Fibroblast Growth Factor Receptors Cooperate to Regulate Neural Progenitor Properties in the Developing Midbrain and Hindbrain

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Fibroblast growth factors (FGFs) secreted from the midbrain–rhombomere 1 (r1) boundary instruct cell behavior in the surrounding neuroectoderm. For example, a combination of FGF and sonic hedgehog (SHH) can induce the development of the midbrain dopaminergic neurons, but the mechanisms behind the action and integration of these signals are unclear. We studied how FGF receptors (FGFRs) regulate cellular responses by analyzing midbrain–r1 development in mouse embryos, which carry different combinations of mutant Fgfr1, Fgfr2, and Fgfr3 alleles. Our results show that the FGFRs act redundantly to support cell survival in the dorsal neuroectoderm, promote r1 tissue identity, and regulate the production of ventral neuronal populations, including midbrain dopaminergic neurons. The compound Fgfr mutants have apparently normal WNT/SHH signaling and neurogenic gene expression in the ventral midbrain, but the number of proliferative neural progenitors is reduced as a result of precocious neuronal differentiation. Our results suggest a SoxB1 family member, Sox3, as a potential FGF-induced transcription factor promoting progenitor renewal. We propose a model for regulation of progenitor cell self-renewal and neuronal differentiation by combinatorial intercellular signals in the ventral midbrain.

Key words: fibroblast growth factor; isthmic organizer; midbrain; dopaminergic neuron; neural stem cell; SoxB1

Introduction

Development of structures derived from the embryonic midbrain and rhombomere 1 (r1) is governed by a combinatorial action of intercellular signaling pathways. One source of such signals is the isthmic organizer (IsO), which is located at the midbrain–r1 boundary and secretes signaling molecules, including fibroblast growth factor (FGF) family members (Wurst and Bally-Cuif, 2001; Zervas et al., 2005; Rhinn et al., 2006). Knowledge of the cellular responses to these signals is important for both understanding normal brain development and devising strategies for therapeutic neuronal stem cell differentiation.

FGF signaling has been implied in several aspects of development in the midbrain and r1. Especially, FGF8 can transform cellular identities and induce development of ectopic midbrain and r1 structures (Nakamura et al., 2005; Zervas et al., 2005). Conditional inactivation of Fgf8 in the midbrain–r1 region results in apoptotic cell death and a failure in development of both dorsal and ventral brain structures (Chi et al., 2003). FGF signaling in the midbrain and r1 may control cell proliferation and differentiation of specific neuronal cell types (Ye et al., 1998; Xu et al., 2000; Trokovic et al., 2005). In a rat midbrain explant culture model, FGF8 and sonic hedgehog (SHH) can promote the development of dopaminergic (DA) neurons, whereas inhibition of FGF signaling blocks it (Ye et al., 1998). However, whether and how FGFs regulate the production of the DA neurons in vivo still remains open.

During the midbrain–r1 development in the mouse, FGF receptors Fgfr1, Fgfr2, and Fgfr3 are expressed (Liu et al., 2003; Blak et al., 2005; Trokovic et al., 2005). Fgfr1 transcripts are abundant in the entire midbrain and r1, whereas Fgfr2 and Fgfr3 are not detected at the midbrain–r1 boundary. Consistently, conditional inactivation of Fgfr1 results in midbrain and r1 defects (Trokovic et al., 2003, 2005; Jukkola et al., 2006), whereas inactivation of Fgfr2 or Fgfr3 alone does not interfere with the development of this brain region (Blak et al., 2006). Compared with the conditional Fgfr8 mutants, however, the phenotype of the conditional Fgfr1 mutants is clearly less severe (Trokovic et al., 2003; Jukkola et al., 2006). Target genes of FGF signaling are still expressed in the midbrain and r1 of the Fgfr1 mutants, except in the cells near the midbrain–r1 border (Trokovic et al., 2003, 2005). Because these regions overlap with Fgfr2 and Fgfr3 expression, it is possi-
ble that in Fgfr1 mutants, either Fgfr2, Fgfr3, or both mediate residual FGF signaling.

Here, we have generated mice carrying different combinations of Fgfr1, Fgfr2, and Fgfr3 mutations. Our results demonstrate partly redundant contributions of the three FGF receptors in receiving signals from the IsO, regulating cell survival, and patterning in the developing midbrain and r1. In addition, we suggest that FGF signaling through these receptors in the ventral midbrain controls SoxB1 activity and the decisions about the neural progenitor cell proliferation versus differentiation. Distinct signaling pathways need to be integrated to regulate self-renewal, cell identity, and postmitotic differentiation of neural progenitors into neuronal subtypes, such as DA neurons.

Materials and Methods

Generation and genotyping of mice and embryos. Generation and genotyping of an Engrailed 1 (En1) allele carrying Cre-recombinase knock-in (Kimmel et al., 2000), a conditional Fgfr1 allele (Trokovic et al., 2003), a conditional Fgfr2 allele (Yu et al., 2003), and a Fgfr3 null allele (Colvin et al., 1996) have been described previously. All of the alleles were maintained in an outbred genetic background (ICR).

