry (IHC), embryos were fixed in fresh 4% PFA in PBS for at least 2 days at room temperature. Adult brains were first intracardially perfused by +37°C 4% PFA in PBS. The samples were dehydrated and embedded in paraffin using automated Leica tissue processor, and sectioned at 5 μm.

mRNA in situ hybridization, immunohistochemistry and microscopy

Radioactive mRNA in situ hybridization with ^{35}S cRNA-probes on sections (Wilkinson and Green, 1990) was carried out with probes previously described (Trokovic et al., 2003; Jukkola et al., 2006; Kala et al., 2009). In addition, *Wnt8b* probe was transcribed from clone IMAGp998E1212657Q, Δ*Fgfr2* probe was from David Ornitz (Washington University School of Medicine, St Louis, MO, USA), *Wnt5a*, *Wnt7b* and *FoxA2* from Irma Thesleff (Institute of Biotechnology, Helsinki, Finland), *Pou4f1* from Siew-Lan Ang (MRC National Institute for Medical Research, London, UK), *Lmx1b* from Horst Simon (University of Heidelberg, Germany) and *Corin1* from Yuichi Ono (KAN Research Institute, Kobe, Japan). Non-radioactive in situ hybridization with DIG-labeled probes was based on the same protocol and the signal was visualized by anti-DIG-AP and NBT/BCIP color substrates (Sigma).

Immunohistochemistry was performed essentially as described previously (Jukkola et al., 2006). The primary antibodies were rabbit anti-Aldh1a1 (1:400, Abcam), rabbit anti-β-galactosidase (1:1500, MP Biomedicals), rabbit anti-Cas3 active (1:500, R&D Systems), mouse anti-En1 (1:100, concentrate from DSHB), rabbit anti-FoxP1 (1:400, Abcam), mouse anti-Gad67 (1:500, Millipore), mouse anti-HuC/D (1:500, Invitrogen), rabbit anti-Lmx1a (1:400, from Michael German, University of California at San Francisco, San Francisco, CA, USA), mouse anti-Nkx6.1 (1:1000, concentrate from DSHB), goat anti-Otx2 (1:300, R&D Systems), rabbit anti-phospho-Erk1/2 (1:100, Cell Signaling Technology,), rabbit anti-Pitx3 (1:300, Zymed/Invitrogen), mouse anti-Pou4f1 (1:200, Santa Cruz Biotechnology), rabbit anti-Sox2 (1:400, Millipore), mouse anti-Th (1:500, Millipore) and rabbit anti-Th (1:500, Millipore).

Secondary antibodies were Alexa Fluor conjugated (1:400, Invitrogen) and nuclei were visualized with DAPI (4′,6′-diamidino-2-phenylindole, Sigma). Samples were imaged using Olympus AX70 microscope connected to Olympus DP70 camera, and pictures processed with Adobe Photoshop CS3. In each experiment, a minimum of three mutant and three littermate control embryos were analyzed. Detailed in situ hybridization and immunohistochemistry protocols are available upon request.

Retinoic acid treatment and the statistical analysis

Pregnant females received all-trans retinoic acid (RA; Sigma) essentially as described previously (Jacobs et al., 2007), from the evening of 9.5 days post fertilization until E13.5, when embryos were collected. On average, the mice (n=3) consumed 1.25 mg of RA per day, corresponding to 0.04 mg/g of body weight. Higher doses of RA resulted in high embryonic lethality. RA-treated mutants and controls were from three different litters, non-treated embryos from one litter. The number of $Pitx3^+$ cells was counted throughout the midbrain from three or four sections from littermate controls (no RA, n=3; RA treated, n=3) and nine or ten sections from the mutants (no RA, n=3; RA treated, n=4). The RA-treated mutant midbrain showed higher variation between sections and embryos. The results were analyzed using a standard Student's t-test.

Measurement of striatal dopamine

The amount of dopamine in adult striatal tissue was measured from $DAT^{Cre};Fgfr1^{cko};Fgfr2^{cko}$ (n=5) and control females (n=4) as described (Airavaara et al., 2006). The age of mice ranged from 13 to 17 months, and mice of similar ages were represented in both groups. Dopamine amount in nanograms was compared with the weight of the striatal tissue, and the results compared using a standard Student's t-test.

RESULTS

FGF signaling is active in midbrain DA progenitors

In $En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}$ and $En1^{Cre};Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}$ embryos, DA neurons begin to develop and express TH but their maturation fails, and the majority of TH^+ neurons are lost by E15.5 (Saarimaki-Vire et al., 2007). FGF signaling could affect proliferative DA progenitors, postmitotic DA precursors, or both. To investigate this, we analyzed the expression of

Fgfr1 and *Fgfr2*, the FGF targets *Erm* (*Etv5*), *Pea3* (*Etv4*) and *Dusp6*, as well as phosphorylated ERK1/2 in the developing midbrain.

At E10.5, *Fgfr1* was detected in the proliferative progenitors of the ventricular zone (VZ) throughout the midbrain, whereas *Fgfr2* was restricted to the anterior midbrain, consistent with earlier results (Fig. 1A,B,E,F) (Trokovic et al., 2005; Blak et al., 2005). *Erm*, *Pea3* and *Dusp6*, all of which strongly expressed in the midbrain-hindbrain boundary region, appeared undetectable more anteriorly (Fig. 1I,M,Q). *Dusp6* was detected throughout the basal plate (Fig. 1R), whereas *Erm* and *Pea3* showed strongest signal in the *Aldh1a1*-expressing DA progenitor domain (Fig. 1J,N,U).

At E11.5 and E12.5, midbrain DA progenitors expressed *Fgfr1* and *Fgfr2*, but had downregulated the FGF targets (Fig. 1C,D,G,H,K,L,O,P,S,T). The postmitotic DA precursors lacked strong expression of both targets and receptors. DA progenitors still showed pERK1/2 expression at E11.5 (Fig. 1V), but the signal decreased later (Fig. 1W). Thus, the expression of FGF signaling components appears to be restricted to the caudal midbrain VZ, and to an early stage of DA neuron development. Although the caudal diencephalon VZ expresses *Fgfr1* and *Fgfr2*, it lacks FGF targets.

Distinct DA precursor populations in the midbrain and diencephalon

In the midbrain, DA neurons are generated in the most ventral domain, marked by *Lmx1a* and *FoxP1* expression (called m7) (Nakatani et al., 2007). This domain is flanked by Nkx6.1^{+} and Pou4f1^{+} cells (domain m6), which give rise to Islet1^{+} motoneurons and glutamatergic Pou4f1^{+} red nucleus (Agarwala and Ragsdale, 2002; Prakash et al., 2009).

In addition to the midbrain, *Th*-expressing cells have been identified in prosomeres (p) 1-3 of the developing diencephalon (Marín et al., 2005). The most caudally located populations associate closely with midbrain DA neurons, and may contribute to SNpc and VTA populations. However, a detailed comparison of the early diencephalic and midbrain DA populations is lacking. To analyze the mesodiencephalic DA domain in more detail, we investigated the Lmx1a^{+} region in E12.5 sagittal sections. The location of the mesodiencephalic boundary (arrowheads in Fig. 2A-C) was deduced from the position of posterior commissure (data not shown), and the midbrain-hindbrain boundary from the caudal limit of Otx2 (Fig. 2B, arrows in 2A-C).

Consistent with the previous study (Marín et al., 2005), we detected TH^{+} cells in the midbrain and in the caudal diencephalon, probably in p1-p2 (Fig. 2A). Unexpectedly, the ventral En1^{Cre}-recombined region, labeled by *R26R*, extended to the caudal diencephalon (Fig. 2A), and some TH^{+} cells were also detected anterior to it (brackets and higher magnification in Fig. 2A). In the diencephalic Lmx1a^{+} domain, TH^{+} DA precursors were intermingled with Pou4f1^{+}FoxP1^{+} cells (Fig. 2C, supplementary material Fig. S1G-G″). Diencephalic Pou4f1^{+} cells also expressed variable amounts of Lmx1a (supplementary material Fig. S1C-C″), although they gradually downregulated it during embryogenesis (data not shown). In contrast to the midbrain Pou4f1^{+} cells, the diencephalic Pou4f1^{+} population lacked Nkx6.1 (data not shown).

