FGF/FGFR2 Signaling Regulates the Generation and Correct Positioning of Bergmann Glia Cells in the Developing Mouse Cerebellum

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Abstract

The normal cellular organization and layering of the vertebrate cerebellum is established during embryonic and early postnatal development by the interplay of a complex array of genetic and signaling pathways. Disruption of these processes and of the proper layering of the cerebellum usually leads to ataxic behaviors. Here, we analyzed the relative contribution of Fibroblast growth factor receptor 2 (FGFR2)-mediated signaling to cerebellar development in conditional Fgf2 single mutant mice. We show that during embryonic mouse development, Fgf2 expression is higher in the anterior cerebellum primordium and excluded from the proliferative ventricular neuroepithelium. Consistent with this finding, conditional Fgf2 single mutant mice display the most prominent defects in the anterior lobes of the adult cerebellum. In this context, FGFR2-mediated signaling is required for the proper generation of Bergmann glia cells and the correct positioning of these cells within the Purkinje cell layer, and for cell survival in the developing cerebellar primordium. Using cerebellar microexplant cultures treated with an FGFR agonist (FGF9) or antagonist (SU5402), we also show that FGFR9-mediated signaling inhibits the outward migration of radial glia and Bergmann glia precursors and cells, and might thus act as a positioning cue for these cells. Altogether, our findings reveal the specific functions of the FGFR2-mediated signaling pathway in the generation and positioning of Bergmann glia cells during cerebellar development in the mouse.


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Introduction

During vertebrate development, the cerebellum is folded into lobes and lobules with a well-defined cellular architecture comprising three cell layers, namely the outer molecular layer (ML), the Purkinje cell layer (PCL) containing Purkinje cells (PCs) and Bergmann glia (BG), and the granular layer (GL) made up of granule cells (GCs) [1,2]. The aberrant generation during embryonic development or degeneration during adulthood of these main cerebellar layers and cell types can cause ataxic behaviors, thus underscoring the essential role of the cerebellum for motor coordination in vertebrates [3]. At around embryonic day (E) 9.0 in mice, the cerebellar anlage (Cba) is specified in the dorsal part of the anterior hindbrain under the influence of the isthmic organizer located at the boundary between the midbrain and the hindbrain [4,5]; reviewed by [1,2,6]. Shortly after, at around E10–E12.5, neurons of the deep cerebellar nuclei are among the first cells generated in the Cba [7,8]. Between E10 and E15, PCs are born in the cerebellar ventricular zone (VZ) lining the fourth ventricle, and migrate radially into the Cba along radial glia (RG) fibers spanning from the ventricular to the pial surface of the Cba [7,9–12]. PCs accumulate in a multilayer underlying a second germinal zone in the outer Cba (the external granular layer (EGL)) and later form a monolayer, the PCL, in the adult cerebellar cortex. The EGL consists of granule cell precursors (GCPs) deriving from the rhombic lip at around E12 and migrating tangentially over the Cba surface until approx. E16 in mice [7,11]. BG precursors are born in the cerebellar VZ at
around E13, and migrate radially into the CBa from E14 onwards to settle among the PCs in the PCL [13]. Around birth, GCPs begin to generate postmitotic GCs that migrate along the unipolar fibers of mature BG cells past the PCs to the internal granular layer (IGL), giving rise to the GL of the adult cerebellum. The ML, containing postnatally born stellate and basket interneurons and BG fibers enshathing the GC axons and PC dendrites, is established as the outer layer of the adult cerebellum when the inward migration of GCs has ceased [1,2].