Mice carrying these alleles were intercrossed to generate En1Cre;Fgfr1flox/flox;Fgfr2flox/flox;Fgfr3flox/flox, En1Cre;Fgfr1flox/flox;Fgfr2flox/flox;Fgfr3null/null (Fgfr3null), En1Cre;Fgfr1flox/flox;Fgfr2null/null;Fgfr3flox/flox (Fgfr2null), En1Cre;Fgfr1flox/flox;Fgfr2null/null;Fgfr3null/null (Fgfr2null,Fgfr3null), and En1Cre;Fgfr1flox/flox;Fgfr2null/null;Fgfr3null/null (Fgfr1cko,Fgfr2cko,Fgfr3null) embryos.

Table 1. Summary of the midbrain–r1 defects observed in Fgfr compound mutant embryos

| Genotype                        | Phenotype                  | E9.5          | E12.5         | E18.5          |
|--------------------------------|----------------------------|---------------|---------------|---------------|
| Fgfr1flox                       | Boundary defect            | n.a.          | n.a.          | n.a.          |
| Fgfr2flox                       | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr3null                       | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr1cko                       | Apoptosis, mispatterning, no IsO gene expression dorsally | n.a.          | n.a.          | n.a.          |
| Fgfr1cko,Fgfr2flox             | Apoptosis, mispatterning, no IsO gene expression dorsally | n.a.          | n.a.          | n.a.          |
| Fgfr1cko,Fgfr3null             | Apoptosis, mispatterning, no IsO gene expression dorsally | n.a.          | n.a.          | n.a.          |
| Fgfr2flox,Fgfr3null             | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr2flox,Fgfr3null             | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr3null                       | n.a.                       | n.a.          | n.a.          | n.a.          |

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| Genotype                        | Phenotype                  | E9.5          | E12.5         | E18.5          |
|--------------------------------|----------------------------|---------------|---------------|---------------|
| Fgfr1flox                       | Boundary defect            | n.a.          | n.a.          | n.a.          |
| Fgfr2flox                       | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr3null                       | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr1cko                       | Apoptosis, mispatterning, no IsO gene expression dorsally | n.a.          | n.a.          | n.a.          |
| Fgfr1cko,Fgfr2flox             | Apoptosis, mispatterning, no IsO gene expression dorsally | n.a.          | n.a.          | n.a.          |
| Fgfr1cko,Fgfr3null             | Apoptosis, mispatterning, no IsO gene expression dorsally | n.a.          | n.a.          | n.a.          |
| Fgfr2flox,Fgfr3null             | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr2flox,Fgfr3null             | n.a.                       | n.a.          | n.a.          | n.a.          |
| Fgfr3null                       | n.a.                       | n.a.          | n.a.          | n.a.          |

Table 1. Summary of the midbrain–r1 defects observed in Fgfr compound mutant embryos

1D, Dorsal motor nucleus; 1V, trochlear nucleus; DR, serotonergic neurons of the dorsal raphe; IC, inferior colliculus of the midbrain; LC, locus ceruleus; PPT, posterior pretectum; SC, superior colliculus of the midbrain; Ve, vermis of the cerebellum; n.a., not analyzed; –, no phenotypical defects observed.

Results

Anatomical defects in the midbrain and rhombomere 1 derivatives of the compound Fgfr mutants

To address the possible redundancy among Fgfr1, Fgfr2, and Fgfr3 in the regulation of the midbrain–r1 development, we generated embryos carrying different combinations of En1Cre, conditional Fgfr1 (Fgfr1flox), conditional Fgfr2 (Fgfr2flox), and Fgfr3 null (Fgfr3null) alleles (Table 1, Fig. 1). The Cre-recombinase-mediated inactivation of Fgfr1 and Fgfr2 gene expression in the midbrain and r1 was observed between E8.5 and E9.5 [by 10 somite stage (ss) in the case of Fgfr1 (Trokovic et al., 2003; Blak et al., 2006) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material)]. In contrast to the Fgfr1 mutants, in which Fgfr1 was conditionally inactivated by En1Cre (hereafter referred to as Fgfr1cko) (Trokovic et al., 2003), neither the conditional inactivation of Fgfr2 by En1Cre (Fgfr2cko) nor the null mutation of Fgfr3 (Fgfr3null) disturbed the normal development of the midbrain–r1 region (Table 1) (Blak et al., 2006). Even Fgfr1cko,Fgfr2cko,Fgfr3null mutants had no defects at E18.5 (Fig. 1C). Thus, FGFRI clearly is the primary FGF receptor receiving signals from the IsO.

However, the other FGFs play a role as well. The phenotype of E18.5 Fgfr1cko,Fgfr2cko,Fgfr3null mutants was strikingly more severe than that of the Fgfr1cko mutants (Fig. 1B, D). Unlike the Fgfr1cko mutants, the Fgfr1cko,Fgfr2cko mutants did not survive after birth. In the Fgfr1cko,Fgfr2cko mutants, most of the dorsal midbrain, including the superior and inferior colliculi, was deleted. Similarly, whereas the Fgfr1cko embryos lack only the vermis of the cerebellum, derived from the dorsorostral anteri r1, the Fgfr1cko,Fgfr2cko double mutants lack the entire cerebellum. In addition to these dorsal derivatives of the midbrain and r1, the developmental defects of many of the ventrally derived nuclei were clearly more severe in the Fgfr1cko,Fgfr2cko,Fgfr3null mutants than in the Fgfr1cko mutants (Table 1;
see below). In contrast, we did not observe phenotypical differences between the Fgfr1cko;Fgfr3null and Fgfr1cko mutants at E18.5 (Fig. 1B, E).