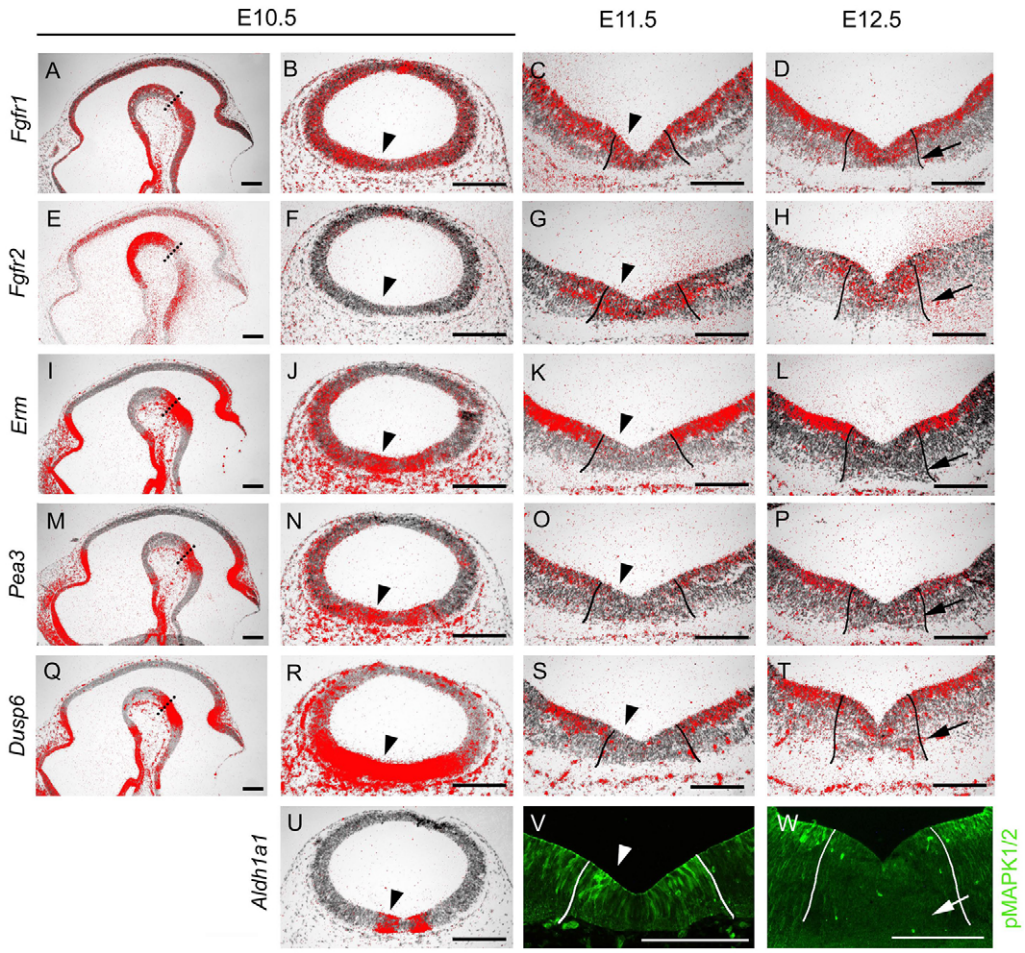

Fig. 1. *Fgfr1*, *Fgfr2* and FGF targets are expressed in midbrain DA progenitors. (**A-U**) Expression of *Fgfr1*, *Fgfr2*, *Erm*, *Pea3*, *Dusp6* and *Aldh1a1* in E10.5-E12.5 wild-type embryos examined using radioactive in situ hybridization. Parallel sections of E10.5 embryos are depicted. Black dotted line in E10.5 sagittal sections indicates the plane of coronal sections. (**V**,**W**) Immunohistochemistry for pERK1/2. Arrowheads indicate DA progenitors and arrows indicate postmitotic DA neurons. Lines indicate DA region in E11.5 and E12.5 sections. Scale bars: 200 μm.

