FGF signaling gradient maintains symmetrical proliferative divisions of midbrain neuronal progenitors

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ABSTRACT

For the correct development of the central nervous system, the balance between self-renewing and differentiating divisions of the neuronal progenitors must be tightly regulated. To maintain their self-renewing identity, the progenitors need to retain both apical and basal interfaces. However, the identities of fate-determining signals which cells receive via these connections, and the exact mechanism of their action, are poorly understood. The conditional inactivation of Fibroblast growth factor (FGF) receptors 1 and 2 in the embryonic mouse midbrain– hindbrain area results in premature neuronal differentiation. Here, we aim to elucidate the connection between FGF signaling and neuronal progenitor maintenance. Our results reveal that the loss of FGF signaling leads to downregulation of Hes1 and upregulation of Ngn2, Dll1, and p57 in the ventricular zone (VZ) cells, and that this increased neurogenesis occurs cell-autonomously. Yet the cell cycle progression, apico-basal-polarity, cell–cell connections, and the positioning of mitotic spindle in the mutant VZ appear unaltered. Interestingly, FGF8–protein is highly concentrated in the basal lamina. Thus, FGFs may act through basal processes of neuronal progenitors to maintain their progenitor status. Indeed, midbrain neuronal progenitors deprived in vitro of FGFs switched from symmetrical proliferative towards symmetrical neurogenic divisions. We suggest that FGF signaling in the midbrain VZ is cell-autonomously required for the maintenance of symmetrical proliferative divisions via Hes1–mediated repression of neurogenic genes.

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Introduction

The central nervous system develops from a pseudostratified layer of neuronal progenitors. They both self-renew and produce postmitotic precursors, which leave the ventricular zone (VZ) to form the marginal zone (MZ). Controlling the stoichiometry between symmetric and asymmetric divisions – which produce either two progenitors, a progenitor and a neuronal precursor, or two neuronal precursors – is crucial. Excessive proliferation results in tumor formation (Fan and Eberhart, 2008), whereas premature differentiation depletes the stem cell pool.

Factors suggested to contribute to cells adopting neuronal fate include decelerated cell cycle, change in the mitotic spindle orientation, loss of apico-basal cell polarity, and depletion of the apical membrane components (Calegari et al., 2005; Knoblich, 2008; Zhong and Chia, 2008). In addition, the connection between the neuronal progenitors and the basal lamina via basal processes likely maintains progenitor cell fate (Konno et al., 2008; Kosodo and Huttner, 2009).

The regulation of proliferation vs. differentiation–balance involves several transcription factors. These include proneural bHLH factors Ngn2 and Ascl1/Mash1 and their antagonists: the members of the Sox81 and Hes families (Ross et al., 2003; Holmberg et al., 2008; Wegner and Stolt, 2005). However, the exact identity and role of upstream signals controlling these effectors remain obscure (Gotz and Huttner, 2005). Of the various signaling pathways implied in the progenitor maintenance, Notch has been described most extensively (Lathia et al., 2008). Since it acts via lateral inhibition, the regulation of patterning and cell fate likely involves also longer–range signals.

In the developing midbrain and hindbrain, FGF signaling, which consists of FGF8, FGF17, and FGF18 from the isthmic organizer (IsO) at the midbrain–hindbrain boundary (MHB), regulates cell survival, proliferation, and differentiation (Trokovic et al., 2003; Ye et al., 1998; Xu et al., 2000). During the stages of IsO activity, three of the four mammalian FGF receptors, Fgfr1–3, are expressed in the midbrain–hindbrain region (Blak et al., 2005; Liu et al., 2003; Trokovic et al., 2005). Midbrain neurogenesis begins rostrally in a gradient-like fashion, in an opposite pattern to FGF gradients suggested to form around IsO (Chen et al., 2009). The conditional inactivation of Fgfr1–mediated signaling shifts neurogenesis towards MHB (Jukkola et al., 2006). The compound mutagenesis of Fgfr1, Fgfr2, and Fgfr3 affects dorsal and ventral regions differently – the dorsal tissue dies apoptotically whereas the number of progenitors in the ventral midbrain decreases, possibly due to premature neurogenesis (Saarimaki-Vire et al., 2007).
Here we aimed to understand why the loss of FGF signaling depletes neuronal progenitors in the ventral midbrain, and investigate the function of FGF8-protein in the formation of the signaling gradient from the isthmus. We show that the inactivation of FGF signaling results in the loss of Hes1 expression, which renders the VZ progenitors to become more neurogenic in a region-specific and cell-autonomous manner. Despite accelerated neurogenesis, the FGF-deficient neuronal progenitors display normal progression of the cell cycle, cell polarity, cell–cell contacts, and the mitotic spindle orientation. Instead, our results suggest that the loss of FGF signaling increases the number of symmetric neurogenic divisions. Furthermore, FGF8 protein appears to localize primarily in the extracellular matrix of the basal lamina, generating a gradient which may be important for supporting the proliferative progenitors. We speculate that FGFs may normally enter the progenitors via their basal processes, supporting them to retain their progenitor status.

Materials and methods

Generation and genotyping of mouse embryos

The generation and genotyping of Engrailed1-Cre (Kimmel et al., 2000), Sonic hedgehog-Cre (Harfe et al., 2004), R26R (Soriano, 1999), Fgfr1 flox (Trokovic et al., 2003), Fgf2 flox (Yu et al., 2003), Fgf1 IICn (Partanen et al., 1998) and Fgf3 null (Colvin et al., 1996) mouse strains were previously described. All alleles were maintained in an ICR outbred genetic background. These strains were intercrossed to generate En1-Cre+/+, Fgf1 flox/flox (Fgf1 flox), En1-Cre+/, Fgf1 flox/flox; Fgf2 flox/flox (Fgf1 flox, Fgf2 flox), En1-Cre+/, Fgf1 flox/flox; Fgf2 flox/flox; Fgf3 null (Fgf1 flox, Fgf2 flox, Fgf3 null), and Shh-Cre+/. Fgf1 flox/flox; Fgf2 flox/flox (Shh-Cre, Fgf1 flox, Fgf2 flox) embryos. Chimeric embryos were aggregated from wild-type (ICR) and En1-Cre+/, Fgf1 flox/flox; IICn; Fgf2 flox/flox; R26R/R26R morulae. The embryonic day (E) 0.5 was the noon of the day of the vaginal plug. The embryonic age was determined more precisely by counting the somites. All the experiments were approved by the national committee of experimental animal research in Finland.