The phenotype of E18.5 Fgfr1cko; Fgfr2cko;Fgfr3null triple mutants resembled that of the Fgfr1cko;Fgfr2cko double mutants, but only in the triple mutants did the dorsal deletion include the posterior pretectum (Fig. 1D, F). The deletions in the ventral brain region may also be more severe in the triple mutants, but this phenotypic characteristic was difficult to measure quantitatively. More convincing differences between the Fgfr1cko;Fgfr2cko;Fgfr3null and Fgfr1cko; Fgfr2cko mutants were seen in the early gene expression patterns and numbers of DA neurons (see below). Overall, the phenotype of the Fgfr1cko;Fgfr2cko;Fgfr3null mutants at E18.5 appeared very similar to the Fgf8 conditional mutants (Chi et al., 2003).

FGF target gene expression

To study the effects of Fgfr mutation on FGF signaling in embryos, we analyzed the expression of the Ets-family transcription factors Erm and Pea3, as well as the feedback antagonist Sprouty1 (Spry1). All of these genes are considered to be general and early transcriptional targets of the FGF signaling pathway. Compared with the wild type, in Fgfr1cko;Fgfr2cko double mutants Erm (Fig. 1G, J), Pea3 (Fig. 1K, L), and Spry1 (Fig. 1M, N) were markedly downregulated by E9.0 (20–23 somite stage). This is in contrast with the same stage Fgfr1cko mutants, in which these genes are downregulated only in a narrow stripe of cells close to the midbrain–r1 boundary (Fig. 1H) (Trokovic et al., 2005). In the Fgfr1cko;Fgfr2cko mutants, some expression of Erm still existed in the ventral neuroectoderm at the midbrain–r1 boundary (Fig. 1H) (Trokovic et al., 2005). In the Fgfr1cko;Fgfr2cko mutants, some expression of Erm still existed in the ventral neuroectoderm at the midbrain–r1 boundary (supplemental Fig. S2P, available at www.jneurosci.org as supplemental material). In contrast, we found no neuroectodermal Erm expression in the Fgfr1cko;

Figure 1. Anatomical defects and FGF target gene expression in the compound Fgfr mutants. A–F, Midsagittal sections of E18.5 wild-type (WT; A), Fgfr1cko (R1cko; B), Fgfr2cko;Fgfr3null (R2cko;R3null; C), Fgfr1cko;Fgfr2cko (R1cko;R2cko; D), Fgfr1cko; Fgfr3null (R1cko;R3null; E), and Fgfr1cko;Fgfr2cko;Fgfr3null (R1cko;R2cko;R3null; F) brains stained with hematoxylin-eosin. G–Z, Expression of FGF-signaling target genes Erm (G–J), Pea3 (K, L), and Sprouty1 (M, N), Fgf8 (O, P, S–V; dorsal view, O’, P’, S’), Fgf17 (Q, R, and En1 (W–Z) in WT, Fgfr1cko;Fgfr2cko, and Fgfr1cko; Fgfr2cko;Fgfr3null embryos as detected by whole-mount in situ hybridization at E8.5 (11 ss)–E9.5 (20–23 ss). Red arrows indicate dorsal structures that are affected in mutant brains and loss of gene expression in mutant embryos. Red arrowheads point to ventral expression domains that remain in Fgfr1cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants. White arrowheads mark the location of the midbrain–r1 border in WT embryos in all of the figures. Cb, Cerebellum; CP, choroid plexus; IC, inferior colliculus; PC, posterior commissure; PN, pontine nuclei; PPT, posterior pretectal nucleus; SC, superior colliculus. Scale bar, 1 mm.
Fgfr2<sup>cko</sup>;Fgfr3<sup>null</sup> mutants at the same stage (E9.5, 23 ss) (Fig. 1; supplemental Fig. S2Q, available at www.jneurosci.org as supplemental material).

Next, we analyzed the expression of other downstream targets of FGF signaling, such as Fgf8, Fgf17, and Fgf18 themselves, as well as En1. In contrast to the Fgfr1<sup>cko</sup> mutants (Fig. 1T,X), the expression of all of these genes was abolished in the Fgfr1<sup>cko</sup>;Fgfr2<sup>cko</sup> embryos between E8.5 (11 ss) (Fig. 1O,P) and E9.5 (20 –25 ss) (Fig. 1Q–Z) (data not shown), except for a small ventral domain. Similarly to the Erm expression, the downregulation of both Fgf8 and En1 in the Fgfr1<sup>cko</sup>;Fgfr2<sup>cko</sup>;Fgfr3<sup>null</sup> mutants at the same stage was even more striking and almost complete (Fig. 1V,Z). Thus, the inactivation of all Fgfr1, Fgfr2, and Fgfr3 results in an early failure in Iso signaling. Also, the comparison of the gene-expression patterns between Fgfr1<sup>cko</sup>;Fgfr2<sup>cko</sup> and Fgfr1<sup>cko</sup>;Fgfr2<sup>cko</sup>;Fgfr3<sup>null</sup> mutants reveals a role for Fgfr3, especially in the ventral midbrain–r1 region.

Cell death
Because the loss of Iso signaling in the conditional Fgf8 mutant and in the Wnt1 null mutant embryos results in elevated cell death at E8.5–E9.5 (Chi et al., 2003), we next analyzed apoptotic cell death in the compound Fgfr mutants. Whole-mount TUNEL staining revealed an increased number of apoptotic cells in the dorsal midbrain–r1 region of Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup> and Fgfr1<sup>cko</sup>;Fgfr2<sup>cko</sup>;Fgfr3<sup>null</sup> embryos at E8.5–E9.0 (12 and 16–18 ss) (Fig. 2A–I).