The Sonic hedgehog (SHH) and Fibroblast growth factor (FGF) signaling pathways play a particularly prominent role during cerebellar development. SHH secreted from PCs controls the proliferation and subsequent differentiation of GCPs [14-18]. The specific functions of the FGF/FGF receptor (FGFR) signaling pathway(s), by contrast, still remain unclear due to the overlapping expression domains of several FGFs and of the four known FGFRs in the developing cerebellum [19], and because of the multiple postnatal cerebellar defects in the corresponding mouse mutants [20–22]. Conditional ablation of the Fgfr2 in neural progenitors and RG cells results in similar postnatal cerebellar phenotypes [20,21], suggesting that FGF9 is one of the principal FGF1/FGF2 ligands in the developing cerebellum. In other cellular contexts, neuron-derived FGF9 binds to FGFR2 expressed on glial cells and acts as a potent survival factor [23–26].

We show here that the transcription of Fgf2 within the developing CBa initiates after E14.5 and comprises mostly cells within the anterior CBa of the developing mouse embryo. Conditional ablation of Fgf2 in neural progenitors results in a strong reduction of FGF signaling, in a reduced generation of BG cells and their aberrant positioning within the EGL, and in a decreased cell survival in the anterior CBa, leading to BG and PC defects already during prenatal cerebellar development. We also show that FGF9/FGFR2-mediated signaling inhibits the outward migration of RG/BG cells in vitro, and might thereby control their proper positioning within the PCL during cerebellar development in vivo.

Materials and Methods
Mice
Generation and genotyping of Fgf2lox/lox, Nestin-Cre and R26R mice was described previously [27–29]. Mice were kept on mixed (C57BL/6j, CD-1 and 129Sv) genetic backgrounds. CD-1 mice were purchased from Charles River (Kisslegg/Germany). Nestin-Cre mice were mated to Fgf2lox/lox mice and the resulting Nestin-Cre;Fgf2lox/lox offspring was intercrossed to obtain Nestin-Cre;Fgfr2lox/lox animals, which were maintained by mating to Fgf2lox/lox mice. Adult (2–7 months old) Nestin-Cre;Fgh2lox/lox mice (n = 25 for histological analysis) were compared to their Fgf2lox/lox littermate controls (n = 17 for histological analysis). For embryonic analyses, Fgf2lox/lox,R26R/R26R females were mated to Nestin-Cre;Fgfr2lox/lox,R26R/+ males, and the Nestin-Cre;Fgfr2lox/lox,R26R/+ mutant offspring (n = 28) was compared to Nestin-Cre;Fgfr2lox/lox,R26R/+ heterozygote controls (n = 12). The CBa phenotype of the analyzed Nestin-Cre;Fgfr2lox/lox,R26R/+ mutants was routinely checked by in situ hybridization of serial sections from these embryos with a Tenascin C (Tnc) riboprobe. Collection of embryonic stages was done from timed-pregnant females, noon to dusk, and embryos were dissected 24 h later and processed for EdU detection on paraffin sections according to the manufacturer’s instructions (Click-IT EdU Alexa Fluor 488 Imaging Kit, Invitrogen).

Radioactive in situ hybridization (ISH)
Serial paraffin sections (8 μm) from embryonic and adult mouse heads or brains were hybridized with radioactive [35S]UTP, GE Healthcare/USA or Digoxigenin (DIG RNA Labeling Mix, Roche/Germany) labeled riboprobes as described previously [31–33]. Riboprobes were used for Fgf2 exon 5 and Etn5 (Emn) [27], Gad2 (GAD65) and VachT (Slh) [28]. Fgf1, Fgf2, Fgf3 and Fgf4 [35], Tenascin C (Tnc) [36], Atoh1 (Math1) [37], Shh and Scl (Scl64) [31], Shh [38], and Patched 1 (Pch1) [39]. Images were taken with an Axioplan2 microscope or Stereoview stereo microscope using bright- and darkfield optics, Axioscam MRc camera and Axiosvision 4.6 software (Zeiss/Germany), and processed with Adobe Photoshop CS5 software (Adobe Systems Inc./USA).