Histology

For in situ hybridization and immunohistochemistry, the embryos were dissected in Dulbecco’s, fixed in 4% PFA in PBS for at least overnight at RT, dehydrated and embedded in paraffin. Embryos were sectioned at 5 μm, except for the cell division plane analysis the sectioning was done at 12 μm. For the electron microscopy, the sections were fixed 2 h at RT, post-fixed in 1% osmium tetroxide and embedded in Epon (Taab embedding resin). 60 nm coronal sections were cut and viewed in Jeol 1200–EX II microscope.

mRNA in situ hybridization and immunohistochemistry

mRNA in situ hybridization on sections was carried out as described (Wilkinson and Green, 1990) using 35S or digoxigenin-labeled cRNA-probes described in Saarimaki-Vire et al. (2007) and Jukkola et al. (2006). Tis21-probe was a gift from Wieland Huttner, and Hes1-probe from Irma Thesleff. Immunohistochemical staining on paraffin sections was performed essentially as described (Jukkola et al., 2006). The antibodies used were mouse anti-BrdU (1:400, GE Healthcare), rabbit anti-BrDU (1:400, 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), rabbit anti-pS7 (1:500, Neomarkers), rabbit anti- Cyclin D1 (1:400, Neomarkers) mouse anti-pancadherin (1:500, Abcam), rabbit anti-laminin (1:800, Abcam), rabbit anti-λ-tubulin (1:500, Sigma), mouse anti-ZO1 (1:500, Zymed), rabbit anti-ZO2 (1:500, Santa Cruz Biotechnology), rabbit anti-Par3 (1:500, Millipore), mouse anti-αPKCα (1:500, BD Biosciences), mouse anti-β-catenin (1:500, BD Biosciences), rabbit anti-β-galactosidase (1:1500, MP Biomedical), mouse anti-Nestin (1:500, Millipore), rabbit anti-phospho-ERK1/2 (1:100, Cell Signaling Technologies), and mouse anti-Nucleolin (1:50, Santa Cruz Biotechnology). For the goat anti-FGF8 (1:800, R&D Systems), the protocol was modified to use TBS-buffer pH 7.4 containing 0.1% TX-100 in all the washes. In addition, the sections were boiled in 0.01 M Tris-EDTA pH 9.0 for 10 min and blocked in the washing buffer containing 10% donkey serum and 1% BSA. According to the manufacturer, FGF8 antibody does not crossreact with other FGFs. Omitting the primary antibody gave no signal, and the antibody specificity was also confirmed using other FGF8-expressing tissues, as well as mouse mutants where Fgf8 was downregulated in the midbrain (see Results). All secondary antibodies were Alexa Fluor–conjugated (1:400, Invitrogen) and nuclei were visualized with DAPI (Sigma).

BrdU and EdU incorporation experiments

The BrdU (bromodeoxyuridine) dosage given to females as intraperitoneal injection was 0.03 mg/g body weight. For the pulse chase study, the E10.5 and E11.5 embryos were collected 24 h after the injection. For the calculation of observed cell cycle length, cumulative BrdU-labeling was used (Takahashi et al., 1995). The females received BrdU every 3 h and the E11.5 embryos were collected 0.5 h, 2 h, 4 h, 6 h, and 8.5 h after the first injection. The ratio of BrdU+ nuclei to Sox2+ nuclei (labeling index, LI) was calculated and a linear regression line was fitted to the graph. The fitted line gave Tc/Td, where Td = Tc + T cell cycle length, and Tc, S-phase length, can be calculated.

For the BrdU and EdU double injections, females were first injected with 5-ethyl-2'-deoxyuridine (EdU, 100 μg) i.p. followed by BrdU injection 12 h (approximately one cell cycle) later. The embryos were collected 12 h after BrdU injection (E11.5). BrdU incorporation was visualized with anti-BrdU antibody and EdU with Click-IT EdU imaging kit (Invitrogen). The VZ was identified with anti-Sox2 and ventral midbrain with anti-Lmx1a on parallel sections. The total number of EdU–BrdU double positive cells was calculated and divided with the cell number in the proliferative (Sox2−) layer separately in Lmx1a+ and Lmx1a− areas. Three wild-type and four mutant embryos, and four sections throughout the midbrain, were counted from each embryo.

Cell cultures and the pair-cell assay

Midbrain tissue from NMR E9.5 embryos was dissected in Dulbecco’s and the cells were enzymatically dissociated into a single-cell suspension using papain dissociation system (Worthington) followed by trituration. For FGF and laminin assay, cells were plated in 200 μl volume on poly-l-lysine (Sigma P4832) or poly-l-lysine and laminin-coated (10 μl/ml) 8-well Permanox slides (Lab-Tek) and cultured for 2 h and 21 h in +37 C, 5% CO2 and 100% humidity. Pair-cell assay was modified from Shen et al. (2002). Briefly, single cells were plated in 12 μl volume on poly-l-lysine-coated Terasaki plates, approximately 20–30 cells per well, monitored after 2 h to verify the presence and position of single cells and then let grow for 21 h. All experiments were done in serum-free culture medium: DMEM with B-27, N-2, l-glutamine, sodium pyruvate ( Gibco), and 1 mM N-acetyl-cysteine (Sigma) and for the FGF treatment both 20 ng/ml bFGF (Gibco) and 2 μg/ml heparin (Sigma) were added. The cells were fixed in 4% PFA for 30 min in +37 C, washed twice in PBS + 0.1% TX-100 and blocked in 10% goat serum, 1% BSA in PBS + 0.1% TX-100 for at least 1 h RT, then treated with antibodies against Sox2 and HuC/D overnight at +4 C, washed three times in PBS + 0.1% TX-
100 and incubated in secondary antibodies 3 h RT, stained with DAPI, washed in PBS and mounted in Mowiol.