To further quantify the apoptotic cell death, we performed TUNEL staining on sections of E9.0 (17–20 ss) wild-type and Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup> double-mutant embryos. To determine the borders of the midbrain–r1 region, parallel sections were hybridized with Ptx6 and HoxA2 probes (data not shown). Compared with wild type (n = 2), the number of apoptotic cells was increased in the dorsal midbrain–r1 region of the Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup> embryos (n = 3; p < 0.05) (Fig. 2P–R). Cleaved caspase 3 staining and analysis of semithin plastic sections at E9.0 (14–20 ss) (Fig. 2J–O) (data not shown) also revealed apoptotic cells and a loss of epithelial morphology, especially in the dorsal midbrain–r1 tissue. Thus, the increased cell death presumably contributes to the loss of dorsal midbrain and r1 derivatives. In the ventral midbrain–r1 of the Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup> mutants, the apoptotic cell death showed a small but statistically significant

**Figure 2.** Increased apoptotic cell death in the dorsal midbrain–r1 region. A–I, Increased apoptosis is detected by TUNEL staining in the Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup> (R1cko;R2cko) and the Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup>;Fgfr3<sup>null</sup> (R1cko;R2cko;R3null) embryos compared with wild-type (WT). J–O, Sagittal semithin sections of the E9.0 WT (L, N) and Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup> (K, M, O) dorsal midbrain–r1 region. L–O, Close-ups of dorsal and ventral midbrain of WT (L, N) and Fgfr1<sup>1ko</sup>;Fgfr2<sup>2ko</sup> (M, O) embryos. P–R, The number of apoptotic cells was quantified (mean of total number ± SD) separately from ventral and dorsal midbrain–r1 regions of TUNEL-stained paraffin sections. Red arrows indicate an increased number of apoptotic cells. Mb, Midbrain; hb, hindbrain; ov, optic vesicle; di, diencephalon; DAPI, 4′,6′-diamidino-2-phenylindole. *p < 0.05, analyzed by Student’s t test. Scale bars: J, K, 200 μm; L–O, 30 μm; P, Q, 100 μm.
Anteroposterior patterning

FGF signaling is considered to be involved in establishment of the anterior border of the midbrain and the posterior border of the r1, as well as in the maintenance of the midbrain–r1 border position (Zervas et al., 2005). To determine the borders of the midbrain–r1 region in the Fgfr1cko;Fgfr2cko embryos, we performed a whole-mount in situ hybridization with probes for Pax6, expressed anteriorly in diencephalon, and HoxA2, expressed posteriorly in r2. In E9.5 Fgfr1cko;Fgfr2cko mutants, the size of the midbrain–r1 domain appeared reduced, but Pax6 and HoxA2 expression domains showed no clear signs of spreading or enlargement (Fig. 3A, B). Thus, midbrain–diencephalic and r1–r2 borders appear correctly established in the brain–r1 region in the mutants. Red arrowheads indicate a caudal shift of Otx2 expression toward r2, and red arrow indicates change of gene expression in r1.

Development of neuronal populations in the ventral midbrain–r1 region

Based on the expression of the regional marker genes, such as Pax6, Otx2, En1 and HoxA2, the E9.5–E11.5 Fgfr1cko;Fgfr2cko mutants still have a considerable amount of midbrain–r1 tissue. We therefore wanted to analyze how neuronal differentiation was affected in these mutants, especially in the ventral region, in which apoptotic cell death was not prominent at the early stages.

We found no TH-positive DA neurons in the ventral midbrain of E18.5 Fgfr1cko;Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null embryos (Fig. 4A, B) (data not shown). Analyses for dopamine transporter (Dat), Ptx3 and Nurrl expression confirmed these results (data not shown). In contrast, Fgfr1cko;Fgfr3null mutants had abundant but disorganized DA neurons in the ventral midbrain (data not shown), similarly to the Fgfr1cko mice (Jukkola et al., 2006). In addition, both in the Fgfr1cko;Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null embryos at E18.5, noradrenergic neurons of the locus ceruleus, serotonergic neurons of the dorsal raphe nuclei, and the oculomotor and trochlear motor nuclei, were lacking (supplemental Fig. S2A–N, available at www.jneurosci.org as supplemental material).

Development of the dopaminergic neuron precursors

Because apoptotic cell death or identity transformation seemed unlikely to fully explain the loss of DA neurons in the ventral midbrain, we analyzed their development in the mutants in more detail. Interestingly, in the Fgfr1cko;Fgfr2cko (n = 5) and Fgfr1cko;Fgfr2cko;Fgfr3null (n = 3) embryos at E12.5, few TH-positive neurons existed, although compared with the wild type (n = 7) and Fgfr1cko (p < 0.01) (Fig. 4F–J), their amount was clearly reduced. Between the Fgfr1cko;Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants, the number of TH-positive neurons differed (p < 0.01), as well. Because virtually no TH-positive cells existed in the ventral midbrain of Fgfr1cko;Fgfr2cko mutants at E15.5 (Fig. 4C–E) (n = 3), they are lost soon after E12.5.