Immunohisto-/cytochemistry (IHC/ICC)
Antigens were detected on paraffin sections (8 μm), free-floating cryocryosections (40 μm), or microexplant cultures as reported by [31,40,41]; minor modifications are available upon request.
Primary antibodies used were mouse anti-Gfap (glial fibrillary acidic protein) (1:1000; Sigma/Germany), anti-Calb1 (calbindin) (1:200; Swant/Switzerland, CB300), anti-Bdp1 (Brain lipid binding protein, Fabp7) (1:300; Millipore/USA) and anti-Pax6 (1:400; Developmental Studies Hybridoma Bank/Bank USA); rabbit anti-Calb2 (calretinin) (1:2000; Swant), anti-S100b (1:1000; Sigma), anti-Calb1 (1:5000 (embryos), 1:2000 (adult tissues); Swant, CB38a), anti-Gall (High affinity glutamate transporter, Slc1a3, Eaat1) (1:500; Invitrogen) counterstained with 4',6-diamidino-2-phenylindole (DAPI), or coupled to horseradish peroxidase and detected using the Vectastain ABC Elite Kit (Vector Laboratories/USA). Images were taken with an Axiovert 200M or Axioplan 2 microscope and Axioscam MRc camera (Zeiss), or with an

Versuchstierhaltung, ATV) of the Helmholtz Zentrum München. All efforts were made to minimize suffering.

Behavioral tests
12 weeks old male Nestin-Cre,Fgf2lox/lox (n = 12) and Fgf2lox/lox control (n = 15) mice were tested in the modified hole board as described previously [30]. Motor coordination and balance was assessed one week later using the rotating rod apparatus (Rotarod Letica LE 8200, Bioseb/France). The test phase consisted of three trials separated by 15 min intertrial intervals. Per trial, three mice were placed on the rod leaving an empty lane between two mice. The rod was initially rotating at 4 rpm constant speed to allow positioning of all mice in their respective lanes. Once all mice were positioned, the rod accelerated from 4 to 40 rpm in 300 s, and passive rotations or latency and rpm at which each mouse fell off the rod were recorded.

EdU treatments
Pregnant dams were injected intraperitoneally with 10 μg 5-ethyl-2'-deoxyuridine (EdU; Invitrogen/Germany) per gram body weight on E17.5. Embryos were dissected 24 h later and processed for EdU detection on paraffin sections according to the manufacturer’s instructions (Click-IT EdU Alexa Fluor 488 Imaging Kit, Invitrogen).
were normalized to this area (except the EGL area for cCasp3 -expressing cells) using the following procedure: a computer-generated 50×50 μm grid was superimposed on the image on the side of the microexplant where cells had migrated out. The number of Pax6+/Ccnd1+ (RG/BG), Pax6+/Ccnd1+ (GCP) and Pax6+/Ccnd1− (GC) cells (nuclei) within the 0-50, 50-100, 100-150, 150-200, 200-250, 250-300, 300-350 and 350-400 μm bins was counted, and the correct assignment of the cells to each of these bins was inspected using the Neurolucida Explorer software. A minimum of six microexplants were analyzed for each condition, and data are derived from three independent experiments.

Statistical analyses
All values given are mean ± s.e.m., unless otherwise indicated. Statistical analysis was performed with the SPSS v.10.0 (SPSS Inc., Chicago/USA) and GraphPad Prism 6 (GraphPad Inc./USA) software for the behavioral data, and R software [44] for the cell counting and cell migration data. Modified hole board, rotating rod, cell counts and average migrated distance data were analyzed by one-way or two-way ANOVA, and P<0.05 was considered as significant. Student’s t-tests were used for post-hoc comparisons when appropriate. Mann-Whitney U-tests were used for analysis of rearing behavior data. In case of a non-significant interaction, P-values were taken for genotype or treatment factor. For the cell migration data, the proportion of Pax6+/Ccnd1+ RG/BG precursors/cells among all Ccnd1+ and Pax6+ cells was analyzed with a logistic model for the influence of treatments, considering the grouping of the data according to different experiments (generalized linear mixed-effects model); the R package lme4 [45] was used for these calculations. Confidence intervals for the proportions were calculated with a random intercept-only model.