**Microscopy, image analysis, and statistical similarity testing**

All epifluorescence and in situ images were taken using Olympus AX-70 microscope, and the confocal images using Leica TCS SP5 confocal microscope. For the quantification of in situ - results, same exposure times were applied for wild-type and mutant samples (n = 4–6 for each stage and genotype). For each embryo, >10 sections were analyzed. The boundary between ventral and ventrolateral midbrain was determined from Lmx1a immunostainings on parallel sections, images of which were then overlaid on in situ images using Photoshop, and the overlay images were used to draw the boundaries. For DI1, the lateral boundaries were determined to be 400 µm away from the floorplate. For Ngn2, the lateral boundary was same as Ngn2 expression boundary, which was within the same 400 µm limit. Using Image Pro, the images were segmented, setting the threshold manually for each image, and then the surface area of positive cells was compared to the entire VZ area. For BrdU chase experiment, BrdU+ cells were counted manually and Sox2+ cells using Peakcounter. This software was developed based on a local maxima finder in Matlab (Croker and Grier, The University of Chicago, 1997; Dufresne, Yale University, 2005) and the GUI implementation was done by Ville Rantanen (University of Helsinki, 2007). The program finds local maxima for a selectable colour channel and counts the total number of peaks, in this case nuclei, in a user-definable region of interest. Peakcounter was also used for counting the cells in the cell cycle length experiment. The results were statistically tested using Student’s t-test. For the evaluation of cell cycle length and cell division plane results, statistical similarity analysis was performed as described (Rita and Ekholm, 2007). Briefly, the values of potential similarity limit, \( \theta \), were determined from the results using the explorative approach. These values define the boundaries within which the difference between observed values should fit to support the similarity hypothesis — i.e., that the data did not show a statistically significant difference. Due to the similarity hypothesis, 90% confidence interval was used. For estimating the required change in mutant cell cycle length, the following equation was used: 

\[
\frac{C_{Wt} \times 2 \exp(48/\tau_{on}}{C_{Wt} \times 2 \exp(48/\tau_{on})} = 0.5. \]

\( C_{Wt} \) is the number of cells at E9.5, when the Fgffr-alleles are fully inactivated (Trokovic et al., 2003), and when the Sox2 layer has not yet thinned (Cmut = Cwt); 48 is the time in hours between the inactivation and analysis points (E9.5–E11.5); \( \tau_{on} \) and \( \tau_{off} \) (9.15 h) are cell cycle lengths in mutants and wild-types, respectively. The value of equation, 0.5, is the ratio of \( Fgf1^{1-ko};Fgf2^{2-ko} \) and WT Sox2 layer thickness at E11.5.

**Results**

**Inactivation of FGF signaling results in increased neurogenesis**

As our earlier study demonstrates premature neurogenesis in the ventral midbrain of \( Fgf1^{1-ko};Fgf2^{2-ko} \) and \( Fgf1^{1-ko};Fgf2^{2-ko};Fgf3^{null} \) embryos (Saraimaki-Vire et al., 2007), we investigated the alterations in the neurogenic properties of the mutant VZ progenitors. For that, we quantified Ngn2+ and DI1+ cells at E10.5 and E11.5 (Fig. 1A–D, A–D, A′–D′). Because both types of mutants showed qualitatively similar depletion of VZ, we focused on analyzing only \( Fgf1^{1-ko};Fgf2^{2-ko} \) embryos.

We analyzed ventral (Lmx1a+) midbrain, which gives rise to dopaminergic neurons, and ventrolateral (Lmx1a+) midbrain separately (dashed lines visualize their boundaries in Fig. 1A–E, A′–E′). In \( Fgf1^{1-ko};Fgf2^{2-ko} \) embryos the expression of both DI1+ and Ngn2+ increased, more prominently in the ventrolateral region (arrowheads in Figs. 1A–D′).

\( DI1 \) and Ngn2 upregulation implies that more progenitors might also leave the cell cycle. To verify this, we performed immunostainings on E11.5 mutant and wild-type coronal sections with markers associated with cell cycle arrest. While CDK-inhibitor p57Kip2 was weakly expressed both in the VZ and in the MZ, a zone of more strongly p57-expressing cells, corresponding to recently differentiated precursors, was localized in the intermediate zone (IZ). Consistent with the DI1 and Ngn2 results, in the mutant ventrolateral IZ the number of p57+ cells was greatly increased (white arrowheads in Figs. 1E, E′). In addition, neurogenic markers Tis21, Mash1/Ascl1, and Jagged1 in the ventral midbrain VZ showed similar upregulation (Supplemental Fig. S1A–D, A′–D′).

Notch-effector Hes1 represses the expression of Notch-ligands and several proneural genes, such as DI1, Jagged1 and Mash1/Ascl1 (Tobayashi et al., 2009). The cyclical expression of Hes1 has been shown to depend on FGF signaling (Nakayama et al., 2008), and recently Sato et al. (2010) demonstrated how Hes1 maintains neuronal progenitor cells in the developing cortex under FGF-Frs2α-ERK pathway, independently of Notch-signaling.