To test the idea that the residual TH-positive cells may result from an incomplete Cre recombination, we analyzed Fgfr1 expression in E12.5 Fgfr1cko;Fgfr2cko mutant midbrain (Fig. 4T,V). We observed scattered Fgfr1-expressing cells dorsally. These cells may have their origin outside the midbrain and may have moved into this region after the apoptotic death of the dorsal midbrain, because no Fgfr1-expressing cells exist in the dorsal midbrain at earlier stages in either Fgfr1cko or Fgfr1cko;Fgfr2cko mutants (Troccoli et al., 2005). In contrast, the ventral midbrain appeared completely negative for Fgfr1 expression. Thus, for the residual TH-positive cells in the Fgfr1cko;Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants, a mosaic receptor mutation is an unlikely explanation.

To further characterize the TH-positive cells in the ventral midbrain of Fgfr1cko;Fgfr2cko mutant embryos at E12.5, we analyzed the expression of LMX1A (marker of both proliferative progenitors and postmitotic precursors of DA neurons), HuC/D (general marker of postmitotic neural precursors), Ptx3, TH, and Dat (postmitotic and mature DA neurons) on adjacent coronal sections by immunohistochemistry and in situ hybridization. Our results indicate that in the Fgfr1cko;Fgfr2cko mutants, both the proliferative DA neuron progenitors (LMX1A+, HuC/D−) (supplemental Fig. 2R, available at www.jneurosci.org as supplemental material) (p < 0.005) and postmitotic precursors...
failed to express PITX3 and positive for LMX1A, HuC/D, and TH failed to express PITX3 and Dat (Fig. 4O–R, S, U). Thus, in the Fgfr1cko;Fgfr2cko mutants, the maturation of the DA neurons is also disturbed.

Next, we studied the generation of DA neuron precursors at E10.5–E11.5. Aldh1 is one of the earliest specific markers of DA neurons, expressed in their proliferative progenitors, postmitotic precursors, and mature neurons (Wallen et al., 1999). Consistent with the observed r1-to-midbrain transformation, the Aldh1 expression shifted posteriorly in the Fgfr1cko, Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants at E10.5 and E11.5, but the overall amount of Aldh1-positive cells was clearly reduced (Fig. 5A–L). In agreement with the number of TH-positive neurons at E12.5, the Aldh1 expression domain in the midbrain of the Fgfr1cko;Fgfr2cko;Fgfr3null embryos was consistently smaller than in the Fgfr1cko;Fgfr2cko mutants (Fig. 5C, D, I, J). Similarly to Aldh1, we detected very limited expression of Pitx3 in the postmitotic DA neuron precursors in the E11.5 Fgfr1cko;Fgfr2cko mutants (Fig. 5M–O). In conclusion, in the Fgfr1cko;Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null embryos the amount of early DA neuron precursors is markedly reduced.

Expression of proneural genes and ventral signaling molecules
To test the hypothesis that decreased neurogenesis in the ventral midbrain could contribute to the loss of DA neurons, we analyzed the expression of proneural genes Ngn2 and Mash1 (Fig. 6A–M). In the ventral midbrain of E10.5 Fgfr1cko;Fgfr2cko mutants, both were expressed at normal levels. Their expression had shifted posteriorly, likely as a result of the posterior shift of the midbrain–r1 border. This is in contrast to the Fgfr1cko mutants, which have a gap in Mash1 expression in the ventral r1 (Fig. 6J), possibly reflecting a failure in the differentiation of the most anterior serotonergic neurons (Jukkola et al., 2006). Analysis of Ngn2 and Mash1 expression on E11.5 coronal sections revealed an apparently normal dorsoventral expression pattern, as well (Fig. 6G, H, L, M).

Consistent with the unaltered Ngn2 and Mash1 expression in the E11.5 Fgfr1cko;Fgfr2cko mutants, Gata3 and Ptxd4l were also expressed in their correct ventrolateral domains (Fig. 6N–W). In (LMX1A+, HuC/D+) are reduced (Fig. 4K–N; supplemental Fig. 2R, available at www.jneurosci.org as supplemental material) (p < 0.01). Interestingly, the cells positive for LMX1A, HuC/D, and TH failed to express PITX3 and Dat (Fig. 4O–R, S, U). Thus, in the Fgfr1cko;Fgfr2cko mutants, the maturation of the DA neurons is also disturbed.

Figure 4. Failure in the development of the midbrain dopaminergic neurons. A–J, Immunohistochemical detection of TH expression on midsagittal sections of wild-type (WT) and Fgfr1cko;Fgfr2cko (R1cko;R2cko) and Fgfr1cko;Fgfr2cko;Fgfr3null (R1cko;R2cko;R3null) mutant brains at E18.5 (A, B), E15.5 (C, D; quantification in E; mean of total number = SD), and E12.5 (G–J; quantification in F; mean of total number = SD). K–V, Immunohistochemical analysis of the expression of LMX1A (K, M), LMX1A plus HuC/D (L, N), PITX3 (G, Q), and PITX3 plus TH (P, R); and in situ hybridization with Dat (S, U) and Fgfr1ΔA (T, W) probes in WT and Fgfr1cko;Fgfr2cko;Fgfr3null mutant embryos at E12.5. *p < 0.05 and **p < 0.01, analyzed with Student’s test. A13, DA cell group A13 in diencephalon; LC, locus ceruleus; SN, substantia nigra; VTA, ventral tegmental area. Red and black arrows indicate lost or decreased expression of DA markers or Fgfr1. Black arrowheads indicate DAT expression in WT ventral midbrain (S) and Fgfr7 expression in dorsal tissue of the Fgfr1cko;Fgfr2cko mutants (V). Scale bars: C, D (in O, 500 μm); G–V (in G, K, M, O, S, U), 100 μm.
contrast to the specific markers of the DA neuron precursors, Aldh1 and Pitx3, the expression level of other genes important for DA neuron development and maturation, such as Nurr1, Lmx1b, and Lmx1a (Andersson et al., 2006b), was unchanged at E10.5–E11.5 in the Fgfr1cko;Fgfr2cko mutants (Fig. 6X–e) (data not shown).