Results
Fgfr2 deficiency leads to locomotor deficits and an aberrant cerebellar organization in adult mice
To establish the function of FGFR2 in developing neural progenitors, we generated Nestin-Cre:Fgfr2fl/fl conditional knock-out (henceforth designated Fgfr2 cKO) mice, in which deletion of exon 5 leads to a premature stop codon in exon 6 and truncation of the Fgfr2 protein at the extracellular Ig-like II domain [27]. Fgfr2 exon 5-specific mRNA was only detected in the choroid plexus (ChP) but not in the cerebellum of adult (>8 weeks old) Fgfr2 cKO mice (Figure 1A–D), and full-length Fgfr2 protein was not detectable in the brains of these mice using an antibody raised against the C-terminus of FGFR2 (Figure 1E). Adult Fgfr2 cKO mice were viable and fertile; to assess their locomotor abilities, we tested these mice on the modified hole board (mHB) [30] and rotating rod (Rotarod). In the mHB test, Fgfr2 cKO males showed a significantly decreased maximum velocity and reduced total distance travelled (Figure 1F,G; Table S1 in File S1), indicating an altered horizontal locomotion. The mutant males also showed a significantly increased latency to the first rearing and reduced rearing frequency on board in the middle of the arena (Figure 1H,I; Table S1 in File S1), indicating an altered vertical locomotion when this was not supported by the arena wall. However, the Rotarod performance (latency to fall) of the Fgfr2 cKO males was not significantly different from control males (Figure 1J; Table S1 in File S1), although we noted some variation in the latencies to fall from the Rotarod among the Fgfr2 cKO males (Figure S1 in File S1). Because of these results, we focused our further analyses on the cerebellum of the Fgfr2 cKO mice, although we cannot exclude that the loss of FGFR2 in other brain regions of these mice also contributed to the observed locomotor phenotype. Nevertheless, we did not observe any

Cell countings
Cells were counted in an area corresponding to the anterior part (anterobasal lobe) of the CbA on 5 to 8 mid-sagittal sections from E16.5 and E18.5 control and Nestin-Cre:Fgfr2fl/fl; R26R/+ embryos using Stereo Investigator 5.0.5 software (MBF Bioscience/USA). Tnc(+/−) and cCasp3 (cCasp3+) -expressing cells were normalized to this area (except the EGL area for cCasp3+ cells); Pax6+ (Pax6+) and EdU+ (EdU+) -expressing cells were normalized to the Pax6+ EGL area; and pH3 (pH3+) -expressing cells were normalized to the VZ area of the cerebellum.

Western blot
Brain tissues were isolated from 8 weeks old Fgfr2fl/fl (control), heterozygote Nestin-Cre:Fgfr2fl/+ and homozygote Nestin-Cre:Fgfr2−/− mice, and homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Sodium deoxycholate, 1 mM EDTA, and Complete protease inhibitors (Roche)). Total protein concentration was determined with the Pierce BCA Protein Assay (Thermo Fisher Scientific), and 30 μg total protein per sample were separated in 10% NuPAGE Novex precast gels (Invitrogen) and blotted onto PVDF membranes (Hall/USA). Blots were blocked in 4% skim milk in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) and probed with rabbit anti-Fgfr2 (1:300; sc-122) and anti-hyoxanphine guanine phosphoribosyl transferase (Hprt) (1:400; FL-218) antibodies (both from Santa Cruz Biotechnology). Membranes were developed in ECL substrate and exposed to Hyperfilm ECL (GE Healthcare).