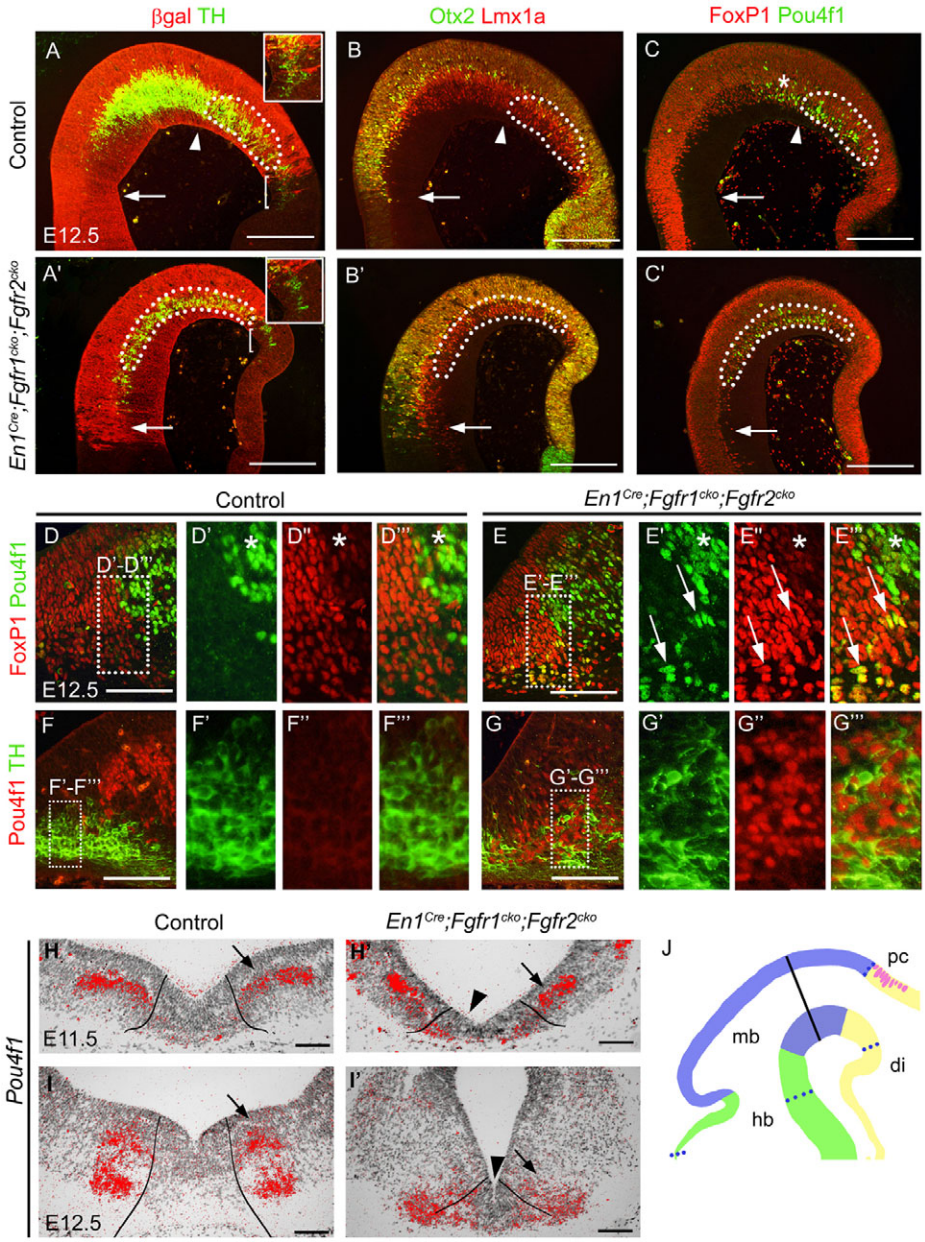

Fig. 2. Distinct cellular composition in the midbrain and diencephalic DA domains in control and *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos. (**A-C′**) E12.5 sagittal sections of ventral midbrain and diencephalon, with midbrain-hindbrain boundary indicated (arrows). The En1^{Cre}-recombined domain (A,A′) extended ventrally from rhombomere 1 to diencephalon. In mutants, the diencephalic Pou4f1^{+}FoxP1^{+} population expanded caudally. Some TH^{+} cells developed anteriorly to the β-galactosidase^{+} domain (brackets and enlargement in A,A′). The position of the mesodiencephalic boundary (arrowheads) was deduced from the position of the posterior commissure. Dotted lines outline the Pou4f1^{+} population in the diencephalic Lmx1a^{+} domain. Asterisk in C indicates Pou4f1^{+}FoxP1^{+} precursors detected in the caudal side of the posterior commissure in very few sections. (**D-G‴**) The midbrain DA domain in mutants contained Pou4f1^{+} cells, which co-expressed FoxP1 (arrows in E′-E‴) and intermingle with TH^{+} cells. Asterisks in D′-D‴,E′-E‴ indicate weak co-expression of FoxP1 and Pou4f1 in few m6 cells. (**H-I′**) *Pou4f1* mRNA upregulation (arrowheads) in mutant Lmx1a^{+} domain (black lines). Arrows indicate m6 precursors. (**J**) Schematic view showing the coronal sectioning plane and the boundaries of En1^{Cre} (dotted lines). Radioactive in situ hybridization is shown in H-I′; immunohistochemistry in A-G‴. Scale bars: 200 μm (sagittal sections); 100 μm (coronal sections). mb, midbrain; di, diencephalon; hb, hindbrain; pc, posterior commissure.

Isthmic FGF8 regulates AP patterning of ventrolateral midbrain structures, such as oculomotor neurons and the red nucleus in m6 (Fedtsova and Turner, 2001; Agarwala and Ragsdale, 2002). To test whether the loss of FGF signaling might similarly affect patterning in m7, we analyzed this region in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos. Supporting our previous results (Saarimaki-Vire et al., 2007), E12.5 mutant midbrain m7 contained TH^{+} cells in a domain of approximately a similar size to that in the control (Fig. 2A′). However, these cells were now intermingled with FoxP1^{+}Pou4f1^{+} cells throughout the midbrain (Fig. 2C′,D-G). Similarly to the Pou4f1^{+} cells in the control diencephalon, FoxP1^{+}Pou4f1^{+} co-expressed variable amounts of Lmx1a (data not shown). Furthermore, *Pou4f1* mRNA was clearly already upregulated in mutant m7 by E11.5 (Fig. 2H-I′).

To test the possibility that Pou4f1^{+} cells emerge in mutant m7 due to dorsoventral mispatterning or migration from m6, we analyzed *Corin1* and Nkx6.1. In *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* m7, no ectopic Nkx6.1^{+} cells were observed and *Corin1* appeared unaltered (supplementary material Fig. S1H-J′). Thus, the dorsoventral pattern in the ventral midbrain remained unaltered, and the excess Pou4f1^{+} cells in the mutant Lmx1a^{+} region had probably not migrated from more lateral regions.

Taken together, these data indicate that mesodiencephalic DA precursors show a distinct AP pattern, where diencephalic DA precursors form a separate population intermingling with Pou4f1^{+}FoxP1^{+} non-DA cells. The emergence of these Pou4f1^{+} cells among *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain DA precursors suggests that this region may have acquired diencephalic characteristics.

DA precursors in the midbrain and diencephalon differ molecularly, and FGF signaling is required for midbrain-specific gene expression

To investigate whether the diencephalic and midbrain DA precursors differ molecularly from each other, we compared several markers of postmitotic DA precursors between these

populations. Already at E10.5, TH$^+$ cells were detected in the wild-type diencephalon but were nearly undetectable in the midbrain (Fig. 3A), corresponding to previous results (Marín et al., 2005). For analyses of E12.5 embryos, the diencephalic DA population was identified by TH and Pou4f1 immunohistochemistry and *Lmx1a* in situ hybridization on parallel slides (dotted line in Fig. 3B-I′). At this stage, DA precursors in the control diencephalon appeared to contain slightly less TH than the ones in the midbrain, and they lacked Pitx3 entirely (Fig. 3B,C, supplementary material Fig. S1D,E). By contrast, *Nurr1* and *Lmx1b* were expressed in both domains (Fig. 3D,E). In addition, diencephalic population lacked *DAT* and expressed less *Ddc* (Fig. 3F,G). Similarly, *En1* was expressed in the diencephalic precursors at a very low level, and *En2* was absent (Fig. 3H,I). Both *En1* and *En2* were still expressed in the caudal midbrain VZ (red arrows in Fig. 3H,I).

In *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* mutants, TH$^+$ cells were found throughout the midbrain already at E10.5 (Fig. 3A′). This may reflect either premature neurogenesis in this region (Lahti et al., 2011), or indicate transformation towards diencephalic DA phenotype. At E12.5, mutant midbrain TH$^+$ cells lacked Pitx3, but retained *Nurr1* and *Lmx1b* (Fig. 3B′-E′, supplementary material Fig. S5C-E′). Further resembling the diencephalic expression, *DAT*, *En1* and *En2* were absent, and *Ddc* was weakly expressed (Fig. 3F′-I′). Thus, consistent with the change in the cellular composition of the DA precursor population, mutant midbrain DA precursors showed molecular characteristics that highly resembled those of their diencephalic counterparts.

En1 and En2 expression in DA progenitors and postmitotic DA precursors in the absence of FGF signaling

Because at E12.5 we could also see a loss of *En1/2* in the VZ, the midbrain DA domain in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos might already have adopted a diencephalic identity at the progenitor stage. To study this possibility, we first analyzed how early *En1/2* are downregulated in DA domain. At E9.5, *En1* was expressed in Aldh1a1$^+$ region, whereas *En2* was detected only in lateral and dorsal midbrain (supplementary material Fig. S2A,B; data not shown). Notably, already at this stage the most anterior Aldh1a1$^+$ domain lacked *En1* (supplementary material Fig. S2A,B, brackets). At E10.5, En1 protein and *En1/2* transcripts were still detected in DA progenitors in the wild-type midbrain, but not in the diencephalon (supplementary material Fig. S2C-F). In E10.5 *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain, both *En1* and *En2* were still present in the caudal midbrain VZ, in the *Aldh1a1*-expressing domain (supplementary material Fig. S2D-F′).