Therefore, we investigated if the loss of FGFs in the developing midbrain VZ also affected Hes1. In the wild-type, Hes1 was detected in the ventricular zone in both Lmx1a+ and Lmx1a– areas (Fig. 1F, G). In E10.5 mutants, the expression was restricted in Lmx1a+ domain and lost in more lateral regions (Fig. 1F). One day later, all Hes1-expression was lost in the mutant midbrain (Fig. 1G). Thus, the loss of FGF signaling in the ventral midbrain leads to gradual downregulation of Hes1.

**FGF receptor removal affects the neurogenic properties in the ventricular zone cell-autonomously**

The inactivation of FGF signaling might either directly regulate the progenitors or generally alter the properties of midbrain–hindbrain area, thus indirectly rendering the progenitors more neurogenic. To study whether FGFs affect neuronal progenitors cell-autonomously, we aggregated wild-type and \( Fgf1^{1-ko};Fgf2^{2-ko} \) mutant morulae to generate chimeric embryos. The midbrain of mutant chimera consisted of tissue mosaic, where clusters of mutant cells deprived of FGF signaling were intermingled among wild-type clusters (Fig. 2A, C). Mutant cells, as identified by their beta-galactosidase expression, showed increased HuC/D expression (Fig. 2A–A′), and they contained more p57-positive cells, especially near the borders between wild-type and mutant areas (Fig. 2C–C′). In the wild-type chimera, the thickness of the HuC/D+ layer was uniformly even and there were less p57+ cells (Fig. 2B, D). Similar results were obtained using a Shh-Cre-line, which inactivated Fgf1 and Fgf2 conditional alleles in the midbrain basal plate only partially. Thus, the ventral midbrain in Shh-Cre: \( Fgf1^{1-ko};Fgf2^{2-ko} \) embryos also contained tissue mosaic (Supplemental Fig. S2A). The mutant clusters began to upregulate Ngn2 (Fig. S2B), whereas the most prominent p57 expression appeared in the mutant clusters near the wild-type tissue (Fig. S2C). Taken together, these results suggest that FGF1/2-mediated signaling in the VZ acts cell-autonomously.

**Mutant neuronal progenitors show increased cell cycle exit**

To verify the increased cell cycle exit in the \( Fgf1^{1-ko};Fgf2^{2-ko} \) mutants, we performed a 24-h BrdU pulse-chase study (Fig. 3). At E9.5–E10.5, the 5-phase progenitor cells were labeled with BrdU, which allowed us to follow their fate. One day later, we measured the number of labeled cells which had exited the cell cycle. For this, we determined the number of BrdU+ nuclei in the MZ, which were negative for the proliferative marker Sox2 (Figs. 3A, A′, B, B′, close-ups in C–C′, D–D′). We analyzed separately the ventrolateral (Lmx1a+) and ventral midbrain (Lmx1a+) in E10.5 (Figs. 3A, A′) and in E11.5 (Figs. 3B, B′) embryos. The number of BrdU+ Sox2− cells was divided
by the number of proliferative (Sox2+) cells in both Lmx1a+ and Lmx1a− areas.

In E10.5 Fgfr1cko;Fgfr2cko and Fgfr1cko;Fgfr2cko;Fgfr3null mutants, the ratio of postmitotic BrdU+ cells to Sox2+ cells displayed no increase in the ventral area (Fig. 3A”). In contrast, in the ventrolateral midbrain the number of progenitors which had left the cell cycle was clearly increased. At E11.5, the phenomenon was even more pronounced in the ventrolateral part (Fig. 3B”). In addition, now the Lmx1a+ area

Fig. 1. Upregulation of proneural genes Dll1, Ngn2 and CDK-inhibitor p57, and downregulation of Hes1. In situ hybridization with DIG-labelled probes for Ngn2 (A–B, A’–B’) Dll1 (C–D, C’–D’), and 35S-labelled probe for Hes1 (F–F’, G–G’) and immunohistochemistry with anti-p57 (E–E’). Coronal midbrain sections of wild-type and Fgfr1cko;Fgfr2cko mutants, both in E10.5 (A, A’, C, C’, F, F’) and in E11.5 (B, B’, D, D’, E, E’, G, G’) developmental stages. Quantified results are shown in A”–E”. The increase in neurogenesis is more apparent in the lateral parts of ventral midbrain (Lmx1a−, arrowheads) than in the most ventral part (Lmx1a+). The dashed lines visualize the boundaries of the quantified areas. Black lines in F, G, G’ mark Lmx1a+ area from a parallel section. Scale bars are 200 μm except in F, G they are 100 μm. *P<0.05, **P<0.01, ***P<0.001. For all stages and genotypes, n = 4–6, and at least 10 sections of each embryo were analyzed. In A”–D”, Y-axis is the percentage of positive in situ signal surface area compared to the entire VZ area, and for E”, Y-axis describes the percentage of p57+ cells compared to the Sox2+ cells (see Methods for details).
differed between wild-types and \textit{Fgfr1cko;Fgfr2cko} embryos. The results support the data from \textit{Dll1}, \textit{Ngn2}, and p57 analyses and as a whole, they indicate that the inactivation of FGF signaling causes the progenitors to become neurogenic and exit the cell cycle. Furthermore, the requirement for FGF signaling in maintenance of the proliferative progenitor pool in the midbrain is region-specific, being less pronounced in the most ventral midbrain which gives rise to dopaminergic neurons.