CbA microexplant cultures
CbA microexplant cultures were prepared essentially as described by Kunemund et al. (1988) [42], with some modifications to account for the embryonic tissues and the smaller size of the CbA. Briefly, cerebellar primordia were isolated from E16.5 CD-1 embryos, cut into small pieces of equal size (approx. 750 μm diameter), and plated onto poly-D-lysine (50 μg/ml; Millipore) and laminin (2 μg/ml; Roche) coated coverslips (1 microexplant/coverslip) in Neurobasal medium supplemented with 2 mM L-glutamine, 1x B27 nutrient mixture, 100 Units/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen), and 200 nM ascorbic acid (Sigma). Immediately after plating, 100 ng/ml recombinant human FGFR9 protein (R&D Systems/USA) in bovine serum albumin (BSA), 20 μM InSolution SU5402 (Merck Chemicals/Germany) in dimethyl sulfoxide (DMSO) or 0.001% BSA and 0.1% DMSO (control) were added to the medium. Microexplants were incubated at 37°C in a humidified 5% CO2 atmosphere for 36 h, and then fixed in 4% paraformaldehyde, processed for Ccnd1 and Pax6 ICC as described before and counterstained with DAPI.

Migration assays
To measure the outward migration of cells from the core of the CbA microexplants, we employed a method that was originally described by Chou et al. (2000) [43]. Briefly, the border of the microexplant was outlined and the average distance migrated by Pax6+/Ccnd1+(RG/BG), Pax6+/Ccnd1+(GCP) and Pax6+/Ccnd1− (GC) cells (nuclei) from the border on one side of the microexplant was measured using the Neurolucida 6 and Neurolucida Explorer software (MBF Bioscience). The distribution of migrating cells was categorized in 50-μm bins (distance migrated from the border of the microexplant) using the following...
obvious alterations in the ventral mid-/hindbrain region (MHR) of the Fgfr2 cKO mice, and dopaminergic, noradrenergic, serotonergic and cholinergic neurons located in this region appeared unaffected by the ablation of Fgfr2 in their progenitors (Figure S2 in File S1).

A detailed histological analysis of 25 adult Fgfr2 cKO cerebella revealed that the cellular architecture of the cerebellum, particularly in the anterior lobules (lobuli II and III), was severely disrupted in 15 (60%) mutants (Figure 2C,F,I) and was less severely affected in 8 (32%) of the mutant mice (Figure 2B,E,H). In the less severely affected mutant cerebella, Calbindin-expressing (Calb1+; Calb1) PCs were misaligned in the presumptive PCL and some PCs were located within the GL, whereas Calretinin-expressing (Calb2+; Calb2) GCs intermingled with the Calb1+ PCs and occupied the gaps devoid of PCs in the PCL (Figure 2E). By contrast, the PCL and GL were completely disrupted in the severely affected Fgfr2 cKO cerebella, and clusters of Calb1+ PCs were surrounded by Calb2+ GCs in these mice (Figure 2F). Because of these strong morphological and cellular alterations in the severely affected Fgfr2 cKO cerebella, we subsequently analyzed only the less severely affected specimens. In these mutant mice, the numbers of S100b+ Bergmann glial cells appeared to be reduced in the anterior lobuli, several S100b+ Bergmann glial cell bodies were ectopically located in the ML, and the Glial fibrillary acidic protein (GFAP)+ radial fibers of the BG cells did not reach the pial surface of the adult Fgfr2 cKO cerebellum (Figure 2J–M). We thus concluded that the inactivation of Fgfr2 in neural progenitors of the developing mouse cerebellum leads to locomotor deficits and the disruption of the normal cellular organization and layering of the adult cerebellum at a variable penetrance. Because a similar albeit stronger postnatal cerebellar phenotype has been reported by Lin et al. (2009) [20] in Fgfr1/Fgfr2 double mutant mice generated with the same conditional mutagenesis approach, we focused our subsequent analyses on the relative contribution of FGFR2 signaling to the developmental defects underlying these phenotypes.