From E11.5 onwards, when first TH$^+$ precursors appeared in Lmx1a$^+$ region, *En1/2* expression was detected in postmitotic DA precursors (Fig. 4A-C,E-G). In E11.5 *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}*

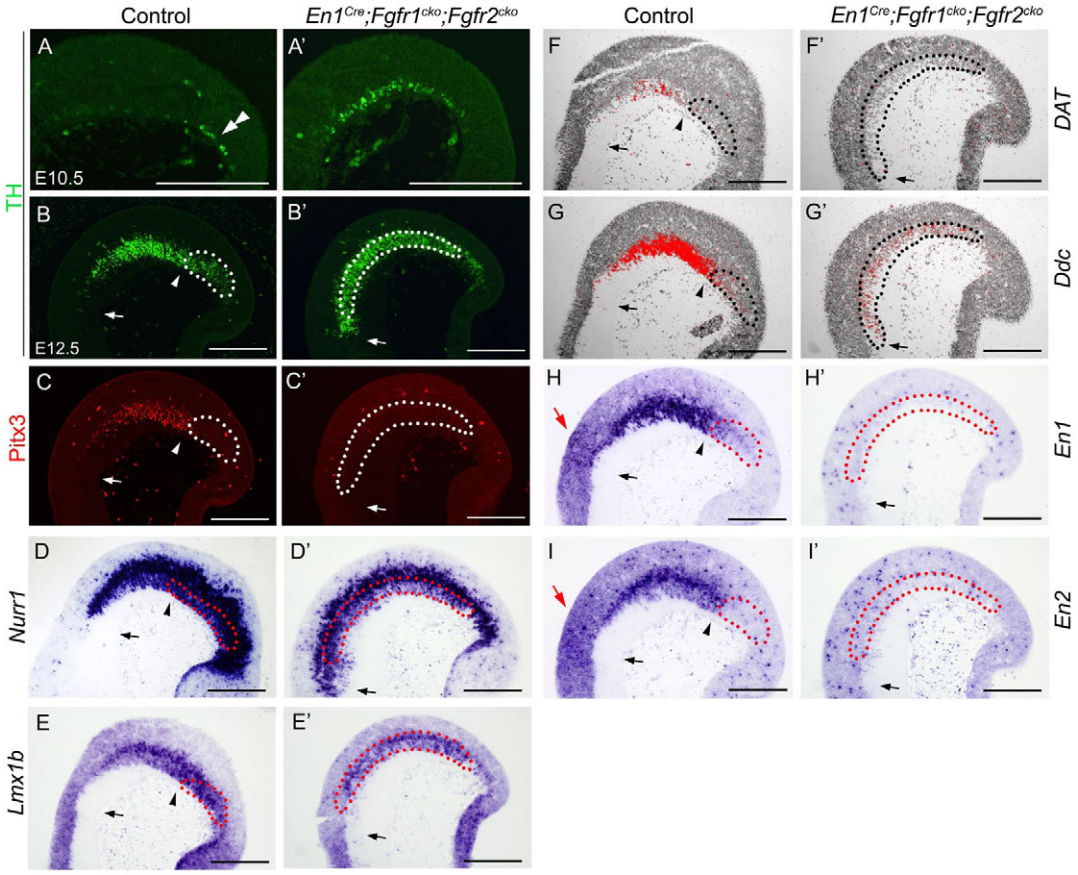

Fig. 3. Differential molecular characteristics in the midbrain and diencephalic DA precursors in control and *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos. (**A**,**A′**) E10.5 midbrain-diencephalon. (**B-I′**) E12.5 ventral midbrain-diencephalon. Dotted lines outline the Pou4f1$^+$ population in the diencephalic Lmx1a$^+$ region, identified on parallel sections. Sagittal sections, anterior rightwards. The location of the mesodiencephalic boundary (arrowheads) was based on the position of the posterior commissure. Black and white arrows show the midbrain-hindbrain boundary, white double arrowhead in A indicates TH$^+$ cells in wild-type diencephalon. Red arrows indicate En1/2 expression gradients in the control VZ. Immunohistochemistry is shown in A-C′; in situ hybridization in D-I′. Scale bars: 200 μm.

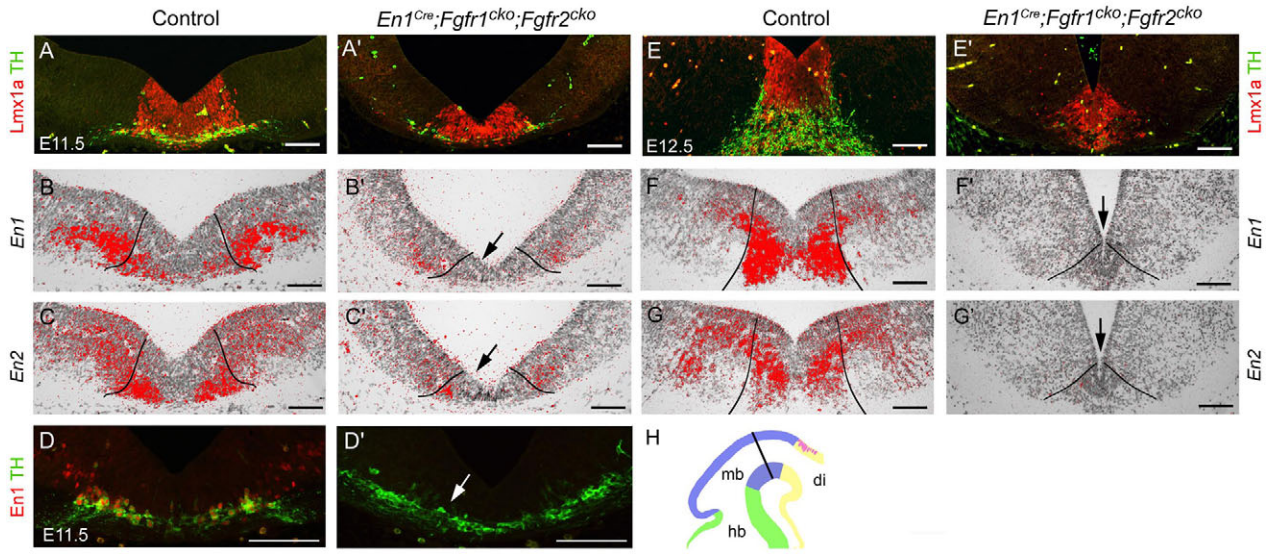

Fig. 4. *En1* and *En2* are absent in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* DA neurons. (**A-C′**,**E-G′**) Coronal sections of control and *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain. *En1* and *En2* were downregulated (arrows) in the mutant midbrain Lmx1a^{+} region (black lines), but were still detected laterally at E11.5. (**D**,**D′**) En1 protein was absent in mutant TH^{+} neurons. Arrow indicates downregulated expression. Immunohistochemistry in A,A′,D-E′; radioactive in situ hybridization in B-C′,F-G′. (**H**) Sectioning plane. Scale bars: 100 μm. mb, midbrain; hb, hindbrain; di, diencephalon.

midbrain, both transcripts were detectable in the lateral midbrain but not in the DA region (Fig. 4A′-C′). Concomitantly, mutant TH^{+} neurons lacked En1 protein (Fig. 4D,D′). At E12.5, both *En1* and *En2* were downregulated, except for a small *En2*-expressing domain in the most caudal midbrain (Fig. 4E′-G′ and data not shown).

Thus, together with genetic cell labeling by En1^{Cre}, our results indicate that *En1* is initially expressed in the wild-type midbrain and caudal diencephalon DA progenitors, but then gradually downregulated in the diencephalon. Downregulation of *En1* and *En2* in anterior mutant midbrain by E10.5 indicates that this region may have already adopted a more anterior identity at this stage. However, based on the β-galactosidase expression pattern (Fig. 2A′), most mutant diencephalic DA neurons have expressed *En1* earlier. Remarkably, postmitotic reactivation of *En1* and *En2* in the midbrain DA precursors completely fails in the absence of FGF signaling.

En1 and En2 expression in non-DA post-mitotic precursors in the midbrain

Unexpectedly, in control embryos, both *En1* and *En2* were also widely expressed in post-mitotic cells outside the Lmx1a^{+} region (Fig. 4A-C,E-G). To investigate their expression patterns in more detail, we mapped *En1/2* to dorsoventral domains of midbrain (m1-m7) using a combination of transcription factors as boundary markers, as described previously (Nakatani et al., 2007; Kala et al., 2009).