\textbf{Loss of proliferative progenitors is not due to decelerated cell cycle}

We next analyzed cell biological processes which FGF signaling might regulate in the neuronal progenitors. For the regulation of proliferation versus differentiation-balance, length of the cell cycle, specifically G1-phase, has been suggested to be an important factor (Calegari et al., 2005; Wilcock et al., 2007). To investigate whether the inactivation of FGF signaling leads to changes in the cell cycle length, we performed a cumulative BrdU labeling (Takahashi et al., 1995). The proportion of BrdU$^+$ nuclei among all (Sox2$^+$) ventral midbrain cells constitutes the labeling index, LI, which achieves the maximum value when all the cells have accomplished the S-phase. We calculated labeling indices from the embryos dissected 0.5, 2, 4, 6, and 8.5 h (Supplemental Fig. S3A–E, A'–E') after the first BrdU injection. The slope of the fitted line in the graph (Figs. 3E, E') was 3.8% smaller in mutants. When the statistical noise was considered using the similarity analysis, the difference could be maximally 10%. In terms of cell cycle length, the observed length in \textit{Fgfr1cko;Fgfr2cko} progenitors (12.8 h) was 3.2% longer than in the wild-type (12.4 h). Using the maximum difference of cell cycle speed it could be 14.2 h–14.5% longer than in the wild-type. Because the VZ was 50% thinner in E11.5 mutants compared to wild-types (data not shown), we could estimate the required change in cell cycle length which would explain the phenotype (see Materials and methods for details). This estimated length was 16.7 h; a 35% longer cell cycle in mutants. Thus, it appears likely that cell cycle had not decelerated enough to explain the thinning of the VZ.

To further analyze the cell cycle in \textit{Fgfr1cko;Fgfr2cko} mutants, we performed sequential EdU/BrdU labeling. The embryos first received an EdU-pulse followed by BrdU after 12 h. Embryos were dissected 12 h after BrdU (thus 24 h after EdU) injection. We counted the number of EdU$^+$BrdU$^-$ double positive nuclei (Supplemental Fig. S3F–F') in proportion to the number of Sox2$^+$ nuclei from Lmx1a$^+$ and Lmx1a$^-$ areas in wild-type and \textit{Fgfr1cko;Fgfr2cko} mutants. No statistically significant changes in EdU$^+$BrdU$^-$/Sox2$^+$ -cell ratio were observed between wild-types and mutants. Thus, it appears likely that cell cycle had not decelerated enough to explain the thinning of the VZ.

Using whole mount in situ hybridization, we have previously shown that the mRNA levels of \textit{Cyclins D1}, \textit{D2}, and \textit{B1} are
downregulated in the dorsal Fgfr1cko;Fgfr2cko midbrain already at E9.5 (Saarimaki-Vire et al., 2007), but the ventral midbrain displays some residual expression. In the E11.5 ventral midbrain, CyclinI was still expressed (Supplemental Fig. S4A–C, A'–C'), although at a lower level compared to the wild-type. However, CyclinD1-immunohistochemistry revealed that the lowered expression level was enough to produce relatively normal amount of protein (Figs. S4D–E, D'–E'). Concomitant with this, the anti-PH3-staining indicated that mitoses were still occurring in the apical part of the mutant VZ (Supplemental Fig. S1E, E').

The premature neurogenesis might result from the loss of progenitor status-maintaining factors, such as Myc (Bartlett et al., 1988). However, the Myc target Nucleolin was still present in the mutant VZ (Fig. S1F, F'). Thus, although FGFs can act as potent proliferation-inducing factors, their loss does not prevent cell cycle progression. Furthermore, our results imply that cell cycle in mutants has not slowed enough to explain the thinning of the VZ.

Cell polarity and cellular architecture are maintained in the Fgfr1cko; Fgfr2cko neuronal progenitors

Inactivating one or several components of the polarity-maintaining complex in VZ cells results in abnormally located mitoses, loss of adherens junctions, and alterations in the cell cycle progression (Cappello et al., 2006; Costa et al., 2008; Imai et al., 2006). To understand if the loss of apico-basal polarity was behind the premature neurogenesis in Fgfr1cko;Fgfr2cko mutants, we stained E11.5 coronal sections with antibodies against Par3, aPKC, β-catenin, γ-tubulin, ZO1, and ZO2.

Compared to the wild-type, the mutant apical surface displayed no apparent differences. The members of the polarity-regulating complex Par3 and aPKCα were present, indicating that after the loss of FGF signaling the apical polarity is retained (Figs. 4A, A', B, B'). Tight junctions, visualized by colocalization of ZO1 and ZO2 (Fig. 4C, C'), appeared normal, as did β-catenin-stained adherens junctions (Fig. 4D, D'). γ-tubulin-visualized centrioles remained normally near the ventricle (Fig. 4D, D'). Prominin-1, localized in the apical membrane (Weigmann et al., 1997) and suggested to be an important stemness-supporting factor (Mizrak et al., 2008), was present in the mutants (data not shown).

In addition, other apical structures such as midbodies and primary cilia are important to maintain proper VZ structure and proliferative state of progenitors (Dubreuil et al., 2007). To identify the presence and normal conformation of these apical structures we analyzed ultrathin (60 nm) plastic sections using transmission electron microscopy. General structure of the apical surface in the ventral midbrain of Fgfr1cko;Fgfr2cko mutants appeared normal (Supplemental Fig. S5A'), and contained midbodies (Fig. S5B'), adherens junctions, and primary cilia (Fig. S5C'). Furthermore, midbodies and primary cilia displayed unaltered tubular structures and electron-dense areas (Fig. S5B'C'). Adherens junctions (Fig. S5C'), which existed regularly in the E11.5 ventral midbrain already at E9.5 (Saarimaki-Vire et al., 2007), were present in the mutant VZ (Fig. 3B, B').

FGF signaling does not regulate the orientation of the mitotic spindle

Positioning of the mitotic spindle determines the direction of cytokinesis, which distributes the cytoplasm and cell fate determinants between the daughter cells, thus contributing to their fate (Kosodo et al., 2004). The absence of FGF signaling could randomize the spindle orientation, resulting in excessive asymmetrical divisions. To investigate this, we stained E11.5 coronal sections with DAPI and antibodies against γ-tubulin and pancytokeratin to visualize chromosomes, centrioles, and cell surface, respectively. Only anaphase and telophase cells close to the apical surface and perpendicular to the viewer were considered for the analysis. We measured the angle between apical surface and the cell division plane (α in Fig. 4E) from 53 wild-type and 41 Fgfr1cko;Fgfr2cko neuronal progenitors in the ventral midbrain. Most of the cell divisions occurred vertically, i.e. in an angle between 70 and 90° (Fig. 4F). The number of horizontal divisions, i.e., having the angle less than 20°, was very low.