To study whether the absence of FGF signaling affects other signaling pathways in the ventral midbrain region, we analyzed the expression of WNT and SHH pathway genes. In Fgfr1cko; Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants, Wnt1 was not expressed at the midbrain–r1 boundary either at E9.0 or E10.5, but interestingly the expression in the ventral and dorsal midbrain was still normal (Fig. 7A–D) (data not shown). Thus, the regulation of Wnt1 expression in the ISO and ventral/dorsal midbrain presumably involves different mechanisms. Similarly to Wnt1, the expression of Drapc1 and Axin2, suggested targets of the canonical WNT pathway (Takahashi et al., 2002; Jukkola et al., 2004), still persisted in the ventral midbrain of the Fgfr1cko; Fgfr2cko mutants (Fig. 7E–H,O,P). Also, Shh and its target gene Gli1 were still abundantly expressed in the Fgfr1cko;Fgfr2cko mutants (Fig. 7J–N). Together, these results demonstrate a marked reduction of the DA neurons and their precursors in the Fgfr1cko; Fgfr2cko;Fgfr3null mutants without major changes in the dorsoventral patterning, in the expression of transcriptional regulators of neurogenesis, or in the components of other signaling pathways regulating neuronal development in the ventral midbrain.

Maintenance of proliferative neural progenitors in the ventral midbrain

We next studied whether the loss of FGF signaling results in a defect in the proliferative properties of neural progenitor cells. Both CyclinD1 (Fig. 8A,B,D,E) and CyclinD2 (Fig. 8G,H) were downregulated dorsally, but not ventrally, in the midbrain–r1 region of the Fgfr1cko;Fgfr2cko embryos already at E9.0–E9.5. In the Fgfr1cko;Fgfr2cko;Fgfr3null mutants, the downregulation of CyclinD1 was more pronounced and seen also in the ventral region both at E9.0 and E9.5 (Fig. 8C,F). Cyclin-dependent kinase inhibitor p21 is normally expressed in a narrow midbrain–r1 boundary cell population dependent on FGF signaling (Trokovic et al., 2005). In E9.5 Fgfr1cko;Fgfr2cko embryos, we could not detect any p21 expression (Fig. 8I,J).

Cell proliferation and the maintenance of neural progenitor cell renewal in the developing spinal cord depend on SoxB1 transcription factors, expressed in the ventricular zone throughout the developing CNS (Pevny and Placzek, 2005). In addition, during the neural induction and the generation of the posterior CNS tissue, SoxB1 expression has been suggested to depend on FGF signaling (Streit et al., 2000; Wilson et al., 2000; Takemoto et al., 2006). Therefore, we hypothesized that FGFs from ISO might also regulate neuronal progenitor cell proliferation in the developing midbrain and r1 by maintaining SoxB1 expression. Consistent with this, Sox3 expression was downregulated already at E9.0 in the dorsal midbrain and r1 of the Fgfr1cko;Fgfr2cko embryos (Fig. 8K–L) and both dorsally and ventrally in the Fgfr1cko;Fgfr2cko;Fgfr3null mutants (Fig. 8M). At E9.5, Sox3 was downregulated in the ventral midbrain of both the Fgfr1cko;Fgfr2cko and the Fgfr1cko;Fgfr3null mutants (Fig. 8N–P).

To analyze the neuronal differentiation and cell-cycle exit in the ventral midbrain, we performed immunohistochemistry on coronal sections of E9.5–E11.5 wild-type, Fgfr1cko; Fgfr2cko;Fgfr3null embryos for SOX2 (marker of the proliferative ventricular zone progenitor cells) and HuC/D (marker of the postmitotic neural precursors) (Fig. 9A–I). In contrast to the wild-type embryos, in the Fgfr1cko;Fgfr2cko;Fgfr3null embryos the HuC/D-positive cells were abundant already at E9.5 (Fig. 9C). At E10.5 and E11.5, we detected more HuC/D-positive cells in the Fgfr1cko;Fgfr2cko;Fgfr3null mutants (n = 3) and Fgfr1cko;Fgfr2cko;Fgfr3null (n = 2) embryos than in the wild type (n = 4). Concomitantly, the SOX2-positive ventricular zone was clearly thinner in the mutants (p < 0.001) (Fig. 9D–L; supplemental Fig S3A, available at www.jneurosci.org as supplemental material). Although the
amount of SOX2-positive cells was reduced, the level of SOX2 expression per cell in mutant tissue was apparently similar to the wild type. In contrast, and consistent with its mRNA expression, the SOX3 protein expression in the E11.5 double- and triple-mutant embryos was strongly reduced, especially in the most ventral ventricular zone (Fig. J–L).