At E12.5, both *En1* and *En2* were detected in m6 and m5, and *En2* in even more lateral domains m4 and m3 (supplementary material Fig. S3A-E). En1 was detected in both Pitx3^{+} and Pitx3^{-} cells, and in a part of Pou4f1^{+} m6 (supplementary material Fig. S3F-I). At E14.5, *En1* and *En2* were mainly expressed in the midbrain DA neurons, but also outside the DA domain, for example in Pou4f1^{+} m6, and especially in the caudal midbrain GAD67^{+} GABAergic region (supplementary material Fig. S3K,L,N-V). These data show that although strongest *En1/2* expression was detected in midbrain DA precursors, both genes were also expressed in other postmitotic neuronal precursors, for example in m6.

Loss of FGF signaling does not lead to increased apoptosis of postmitotic DA precursors

The loss of *En1* and *En2* in vitro and in vivo results in the apoptosis of DA neurons (Albéri et al., 2004; Alavian et al., 2009). To study whether the downregulation of *En1/2* in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos results in a similar phenotype, we analyzed the active form of Caspase3 (Cas3) in TH^{+} neurons. Very few TH^{+} cells were Cas3^{+} in either control or mutant embryos between E11.5 and E13.5, and no significant differences were detectable (Fig. 5A-B′ and data not shown; Cas3^{+} eyes served as positive controls, Fig. 5C-F). In addition, the analysis of DAPI-stained nuclei revealed no fragmentation or condensation in TH^{+} cells (data not shown).

To study whether the disappearance of DA neurons results from TH downregulation, rather than cellular death, we analyzed Lmx1a and TH at later embryonic stages (Fig. 5G-I′). Indeed, E13.5 *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* ventral midbrain still contained postmitotic HuC/D^{+}Lmx1a^{+} cells, (Fig. 5G′), but they lost TH by E15.5 (Fig. 5H-I′). A subset of mutant Lmx1a^{+} cells still expressed Pou4f1 (data not shown). In conclusion, the loss of TH^{+} cells in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* mutants does not likely result from apoptosis, but rather from the loss of neurotransmitter identity.

FGF signaling regulates midbrain-specific gene expression in proliferative DA progenitors

Because diencephalic DA progenitors lacked *En1* and *En2* already at E10.5, we investigated whether other DA progenitor markers would show similar differences between the midbrain and diencephalon. Wnt signaling is required for DA neuron induction, proliferation and differentiation in the midbrain progenitors (Prakash et al., 2006; Castelo-Branco and Arenas, 2006; Andersson et al., 2008). In the diencephalon, its role the DA neuron development is less clear.

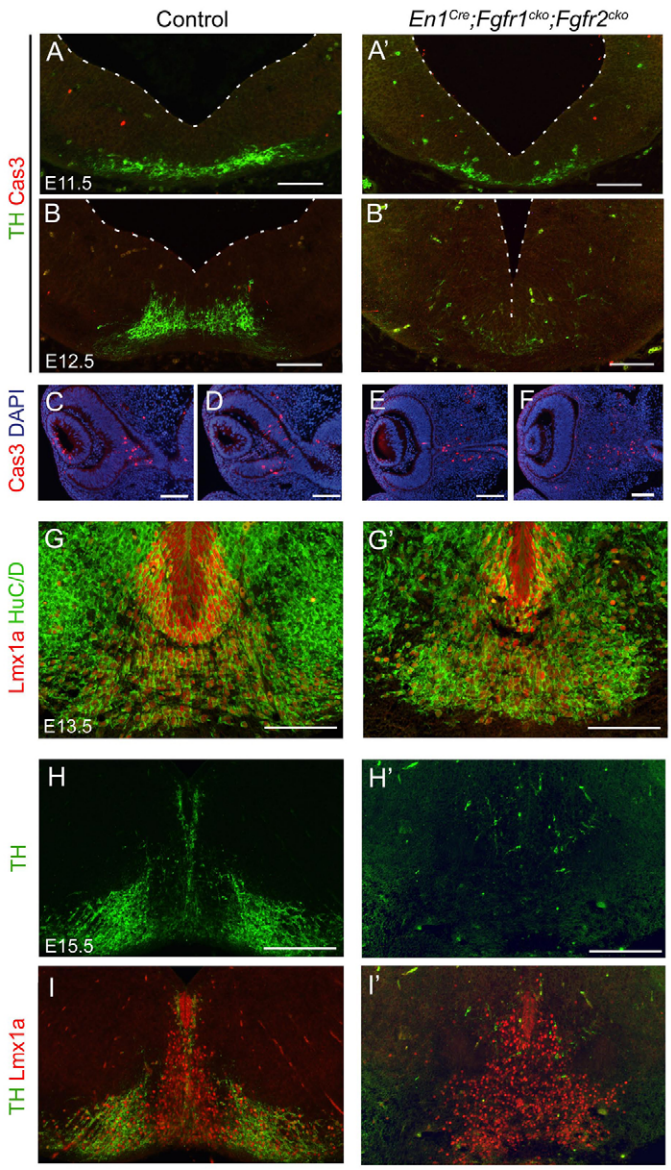

Fig. 5. DA precursors in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* do not display increased apoptosis. (**A-B′**) Apoptotic (activated Cas3^{+}) TH^{+} cells are absent in both control and mutant midbrain. (**C-F**) Eyes from corresponding sections in A-B′ serve as positive staining controls for Cas3. (**G-I′**) Postmitotic HuC/D^{+}Lmx1a^{+} cells lack TH in the mutant midbrain. Immunohistochemistry in all images. Scale bars: 100 μm in A-F; 200 μm in G-H′.

Wnt1 expression in *Aldh1a1*-expressing DA progenitors extended to the diencephalon at E9.5, but by E11.5 both *Aldh1a1* and *Wnt1* were confined to the caudal midbrain (supplementary material Fig. S4A-C,G-K). *Wnt8b* was expressed in the midbrain, but not diencephalic, DA progenitors from E11.5 onwards (supplementary material Fig. S4D-I,L,M). At E12.5, *Wnt1*, *Wnt8b* and *Aldh1a1* were all present in the caudal midbrain DA progenitors but absent more anteriorly (Fig. 6A-C). Wnt-target *Drapc1* (*Apcdd1*) showed stronger expression in midbrain DA progenitors compared with the diencephalon side (Fig. 6D). By contrast, *Wnt5a* and *Wnt7b* were expressed both in the control midbrain and diencephalon (Fig. 6E,F).

If midbrain DA progenitors in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* mutants had adopted more anterior characteristics, they might display diencephalic expression patterns of these genes. Indeed, in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain, *Wnt1*, *Wnt8b* and *Aldh1a1* were downregulated by E12.5 (Fig. 6A′-B′, supplementary material Fig. S4J′-M′). Similarly, *Drapc1* in the mutant midbrain showed p1-like expression (Fig. 6D′, supplementary material Fig. S5I,I′). By contrast, *Wnt7b*, *Wnt5a*, *Shh* and *Foxa2*, which are expressed both in the midbrain and in the diencephalic DA progenitors, continued to be expressed in mutants (Fig. 6G,H,E′-H′, supplementary material Fig. S5A-B′,F-H′). Interestingly, *Wnt7b* was more abundantly expressed in the diencephalon, and in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain DA progenitors its expression appeared slightly stronger. Taken together, these data indicate further differences between diencephalic and midbrain DA progenitors. Furthermore, when FGF signaling was inactivated, not only the postmitotic DA precursors but also DA progenitors in the mutant midbrain acquired diencephalic characteristics.