The data were analyzed using statistical similarity analysis (Rita and Ekholm, 2007). The values for the potential similarity limit, θ,
calculated from the data were 5.36 and 6.18. Because the difference between wild-type (69.57) and mutant (69.98) angle averages was less than one degree (0.41), the results support the similarity hypothesis within 90% confidence interval. Thus we can conclude that based on these data, FGFR1/2-signaling does not seem to affect the positioning of the spindle.

FGF8-protein is localized in the basal lamina

In order to maintain the progenitor status, the neuronal progenitor needs – in addition to retaining the apical constituents – to connect to the basal lamina via the basal process (Konno et al., 2008). To further understand the role of FGF signaling in the neural progenitors, we asked from which side the signal would enter these cells. For that, we investigated the localization of FGF8-protein in tissues using immunohistochemistry. The antibody gave a signal corresponding to areas expressing Fgf8 mRNA. In situ hybridization on parallel sections visualized the Fgf8-expressing cells (Fig. 5B, overlay in C). Fgfr1cko embryos, which retain the morphology of MHB region better than Fgfr1cko;Fgfr2cko mutants but downregulate isthmic Fgf8 by E10.5, served as a negative staining control (Fig. 5C'). FGF8-signal in other Fgf8-expressing areas of mutants, such as in the AER of the limb bud, remained normal (Supplemental Fig. S6M, N).

In the Fgf8-expressing cells, protein was present both in the cytoplasm and on the apical side (Fig. 5A, arrowhead). The brightest signal, however, was observed on the basal side, diminishing away from the expression source. All Fgf8-expressing tissues, such as the limb bud, branchial arches and forebrain, showed similar pattern (Supplemental Fig. S6A,B, E, F, K, L).

If FGF8 indeed reaches the target cells from the basal side, the most likely way is to localize within the extracellular matrix in the basal...
Fig. 5. Basally localized gradient of FGF8-protein and corresponding FGF target gene expression. FGF8 immunostainings of E10.5 midbrain–hindbrain boundary in A, in situ hybridization on parallel section in B, and overlay images of in situ and immunostainings in C, C'. Wild-type tissues depicted, except in C' where Fgfr1cko serves as the negative staining control. Overlay of FGF8 immunostaining (green) and Fg8 in situ hybridization on parallel section (red) in D, and in situ hybridization of FGF target genes Sprouty1 (D') and Dusp6 (D”, close-up in D‴) on parallel sections. Light blue line visualizes the boundaries of Fg8 mRNA, and darker blue shows detectable area of FGF8 protein gradient. Arrows point to spread target gene expression and arrowheads to their mesenchymal expression. Sagittal sections in A–D‴, rostral side towards to the right. Confocal close-up images of the FGF8-expressing cells in the isthmic region in E–E‴, stained with laminin, pan-cadherin, and FGF8. Confocal images of the nestin-stained basal process connections (arrowheads) with the laminin-marked basal lamina in F, F'. Phospho-ERK1/2 immunohistochemistry on coronal E12.5 wild-type tissue in G, arrowhead points to basal processes. Scale bars are 200 μm, except in D–D‴ and G 100 μm. vz, ventricular zone.
lamina. To investigate this, we co-stained the tissues with antibodies against pancadherin, basal lamina -marker laminin, and FGF8 (Fig. 5E–E′). FGF8 appeared to colocalize with laminin and be excluded from the pancadherin-marked cell membranes. Thus, this indicates that FGF8-protein is secreted towards the basal side, where it localizes in the basal lamina.

To remain proliferative the neuronal progenitors require the inheritance of both the apical constituents and the basal process (Konno et al., 2008). Nestin is present the intermediate filaments in radial glia and neuroepithelial cells (Hartfuss et al., 2001), thus visualizing also the basal processes. We could not detect any major differences between the organization and morphology of wild-type and Fgfr1<sup>cko</sup>;Fgfr2<sup>cko</sup> basal processes (data not shown). Mutant progenitors seemed to retain their contacts with the basal lamina normally (Fig. 5F, F′). Thus, our results indicate that in the midbrain basal lamina FGF8-protein forms a concentration gradient. The loss of FGF signaling does not prevent the mutant progenitors from connecting to the basal lamina, which in the wild-type tissue may serve as a source of FGF8.

The activation of FGF receptors leads to a signaling cascade which results in the phosphorylation of ERK1 and 2. In the developing mouse embryo, phosphorylated forms of these kinases are reported to contribute to the FGF signaling pathway, we compared the expression pattern of FGF targets corresponds to the FGF8 protein gradient

To verify that the basally observed FGF8 protein gradient contributes to the FGF signaling pathway, we compared the expression patterns of several known FGF target genes to FGF8 protein distribution.

The mRNA expression of Sprouty1, Dusp6, Pea3 and Erm (Figs. 5D′–D″, Supplemental Fig. S6L, and data not shown) continued in high levels also in areas not expressing Fgf8 mRNA (arrows). The target gene expression level corresponded to the diminishing basal FGF8-gradient (boundaries of the protein gradient are marked by blue lines in Figs. 5D–D″; light-blue lines visualize the Fgf8 mRNA localization). Furthermore, FGF target Dusp6 was also expressed in the mesenchyme, both in the brain (Figs. 5D, D″, black arrowhead), and in other FGF-expressing areas, such as limb buds and branchial arches (Suppl. Fig. S6C, G, black arrowhead). Erm was also detected in both neuroepithelium and mesenchyme of forebrain (Fig. S6J, arrow and arrowhead). Both pERK1/2 and target gene expression were also strongest in regions where the basal FGF8-protein gradient (Fig. S6 A, C, D–H) was detected. Thus, these results suggest that FGF8-protein is secreted to the basal side and FGF8-gradient maintains target gene expression both in neuroepithelium and in the surrounding mesenchyme.