Other transcriptional regulators of the neural stem cell identity and potential targets of FGF signaling include the Hes family members Hes1, Hes3, and Hes5 (Hatakeyama et al., 2004). We found strong Hes5 expression in the ventral midbrain ventricular zone of wild-type embryos. In the Fgfr1cko;Fgfr2cko, Fgfr2cko, Fgfr3null embryos, the Hes5-positive layer was clearly thinner than in the wild type, but the level of Hes5 expression was not reduced (Fig. 9M–O). In the most ventral ventricular zone, a small population of cells that were Hes5-negative yet SOX2-positive was detected (Fig. 9O) (data not shown). However, a similar gap in Hes5 expression existed in the posterodorsal ventral midbrain of the wild-type embryos. Similar to Hes5, Hes1 was also expressed in the ventral midbrain of both wild-type and Fgfr1cko;Fgfr2cko embryos, but at relatively lower levels (supplemental Fig. S3C, available at www.jneurosci.org as supplemental material). Whereas Hes3 was expressed in other regions of the brain, we could not detect its expression in the E10.5–E11.5 ventral midbrain (data not shown).

To study the cell-cycle progression in the mutant embryos at E9.0 and E11.5, we analyzed BrdU incorporation (Fig. 9P–R;T; supplemental Fig. S3, available at www.jneurosci.org as supplemental material) (data not shown). Although in the Fgfr1cko;Fgfr2cko or the Fgfr1cko;Fgfr2cko;Fgfr3null mutants the total number of BrdU-positive cells was reduced, together with the thinning of the ventricular zone, the relative proportion of BrdU-labeled nuclei in the ventricular zone was not altered (Fig. 9P–R;T; supplemental Fig. S3, available at www.jneurosci.org as supplemental material). In contrast to the wild-type embryos, in which the BrdU-labeled nuclei were located mostly in the basal region of the ventricular zone (Z2) because of the interkinetic nuclear migration, in the mutant embryos the BrdU-positive nuclei were also abundant close to the ventricle (Z1).

Together, these results suggest that in the ventral midbrain of the Fgfr mutants, the proliferative neural progenitor population in the ventricular zone is gradually depleted. This is presumably caused by increased differentiation and decreased self-renewal, rather than a decreased rate of cell proliferation in the ventricular zone per se.

**Discussion**

In this study, we have analyzed the contributions of three FGF receptor genes, Fgfr1, Fgfr2, and Fgfr3, to the development of the midbrain and r1 in the mouse. Our results reveal cooperation between the three Fgfrs and provide an explanation for the difference between the phenotypes of midbrain–r1-specific Fgfr1 and Fgfr8 mutants (Chi et al., 2003; Trokovic et al., 2003). Supporting findings in the mouse, chicken, and zebrafish, we show that FGF signaling is involved in the regulation of cell survival and antero-posterior patterning in the midbrain–r1 region (Fig. 10A). In addition, we demonstrate that Fgfr1, Fgfr2, and Fgfr3 together regulate neural progenitor cell properties in the ventral midbrain. We suggest that the loss of FGF signaling results in a failure to maintain normal SoxB1 expression and shifts the balance between the neural progenitor self-renewal and neuronal differentiation. Diverse intercellular signals probably regulate distinct behavioral aspects of precursor populations, such as midbrain DA neurons (for a model, see Fig. 10B).

**FGF1, FGF2, and FGF3 cooperate to receive survival and patterning signals from the IsO**

Our results, together with recent analyses of FGF–FGFR associations (Olsen et al., 2006; Zhang et al., 2006), suggest that the three FGFRs expressed in the neuroectodermal cells receive FGF8/FGF17/FGF18 signals from the isthmic organizer. Although all of these receptors are capable of binding the FGF8 subfamily members, they differ in their in vivo requirements. Fgfr1, followed by Fgfr2, is clearly the main receptor of the isthmic FGF signals.
FGF signaling and development of the dopaminergic neurons

Neuronal differentiation in the ventral midbrain had defects without a loss of tissue or tissue identity, suggesting a more direct role for FGF signaling in the development of neuronal precursor cells themselves. Although Ye et al. (1998) recognized the ability of FGF8 and SHH to induce DA neuron development, the mechanisms involved are unclear. Our results with the Fgfr1cko:Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants demonstrate a marked reduction of DA neurons and their precursors. Apoptotic death in the ventral midbrain may contribute to the loss of DA neurons, but it can unlikely fully explain the loss, because increased apoptosis was prominent only dorsally. Furthermore, around the time of DA neuron induction and differentiation, genes such as Wnt1, Shh, Mash1, and Ngn2 were still expressed in the ventral midbrain of the mutants. This suggests apparently normal dorsoventral patterning and initiation of neurogenesis in the mutants.

The fact that in the Fgfr1cko:Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants both early (Aldh1) and late (Pitx3, TH) markers of DA neurons were downregulated suggests that the defect is not in their later differentiation as in the Ngn2 and Nurr1 mutants (Wallen et al., 1999; Andersson et al., 2006a; Kele et al., 2006), but rather in the very early production of the DA neuron progenitors. Interestingly, Aldh1, an early marker of DA neuron precursors, and Pitx3, a marker of postmitotic precursors, are expressed as opposing gradients, where Aldh1 level is high posteriorly and Pitx3 anteriorly. This would be consistent with the reported anterolateral-to-posteromedial neurogenetic gradient of the mouse midbrain DA neurons (Bayer et al., 1995). Although FGF signaling is necessary for the early development of the DA neuron precursors, this requirement does not appear to be absolute because few DA neurons began to develop even in the Fgfr1cko;Fgfr2cko;Fgfr3null embryos. As an alternative to the induction of the DA neuron identity, our results suggest that FGF signaling promotes the proliferative expansion of the early progenitor cell pool for DA neurons (see below).