Retinoic acid treatment is unable to fully rescue DA neurons in the absence of FGF signaling

The gradual downregulation of *Aldh1a1* in mutants might contribute to the observed DA phenotype, or even to the AP patterning of the Lmx1a^{+} region. All trans-retinoic acid (RA) treatment rescued DA neuron development in *Pitx3^{null}* embryos (Jacobs et al., 2007). To attempt rescue of the mutant midbrain DA neurons, we gave pregnant mice RA-supplemented food from E9.5 to E13.5, and then analyzed the number of TH^{+}Pitx3^{+} neurons. RA treatment increased the number of DA neurons in littermate controls (supplementary material Fig. S6A-C). Untreated *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos lacked Pitx3^{+} cells (supplementary material Fig. S6A′,C), but the effect of RA to mutant DA neurons was very modest (supplementary material Fig. S6B′,C). RA induced some Pitx3^{+}TH^{+} neurons to develop in mutants, but only in the caudal midbrain. These data indicate that a small number of RA-responsive midbrain DA progenitors still exist in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos, and that *Aldh1a1* downregulation may contribute to the loss of *Pitx3* in the DA domain. However, RA treatment cannot rescue the majority of mutant DA precursors.

FGF signaling regulates DA neuron differentiation and midbrain-specific gene expression cell-autonomously

Next, we asked whether FGF signaling regulates properties of the DA domain in the ventral midbrain and caudal diencephalon directly. For this, we aggregated wild-type and *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko};R26R* mutant morulae to create chimeric embryos, the midbrain of which contained wild-type and mutant cell clusters. In E12.5 chimeras, β-galactosidase^{+} (mutant) cells expressed TH, although compared with the wild-type region, its level appeared decreased (Fig. 7A). Consistent with our results with *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos, in the Lmx1a^{+} region only wild-type cells expressed Pitx3 and only mutant cells Pou4f1 (Fig. 7B-B‴). As in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain, the level of co-expressed Lmx1a varied between Pou4f1^{+} cells. *En1* was downregulated in mutant but expressed in neighboring wild-type cells (Fig. 7C-D′), similar to *Wnt8b* and Aldh1a1 (Fig. 7E-H, brackets indicate *Wnt8b* expression domain in a wild-type embryo). As expected, *Shh* and Nkx6.1 showed no differences between mutant and wild-type regions (data not shown). Thus, our results

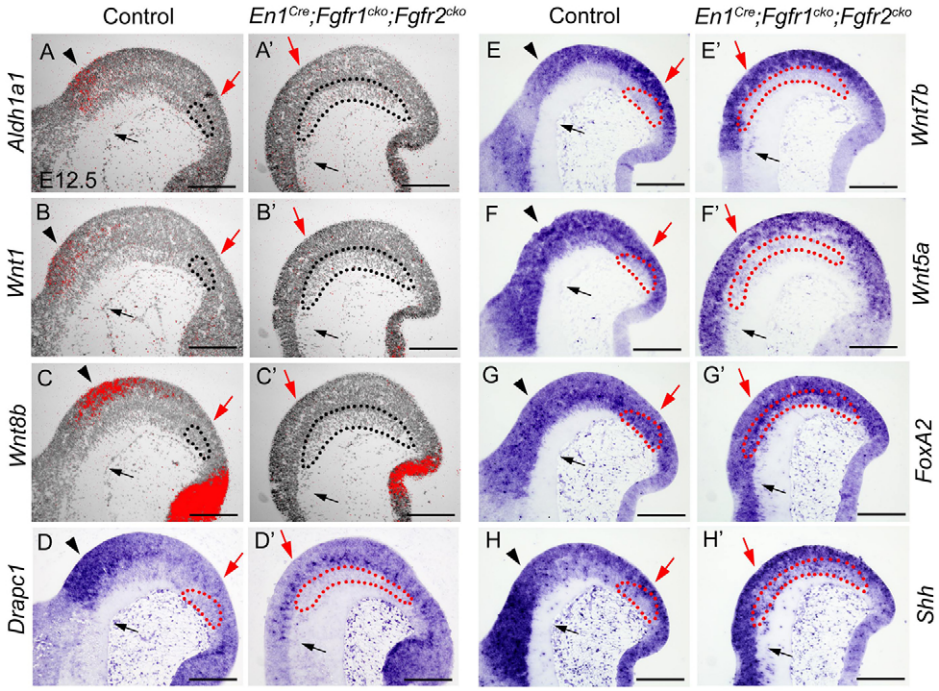

Fig. 6. Gene expression in midbrain and diencephalic DA progenitors in control and *En1*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} embryos. (**A-H′**) Sagittal sections of E12.5 ventral midbrain-diencephalon, anterior rightwards. Dotted lines outline the Pou4f1^{+} population in the diencephalic Lmx1a^{+} region, identified on a parallel section (not shown). In mutants, midbrain VZ gene expression in the DA domain (arrowheads) resembled that of the diencephalon (red arrows). Black arrows indicate the midbrain-hindbrain boundary. Radioactive in situ hybridization in A-C′; non-radioactive in situ hybridization in D-H′. Scale bars: 200 μm.

suggest that FGFR1/2-mediated signaling in the ventral midbrain and caudal diencephalon directly regulates AP patterning and DA neuron differentiation.

Inactivation of FGF signaling in post-mitotic DA precursors does not affect their differentiation or survival

Although the expression of FGFRs and several FGF targets appeared to be absent in postmitotic DA precursors, FGF signaling might still be active in these cells. To study this possibility, we inactivated *Fgfr1* and *Fgfr2* with *DAT*^{Cre}. As *DAT* expression begins at E12.5, we first analyzed the DA phenotype at E15.5 (supplementary material Fig. S7A-D″). Efficient recombination of *R26R* verified that Cre-recombinase was active at this stage (data not shown). *DAT*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} embryos displayed normal *DAT* and *Pitx3* expression, and *Fgfr1* and *Fgfr2* signals were low in both controls and mutants. The receptor expression appeared not to colocalize with *DAT*- and *Pitx3*-expressing cells on parallel sections.

The VTA and SNpc in adult *DAT*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} animals appeared normal despite efficient Cre-recombination, visualized by *R26R* (supplementary material Fig. S7E-E″). Dopamine levels between the control and *DAT*^{Cre} mutant striatum showed no statistically significant difference (supplementary material Fig. S7G). Furthermore, Th^{Cre}-mediated inactivation of *Fgfr1* and *Fgfr2* did not affect DA neuron development or survival by E18.5 (supplementary material Fig. S7F-F″). Both adult *DAT*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} and *Th*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} mice were viable and displayed no obvious behavioral defects. Thus, the loss of FGFR1/2-mediated signaling in the post-mitotic DA precursors has no major effect on their full maturation or survival.

DISCUSSION

DA neurons developing in the caudal diencephalon are thought to merge with midbrain DA populations to form mesodiencephalic DA nuclei. However, compared with their midbrain counterparts, the properties of developing diencephalic DA neurons – such as the use of signaling pathways and transcriptional codes – are less studied. Here, we identified a distinct population of DA precursors in the caudal diencephalon, and compared the early gene expression patterns in developing DA neurons between the midbrain and diencephalon. Midbrain and diencephalic DA populations showed differences in their genetic programs already at the proliferative progenitor stage, and more distinct differences appeared during differentiation of post-mitotic DA precursors. Our results indicate that FGF signaling directly regulates AP patterning in the midbrain DA domain. In its absence, the embryonic midbrain DA domain adopts cellular composition highly similar to the diencephalic DA domain. Furthermore, mutant DA progenitors and precursors show gene expression patterns that resemble those found in the wild-type diencephalic domain.

Distinct properties of DA precursors in the diencephalon and midbrain

Comparisons of gene expression, histology, and morphology in different vertebrate species have lead to a theory that some of the A9 and A10 DA neurons are born in the diencephalon (Smits et al., 2006). The characterization of *Pitx3*^{GFP} and the fate mapping of *Shh*-expressing cells have already identified distinct pools of DA progenitors in the midbrain (Maxwell et al., 2005; Joksimovic et al., 2009; Blaess et al., 2011). However, the origin of heterogeneity among mesodiencephalic DA neurons is still incompletely understood. According to our results, the most medial part of developing caudal diencephalon contains a distinct neuronal precursor population, consisting of DA precursors (TH^{+}) intermingled with non-DA cells (Pou4f1^{+}) (dotted area in Fig. 8B). We speculate that this ventral diencephalic Pou4f1^{+}FoxP1^{+}Lmx1a^{+} population might contribute to the parvocellular red nucleus described previously (Puelles, 1995), whereas Nkx6.1^{+}Pou4f1^{+} neurons in m6 would form the magnocellular part.