The response of neuronal progenitors to FGF benefit from laminin connections in vitro

To investigate whether neuronal progenitors needed a connection to basal lamina components in order to remain proliferative, we plated single cells dissociated from E9.5 wild-type midbrain on plates coated either with poly-γ-lysine or poly-γ-lysine and laminin. We then cultured the cells in the presence or absence of bFGF and heparin. After 2 h, there were no significant differences between the growth conditions, as most of the cells remained Sox2<sup>+</sup> progenitors (Fig. 6A). After 21 h, cells grown on both laminin-coated and poly-γ-lysine-coated plates, but lacking bFGF, showed increased HuC/D expression. The combination of laminin and FGF had the greatest impact on keeping the progenitors in a proliferative state. This suggests that the basal laminin and FGF signaling co-operate to maintain neuronal progenitors.

Absence of FGF signaling shifts the balance of symmetrical divisions from proliferative to neurogenic

In order to understand how FGFs might affect the neuronal progenitors, we analyzed the different cell division types in vitro using a pair–cell assay (Shen et al., 2002). Neuronal progenitors were dissociated from E9.5 wild-type midbrain and plated in a medium either containing bFGF and heparin, or lacking both. The cells were allowed to divide once and the resulting cell duplets were analyzed using Sox2 and HuC/D -immunostaining. All three possible cell division types were present: progenitor–progenitor, progenitor–neuron, as well as neuron–neuron (Fig. 6B). In the presence of bFGF, each type comprised approximately one third of the total divisions. When the cells were deprived of bFGF, 72% of the cells now divided forming neuron–neuron duplets. Thus it appears that in the absence of FGF signaling, the balance shifts from proliferating cell divisions towards symmetrical neuron-generating divisions.

Discussion

In this study we have analyzed the ventral midbrain neuronal progenitors after the conditional inactivation of Fgfr1 and Fgfr2. We show that the loss of FGF signaling leads to the loss of Hes1 and to a region-specific increase in neurogenesis and cell cycle exit, and that FGFs affect the maintenance of neuronal progenitors cell-autonomously. We exclude the possibility that the observed premature neuronal differentiation results from alterations in cell cycle length, apico-basal polarity, cell–cell or cell–basal lamina connections, or from the dysregulation of the mitotic spindle. Instead of affecting the cellular architecture or mechanism of cell division, our results indicate that a key role of FGF signaling is to repress symmetrical neuron–neuron divisions. In addition, we discovered that FGF8 protein primarily localizes on the basal side of VZ, suggesting that FGF signaling may enter the progenitors via their basal lamina contacts.

Neurogenesis and cell autonomy

As the neurogenesis proceeds, the layer of postmitotic neurons in the Fgfr compound mutants excessively thickens at the expense of the progenitor pool (Saarimaki-Vire et al., 2007). The quantification of Dll1<sup>+</sup>, Ngn2<sup>+</sup>, and p57<sup>+</sup> cells, and BrdU chase experiments, demonstrated that this results from a change in the neurogenic properties of the progenitors.

FGF signaling acting via Frs2α maintains Hes1 expression in the developing cortex, where it maintains self-renewal and proliferation of neuronal stem and progenitor cells (Sato et al., 2010). Interestingly, Hes1 expression in the midbrain was detected in the ventral and ventrolateral areas, and both the timing and localization of Hes1-downregulation corresponded to premature neurogenesis observed from E10.5 onwards in FGF receptor compound mutants (Saarimaki-Vire et al., 2007). The loss of Hes1 expression in the midbrain might explain the upregulation of genes normally repressed by Hes1, such as Dll1, p57, and Mash1/Ascl1, which in turn would lead to the observed increase in neurogenesis.
The maintenance of early neuroepithelial cells is regulated by Hes1 and Hes3 before Notch pathway is activated. When neuroepithelial cells become radial glia cells, they activate Notch pathway and Hes5, downregulate Hes3, whereas Hes1-levels remain unchanged (Hatakeyama et al., 2004). Thus other pathways, such as FGFs, may contribute to the regulation of Hes1 expression before and simultaneously with Notch activation. Concomitant with this, all members of Hes-family in the VZ are not affected by the loss of FGFs, as our previous results show that Hes5 is still abundantly expressed in the FGFR-mutant midbrain (Saarimaki-Vire et al., 2007).

Interestingly, neurogenesis is less affected in the ventralmost (Lmx1a +) midbrain, which, regarding both proliferative and
neurogenic properties, appears more quiescent than the surrounding areas. \( DI1 \) and \( Ngn2 \) upregulation in \( Lmx1a^+ \) region is less pronounced at E10.5 than 1 day later, when all \( Hes1 \) expression is gone. This delay is thus possibly due to the residual \( Hes1 \) in the ventralmost region, which might be regulated by other signals than FGFs. Because the interaction of Shh- and Wnt/\( \beta \)-catenin signaling pathways regulates neurogenesis in the floorplate (Joksimovic et al., 2009), FGFs may play a less prominent part there.

Data from both chimeric \( Fgfr1^{\text{cko}};Fgf2^{\text{cko}} \) embryos and \( Shh-Cre \)-created mosaic ventral midbrain tissues show that FGF signaling maintains neuronal progenitor cells autonomously. In areas lacking Fgf receptor 1 and 2 expression, HuC/D\(^+\) and \( Ngn2^+ \) neurogenic cell populations appeared. The strongest \( p57 \) upregulation surrounded the wild-type clusters — likely due to a lower level of lateral inhibition. This suggests that rather than via regulating the general VZ properties, FGF signaling maintains neuronal progenitors directly.