Localized expression of Fgfr2 in the developing mouse CbA

Using a sensitive radioactive ISH method, we first assessed the wild-type (CD-1) expression pattern of Fgfr2 in relation to Fgfr1 and Fgfr3, two other FGFRs expressed during prenatal development [E14.5–18.5] in the murine MHR (Figure 3A–F; [19]). Fgfr2 is transcribed strongly in the ChPl and in the VZ of the dorsal midbrain (tectum), and weakly in the overlying mesenchyme, but not within the CbA of the E14.5 mouse embryo (Figure 3A,B; Figure S3 in File S1). At E16.5, Fgfr2 is expressed in single cells that appear to delaminate from the cerebellar VZ and to migrate towards the cerebellar cortex, because several Fgfr2-expressing cells have already accumulated in the presumptive PCL (Figure 3C,D,H). At this stage, Fgfr2 is also expressed strongly in the ChPl and in the VZ of the dorsal midbrain (comprising the superior and inferior colliculi), and weakly in the overlying
Figure 2. Aberrant cellular layering and organization of the adult Fgfr2 cKO cerebellum. (A–F) Representative confocal images of sagittal sections from adult control (A; n = 2 mice) and Fgfr2 cKO (B,C; n = 5 mice) cerebella, double-immunostained for calbindin (Calb1, green) and calretinin (Calb2, red), and counterstained with DAPI (blue). (D–F) are higher magnifications of the boxed areas in (A–C). White arrowheads in (E) point at sections from adult control (A; n = 2 mice) and in (F) point at Calb1 (H,I; n = 20 mice) cerebella hybridized with a riboprobe for ML, molecular layer; PCL, Purkinje cell layer; II and III, lobuli of the adult cerebellum. Scale bars: 500 μm (A,G), 50 μm (D), 100 μm (J,L).

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mesenchyme of the MHR (Figure S3 in File S1). At E18.5, strongest expression of Fgfr2 is still detected in single cells located within the ChA that appear to migrate towards the cerebellar cortex and to assemble within the forming PCL (Figure 3E,F,I,J). Fgfr2-expressing cells are not detected in the EGL, and only few Fgfr2+ cells are located between the prospective PCL and EGL (the prospective ML) at this stage (Figure 3I,J). Because we detected the majority of the Fgfr2-expressing cells in the anterior ChA at E16.5 and E18.5, whereas relatively fewer Fgfr2+ cells were detected in the posterior ChA (Figure 3C–I), the expression of Fgfr2 within the developing ChA has the appearance of a “graded” anterior→posterior pattern at these stages (Figure 3D,F). At E18.5, transcription of Fgfr2 is still strong in the ChPl but weaker in the overlying mesenchyme (Figure S3 in File S1). Throughout all analyzed stages, Fgfr2 is not detected in the cerebellar VZ or EGL (Figure 3A–J; Figure S3 in File S1). Notably, visualization at high magnification showed that the Fgfr2 ISH signal appeared to co-localize preferentially with intensely Nissl-stained cells at E16.5 and E18.5 (Figure 3G–I), suggesting that this receptor is expressed mainly in glial cells (RG and/or BG precursors and cells) of the ChA at these prenatal stages. In contrast to Fgfr2, Fgfr1 is transcribed strongly in the VZ of the ChA from E14.5 to E18.5 (Figure 3B,D,F; Figure S3 in File S1; [19]). From E16.5 on, Fgfr1 is also expressed in single cells that appear to migrate within the ChA and in cells that have accumulated in the emerging PCL (Figure 3D; Figure S3 in File S1; [19]). Fgfr1 expression becomes most prominent within the PCL at E18.5, although it is still expressed in single cells within the ChA that appear to migrate towards the PCL (Figure 3F; [19]). Fgfr1 is also transcribed in the VZ of the ventral and dorsal midbrain and rostral hindbrain throughout these stages (Figure S3 in File S1). Fgfr3 expression is not detected in the ChA from E14.5 to E18.5 (Figure 3B,D,F), but Fgfr3 is transcribed strongly in the VZ and in scattered cells of the rostral hindbrain, sparing the isthmic region, at these stages (Figure S3 in File S1). We thus concluded that Fgfr2 starts to be transcribed after E14.5 in the developing ChA, and that Fgfr2 is expressed in single cells located mostly within the anterior ChA. These Fgfr2-expressing cells appear to delaminate from the cerebellar VZ and to migrate in direction of the emerging PCL, where they assemble toward the end of the prenatal period. Moreover and based on their Nissl staining, these cells appear to have a glial identity. The partial overlap of Fgfr2 and Fgfr1 expression in the developing ChA at E16.5–18.5 (Figure 3D,F) suggests some functional redundancy between these two FGFRs in cerebellar development. However and in contrast to Fgfr2, Fgfr1 is prominently expressed in the cerebellar VZ, suggesting that Fgfr1 might additionally be involved in the generation and/or maintenance of VZ progenitor cells.
Next, we analyzed the expression of Fgfr2 and of the other three mouse Fgfr genes in the CbA of the Fgfr2 cKO embryos at E18.5 (Figure 3K–R). Transcription of Fgfr2 was completely lost (Figure 3K, L) and Fgfr1 appeared to be reduced in the PCL but not in the VZ of the mutant ChA (Figure 3M, N), whereas the expression of Fgfr3 and Fgfr4 was not altered in the mutants (Figure 3O–R), indicating that the inactivation of Fgfr2 might have affected the expression of Fgfr1 but not of the other two Fgfrs in the developing mouse cerebellum.