Dorsally, the mesodiencephalic boundary is regulated by counter-repression between *En1/2* and *Pax6* (Mastick et al., 1997; Araki and Nakamura, 1999; Matsunaga et al., 2000).

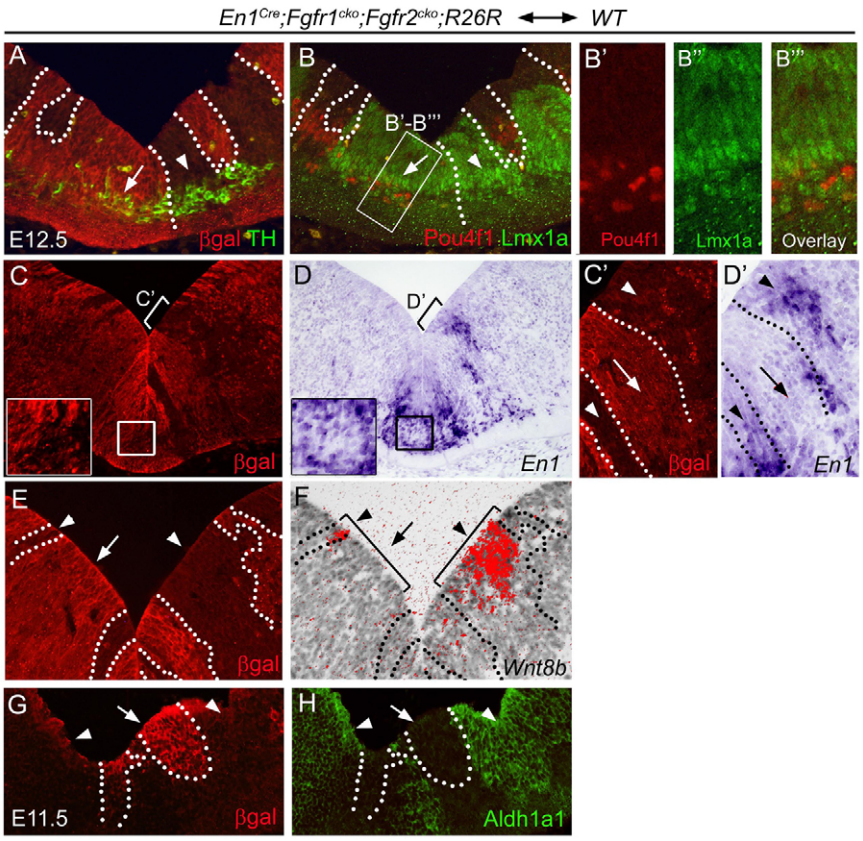

Fig. 7. FGF signaling regulates neuronal development and AP patterning in the midbrain DA domain cell-autonomously. (**A-H**) Coronal sections of ventral midbrain in chimeric *En1*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} ↔ WT embryos. In situ hybridization in D,D′,F; immunohistochemistry in A-C′,E,G,H. β-galactosidase (β-gal) immunohistochemistry, indicated by dashed lines on parallel sections, separates the mutant cells (arrows) from the wild-type cells (arrowheads). Brackets in F indicate the expression domain of *Wnt8b* in a comparable wild-type section – absent in the medial and present in lateral m7.

Consequently, *En1* expression domain is thought to define the anterior border of the midbrain alar plate. Unexpectedly, in the basal plate we located the rostral boundary of En1^{Cre}-mediated recombination in a clearly more anterior region, probably in p1-p2 (Puelles and Rubenstein, 2003). Thus, En1 performs different functions in alar and basal plates. Despite the early *En1* expression, the diencephalic DA progenitors lose *En1* and *En2* by E9.5, *Wnt1* and *Aldh1a1* by E12.5, and lack *Wnt8b* (Fig. 8A). By contrast, although the midbrain DA progenitors downregulate these genes in the most anterior midbrain by E11.5, they express them in the posterior midbrain at E12.5, in a gradient-like manner.

Compared with the midbrain, postmitotic DA precursors in the diencephalon lack *Pitx3* and *DAT*, and express less *Ddc* and *Th*, although the lower TH signal in IHC might also result from a decreased DA precursor density. Furthermore, the diencephalic DA precursors only weakly reactivate *En1*, but not *En2*, expression. Thus, clear differences between these neuronal populations continue to accumulate when DA neurogenesis begins.

Whether these distinct properties will remain later in development, or whether they represent only transient differences, remains unclear. Genetic fate mapping should verify to what extent, if any, the diencephalon-derived TH^{+} cells contribute to the mature mesodiencephalic DA system. Alternatively, the diencephalic precursors may express TH only transiently – a phenomenon observed elsewhere in CNS (Bjorklund and Dunnett, 2007). In the latter case, neither early TH positivity, nor the mere presence of certain transcription factors, can be used as a reliable indicator of successful mesodiencephalic DA neuron generation. Instead, the expression level and temporal expression dynamics of several midbrain DA markers should be monitored.

FGF signaling instructs the proliferative progenitors to produce midbrain-type DA precursors

Given that, in *En1*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} mutants, *Aldh1a1* downregulation was visible already at E9.5, we have previously suggested that FGFs could affect the properties of midbrain DA progenitors (Saarimaki-Vire et al., 2007). On the other hand, observations from the zebrafish have shown that diencephalic DA neurons do not require isthmic FGFs (Holzschuh et al., 2003). Our results on *Fgfr* and FGF target gene expression support both conclusions, as we show here that, during early DA neuron development, FGF signaling is most pronounced in the midbrain proliferative progenitors, whereas in the embryonic diencephalon FGF targets are lacking. Indeed, our analyses of *En1*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} mutants, as well as chimeric embryonic midbrains containing *En1*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} cells, demonstrate that FGF signaling directly regulates DA progenitors to acquire midbrain characteristics, including the expression of genes such as *En1*, *Wnt8b* and *Aldh1a1*.

Reflecting the gene expression changes of the proliferative progenitors in *En1*^{Cre};*Fgfr1*^{cko};*Fgfr2*^{cko} midbrain, post-mitotic mutant DA precursors also appear to have adopted diencephalic characteristics. Indeed, in the absence of FGFR1/2-mediated signaling, midbrain DA neurons become TH^{+}, but are unable to express several midbrain DA markers such as *En1/2*, *Pitx3* or *DAT*. This phenotype resembles that of the wild-type caudal diencephalic neurons (Fig. 8A,B). As patterning genes such as *En1/2* show residual expression in the ventral midbrain of E10.5 mutants, this fate change probably occurs gradually. Downregulation of *En1/2* by E11.5 in the mutant VZ might imprint these cells with a diencephalic fate, and consequently prevent the reactivation of these genes in postmitotic neurons.