**Cell cycle progression**

According to the cell cycle length hypothesis (Calegari and Huttnet, 2003), decelerated cell cycle allows the cell fate determinants to affect longer, biasing the cells towards differentiation. Calegari et al. (2005) concluded that the forebrain neuronal progenitors undergoing neurogenic divisions show a 20% longer cell cycle than the ones undergoing proliferative divisions. In the developing spinal cord proliferative divisions occur also faster than the neurogenic ones, and FGF treatment accelerated the cell cycle (Wilcock et al., 2007). Our results indicate that in \( Fgfr1^{\text{cko}};Fgf2^{\text{cko}} \) VZ, the proportion of neurogenic divisions increased, which would presumably affect the BrdU labeling indices, and thus the cell cycle length. Although the mutant neuronal progenitors displayed no significant change in the cell cycle speed or in BrdU–EdU incorporation, the increase in the proportion of neurogenic divisions may not be large enough to show a detectable difference on a cell population level. In addition, the relative lengths of G1, C2, and M phases remain unclear. Altered duration of one or several of these phases may disturb the maintenance of the proliferative status. Based on the statistical similarity analysis of the cell cycle length, in mutants the cell cycle has become not slowed down enough to explain the thinning of VZ. Thus, similarity analysis of the cell cycle length, in mutants the cell cycle has become unclear. Altered duration of one or several of these phases may disturb these pathways. In addition, FGF signaling has been shown to regulate cell fate development (Neugebauer et al., 2009). However, the cilia in \( Fgfr1^{\text{cko}};Fgf2^{\text{cko}} \) mutants were still present, supporting the earlier results of other signaling pathways remaining unaltered in \( Fgfr1^{\text{cko}};Fgf2^{\text{cko}} \) ventral midbrain (Saarimaki-Vire et al., 2007).

When the regulators of the mitotic spindle orientation are inactivated, the division plane angles change clearly (Fish et al., 2006; Roszko et al., 2006; Sanada and Tsai, 2005). For example, following RNAi knock-down of Aspm, over 20% of the progenitors switch from nearly vertical divisions to horizontal ones, where the angle of division plane is 40° or less (Fish et al., 2006). However, the connection between spindle orientation and cell fate in VZ remains unclear (Siller and Doe, 2009). Indeed, the ventral midbrain progenitors in \( Fgfr1^{\text{cko}};Fgf2^{\text{cko}} \) mutants and wild-types divided at nearly identical angles. Statistical analysis suggests that based on these data, the average values of division plane angles deviate from each other maximally 6°. Thus the increased neurogenesis does not likely result from spindle dysregulation.

**Basal localization of FGF8 suggests a presence of basal-to-apical signaling gradient**

For the generation of diffusion-based FGF concentration gradients from the IsO, basal lamina is an excellent substrate. Indeed, the analysis of FGF8 localization reveals that the protein is mainly secreted towards the basal lamina. Anterior–posterior FGF gradients were recently demonstrated by FGF fusion proteins (Chen et al., 2009) and may regulate both patterning and neurogenesis along the midbrain anterior–posterior axis (Chen et al., 2009; Jukkola et al., 2006). Our results suggest that FGF8 also may form a basal-to-apical gradient, entering the progenitors via their basal processes and phosphorylating ERK1/2, which in turn relay the signal to the apically located cell bodies. Importantly, the mesenchymal expression of several FGF targets supports the idea that FGFs move along the basal lamina.

Although the FGF8-protein gradient is mostly visible in the basal lamina, we cannot rule out the possibility that the biologically active FGF signal might also come from other sources. In addition to basal lamina, FGF8 may be distributed along the apical surface, as the Fgf8-mRNA–expressing cells display a signal also on the apical side. However, as immunostaining cannot reveal these weaker signals in non-expressing cells, further studies would benefit from GFP-tagged ligand and FGF constructs to localize the FGF signaling pathway components.

For the maintenance of progenitor cell status, recent studies have emphasized the importance of basal process (Konno et al., 2008; Kosodo and Huttnet, 2009), which is split between the daughter cells (Kosodo et al., 2008). This would fit our model, in which the neuronal progenitor must retain connection to the source of FGFs in the basal lamina to remain proliferative. Based on the cocoalization of laminin and FGF8 in vivo, and the co-effect of laminin and FGF on cells in vitro, we suggest that FGF signaling has its greatest progenitor-supporting impact if the cells connect to laminin.
Effect of FGFs on cell division types in the ventricular zone

The types of cell divisions in the midbrain VZ in vivo have not been reported previously. Despite midbrain lacks similar basal progenitors found in the developing cortex, some neurons may form from symmetrical neurogenic divisions. We aimed to analyze the different cell division types by using a pair-cell assay, which has been used for similar purposes (Bultje et al., 2009; Shen et al., 2002; Sun et al., 2005). This approach revealed all three possible division types in the developing midbrain – asymmetrical self-renewing, symmetrical proliferative, and symmetrical neurogenic. The loss of FGF signaling shifted the balance, and most of the symmetrical divisions became neurogenic.

In conclusion, we suggest that in a wild-type midbrain VZ, FGF8-protein gradient maintains symmetrical proliferative divisions (Fig. 6C). In Fgfr1−/−;Fgfr2−/− mutants, despite preservation of apico-basal polarity and basal lamina connections, the supply of FGFs to the progenitors ceases, which then triggers symmetrical divisions to become increasingly neurogenic. The loss of FGF signaling leads to increased neurogenesis without a major impact on cell biological parameters, such as cell polarity, cell division plane or cell cycle speed. Instead, our results suggest that FGF signaling is needed for transcription factor Hes1 expression, and the repression of genes driving neurogenic driving center cell exit.

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Appendix A. Supplementary data

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References