In the Fgfr1cko:Fgfr2cko mutants, some TH-positive neurons were detected at E12.5 but not anymore at E18.5. Interestingly, these cells fail to express many markers of mature DA neurons. Thus, FGF signaling may play a role later, in supporting the differentiation and maintenance of the DA neurons. Alternatively, the earlier developmental defects may secondarily lead into abnormal differentiation and neuronal loss.

FGF signaling and maintenance of neuronal progenitor cell renewal

In contrast to the cells close to the midbrain–r1 border (Trokovic et al., 2005), our data suggest that outside the narrow boundary region FGF signaling stimulates CyclinD expression. However, the loss of FGF signaling in the ventral midbrain ventricular zone did not block the S-phase entry. Instead, the balance between the
progenitor self-renewal and postmitotic differentiation was clearly altered. In the spinal cord, the proliferative neural progenitor identity depends on Sox1–3 transcription factors, which counteract the activity of proneural genes (such as Ngn2 and Mash1) and inhibit cell cycle exit and neuronal differentiation (Bylund et al., 2003; Graham et al., 2003). In the developing brain, however, the mechanisms by which the signaling centers regulate the balance between the neuronal progenitor cell self-renewal and differentiation are poorly understood. Because especially Sox3 expression is sensitive to inactivation of FGF signaling, the FGF-mediated maintenance of the proliferative neural progenitors in the midbrain may involve the activity of Sox1–3 transcription factors. Thus, FGFs may act through similar pathways during neural induction, posterior CNS elongation, and the development of the midbrain–r1 region. Interestingly, although Sox3 is broadly expressed throughout the early CNS, a local signaling center, IsO, strictly controls its expression in the midbrain and r1. Thus, despite the widespread expression, SOXB1 activity may be independently regulated in different parts of the developing CNS, as suggested also by enhancer mapping experiments (Brunelli et al., 2003). Given the viable phenotype of the Sox3 null mutants (Rizzoti et al., 2004), Sox3 is unlikely the only FGF-induced mediator of neural progenitor renewal. Other transcriptional regulators of the proliferative progenitor cell identity and potential FGF targets include Hairy and Enhancer of split-related transcription factors of the Hes family (Hirata et al., 2001; Ninkovic et al., 2005; Jukkola et al., 2006). However, our results suggest that in the ventral midbrain at least Hes5 and Hes1 are unlikely critical transcriptional targets of FGF signaling.

Our results support a model in which the combinatorial actions of the intercellular signals guide the production of specialized neuronal subpopulations (Ye et al., 1998; Farkas et al., 2003; Prakash et al., 2006). Different signaling pathways, such as FGF, WNT, and SHH, may regulate distinct molecular cascades and aspects of cellular behavior (Fig. 9B). Although FGFs do not strictly control the expression of WNT and SHH signaling molecules themselves in the ventral midbrain, points of cross talk between the different pathways likely exist further downstream. For example, in cultured neural stem cells, WNT signaling alone (through β-catenin) promotes neuronal differentiation but stimulates cell proliferation in the presence of FGFs (Israsena et al., 2004). A similar mechanism might operate in the ventral midbrain, resulting in the DA neuron progenitor proliferation posteriorly close to the IsO and enhanced differentiation in more anterior regions, in which FGF signaling activity is lower.

Figure 8. Decreased expression of the regulators of proliferative neural progenitor cells. Whole-mount mRNA in situ hybridization analysis of CyclinD1 (A–F), CyclinD2 (G, H, p21 (I, J), and Sox3 (K–P) expression in the wild-type (WT), Fgfr1cko;Fgfr2cko (R1cko;R2cko), and Fgfr1cko;Fgfr2cko;Fgfr3null (R1cko;R2cko;R3null) embryos at E9.0–E9.5. Lateral views (anterior rightwards) are shown. Red arrows indicate downregulated CyclinD, p21, and Sox3 mRNA expression, whereas red arrowheads mark residual expression in the mutants.

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Figure 9. Premature postmitotic differentiation and depletion of proliferative neural precursor cells in the ventricular zone. A–R, Immunohistochemical analysis of SOX2 and HUC/D (A–L), SOX3 and HUC/D (J–L), and BrdU incorporation (P–R), as well as Hes5 nonradioactive in situ hybridization (M–O) on coronal midbrain sections of wild-type (WT), Fgfr1cko;Fgfr2cko;Fgfr3null, and Fgfr1cko;Fgfr2cko;Fgfr3null embryos at E9.5–E11.5. S, The thickness of the ventricular zone (SOX2-positive) and the marginal zone (HUC/D-positive) was quantified (mean ± SD) at E11.5. T, The position at which layer thickness was measured is shown with white lines. For quantification of BrdU incorporation, the ventricular zone was divided into an approximately two-cell-layer-thick periventricular zone (zone1) and basal zone (zone2), visualized with broken lines (P–R). T, The proportion (mean ± SD) of BrdU-positive nuclei in zone1, zone2, and in the entire ventricular zone (total). White arrows point to decreased SOX2-positive layer and increased HUC/D-positive layer (C, E, F, H, I), downregulated SOX3 expression (K, L), and periventricular BrdU-positive cells (Q, R) in the mutants. Z1, Zone1; Z2, zone2. Asterisks indicate Hes5-negative ventral domain. DAPI, 4′,6′-Diamidino-2-phenylindole. Scale bars, 100 μm. *p < 0.05; ***p < 0.001.

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