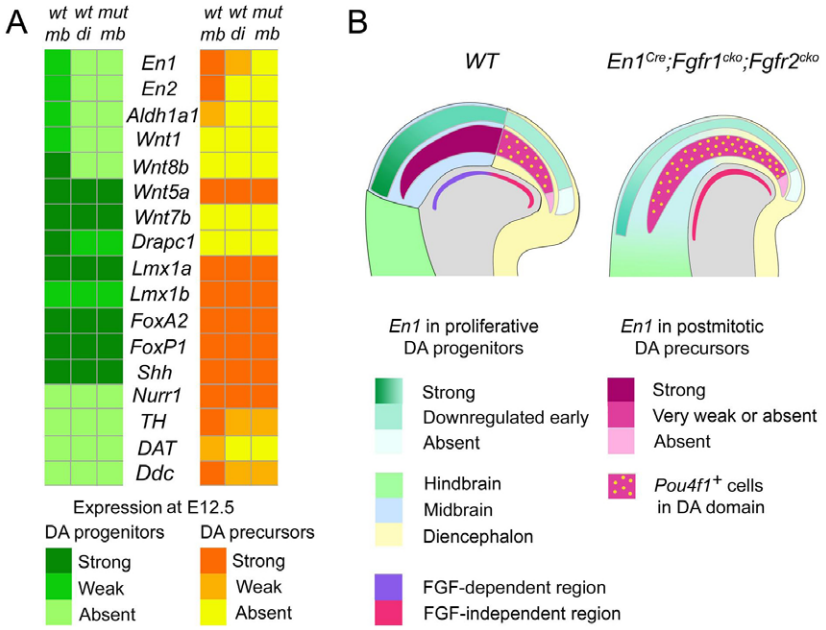

Fig. 8. Midbrain patterning and DA gene expression changes in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos. (**A**) Summary of gene expression in E12.5 DA progenitors (green) and post-mitotic precursors (orange) in wild-type (wt) caudal diencephalon and midbrain, and in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* (mut) midbrain. (**B**) Model of FGF-regulated development of meso-diencephalic DA neurons, focusing on *En1* domains at E12.5. The boundaries were determined according to Puelles and Rubenstein (Puelles and Rubenstein, 2003). DA neurons in the wild-type midbrain, but not in the diencephalon, require isthmic FGFs. The diencephalic DA domain contains also non-DA Pou4f1$^+$ cells (dots). Proliferative midbrain DA progenitors express *En1* in a posterior-to-anterior gradient. All midbrain post-mitotic DA precursors express *En1* strongly, whereas diencephalic DA progenitors lose *En1* early and precursors reactivate it very weakly. A small TH$^+$ neuron population, presumably in p2-p3, develops independently of *En1*. In the absence of FGFR1/2-mediated signaling, midbrain progenitors lose *En1* by E11.5 and precursors fail to reactivate it. DA, dopaminergic; mb, midbrain; di, diencephalon.

Lmx1a$^+$ cells in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain lose TH expression by E15.5. This may result from more complex alterations in *Fgfr* mutants, or reflect the normal development of caudal diencephalic DA neurons (as discussed above). Thus, we suggest that FGFs instruct proliferating progenitors to adopt a midbrain DA neuron identity, which assures the activation of genetic pathway required for full DA neuron phenotype. However, we cannot formally exclude the possibility that the remaining DA precursors in mutants are immature rather than mispatterned, and that early FGF signaling is required for the later maturation of the DA precursors. Such defects have been demonstrated in the forebrain, where early Sox9 function in proliferative neuronal progenitors results in a differentiation defect in post-mitotic precursors derived from them (Scott et al., 2010).

In addition to their role in the midbrain regionalization, *En1* and *En2* are needed for the survival of midbrain DA neurons in a dose-dependent manner (Simon et al., 2001; Albéri et al., 2004; Simon et al., 2004; Alavian et al., 2009). DA neurons die apoptotically both in *En1$^{-/-}$;En2$^{-/-}$* embryos, and after in vitro *En1/2* inactivation in postmitotic wild-type DA precursors. However, a similar phenomenon does not occur in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* compound mutants, which lack *En1* and *En2* in the TH$^+$ precursors. The reason may be temporal: full *En1/2* inactivation affects midbrain and hindbrain earlier than the conditional inactivation of FGFRs. Furthermore, DA precursors in vitro, separated from possible survival-promoting signals in the intact midbrain, may be more sensitive to *En1/2* inactivation than in vivo. Whether the *En1$^{-/-}$;En2$^{-/-}$* mutants show a switch to a diencephalic DA precursor phenotype remains to be studied.

In the *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* midbrain, we observed a posterior expansion of Pou4f1$^+$ population in the Lmx1a$^+$ (m7) region (Fig. 8B). Similarly, as in the wild-type diencephalon, these Pou4f1$^+$ cells intermingled with TH$^+$ precursors and lacked Nkx6.1. This suggests that the excess Pou4f1$^+$ cells do not migrate from m6 to m7 the *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* mutants, but may represent the Pou4f1$^+$ cells observed in the ventral diencephalon. Together with the observed loss of midbrain-specific DA progenitor and precursor markers in mutants, the posterior expansion of the intermingled TH$^+$ and Pou4f1$^+$ populations supports our theory that mutant midbrain acquires diencephalic characteristics.

Alternative to fate transformation, the midbrain DA domain could be lost by cell death in the compound *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* mutants. However, several observations argue against this possibility. First, the size of the overall DA region is similar in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* mutants compared with the wild type. Second, we did not detect apoptosis in the ventral midbrain at any stage studied. This is consistent with the earlier studies, in which no apoptosis was detected in *Fgf8^{cko}* ventral midbrain, and only a slight increase was detected in *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos (Chi et al., 2003; Saarimaki-Vire et al., 2007). Finally, our chimeric analysis demonstrated a transformation of FGF-unresponsive cells in the midbrain, surrounded by wild-type tissue, to adopt diencephalic characteristics. Thus, we suggest that, rather than survival, FGF signaling in the ventral midbrain directly regulates AP patterning.

FGF signaling in post-mitotic DA precursors?

Although *Fgfr1* and *Fgfr2* expression has been reported in the adult rat SNpc (Belluardo et al., 1997), in our analyses *Fgfr1* and *Fgfr2* were nearly undetectable in SNpc and VTA. Furthermore, the few

Fgfr-expressing cells did not appear to colocalize with TH, and were probably oligodendrocytes and astrocytes (Redwine et al., 1997; Reimers et al., 2001). Neither $DAT^{Cre};Fgfr1^{cko};Fgfr2^{cko}$ nor $Th^{Cre};Fgfr1^{cko};Fgfr2^{cko}$ mutants displayed obvious alterations in their midbrain DA neurons or behavior, although controlled behavioral testing may reveal more subtle deficiencies. By contrast, mice carrying a dominant-negative *Fgfr1* under *Th*-promoter have a slightly reduced density of TH^+ cells in SNpc, increased DA transmission in striatum, and display a schizophrenia-like syndrome (Klejbor et al., 2006). Nevertheless, the inactivation of FGF signaling in postmitotic DA neurons does not lead to their disappearance in either conditional or dominant-negative *Fgfr* mutants. These data support the conclusion that, during DA neuron development, FGF signaling regulates primarily the early proliferative progenitors.

Recently, *Erm* and *Pea3* were detected in postnatal DA neurons (Wang and Turner, 2010). Thus, the level of FGF signaling may temporally change during DA neuron development. Alternatively, postnatal *Erm* and *Pea3* may reflect the activity of another signaling pathway in DA neurons.

Conclusions

During development, DA progenitors and precursors in the caudal diencephalon and midbrain employ partly different genetic programs. Moreover, $Pou4f1^+$ non-DA cells and TH^+ DA precursors intermingle in the caudal diencephalon, whereas in the midbrain these cell types separate to m6 and m7. Normally, isthmic FGF signaling regulates AP patterning in this region by suppressing the diencephalic and maintaining the midbrain identity. In the midbrain, FGF signaling induces genetic pathways leading to the activation of midbrain-specific gene expression in proliferative progenitors and postmitotic DA precursors. In the absence of FGF signaling, the midbrain DA domain adopts characteristics of the embryonic diencephalon, including a concomitant caudal expansion of the $Pou4f1^+$ population. Later, mutant DA precursors fail to terminally differentiate and lose their neurotransmitter phenotype. Fate-mapping experiments are needed to demonstrate whether this reflects the normal development of the caudal diencephalic DA precursors, or whether these precursors contribute to specific neuronal subtypes in adult DA nuclei.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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