Reduced numbers and mispositioning of BG cells in the EGL are the primary cerebellar defects in the Fgfr2 cKO embryos

The previous results indicated a strong correlation between the higher expression of Fgfr2 in the developing anterior ChA, particularly in what appeared to be migrating glial cells (Figure 3), and the prominent anterior PC, GC and BG layering defects in the adult Fgfr2 cKO cerebella (Figure 2). These defects are expected to arise between E14.5 and E16.5, because Fgfr2 is not expressed in the ChA before E14.5 (our data and [19]). Indeed, initial defects were apparent at E16.5 in the ChA of the
mutant embryos, and detected in 15 out of 28 (~54%) Fgf2 cKO embryos. Double immunostaining for the neural progenitor marker Sox2, which is also expressed in RG/BG cells [46–49], and the RG/BG marker Blbp (also known as brain fatty acid binding protein), [51] revealed a strong reduction of Sox2- and Blbp-expressing cells in the Cha of the Fgf2 cKO embryos at E16.5 and E18.5 (Figure 4A,D,G,I). A reduction of Sox2+ and Blbp+ neural progenitors and RG/BG precursors was also apparent in the anterior part of the cerebellum VZ of the mutant embryos at E16.5 (Figure 4A,D) whereas at E18.5, Sox2+ and Blbp+ neural progenitors cells appeared to accumulate in the cerebellar VZ of the mutant embryos (Figure 4G,I). Furthermore, only few Blbp+ RG/BG processes reached the pial surface of the mutant Cha, and these fibers were frequently arranged in a parallel (tangential) rather than perpendicular (radial) manner relative to this surface in the Fgf2 cKO embryos (Figure 4B,C,E,F,H,J). Notably, we also detected an increased number of ectopically positioned Sox2+/Blbp+ BG cells within the EGL of the mutant embryos compared with the control embryos at both stages (Figure 4B,C,E,F,H,J). Because the strong reduction of Sox2+ and Blbp+ cells already indicated a defective generation and/or differentiation of BG cells in the Fgf2 cKO embryos, we also determined the expression of Tenascin C (Tnc), an extracellular matrix glycoprotein whose mRNA is localized to the somata of RG precursors and BG cells and considered as one of the earliest marker for nascent BG [36,47]. Tnc is transcribed in cells located in the cerebellar VZ, in single cells within the Cha, and in cells that begin to align within the PCL along the entire anterior-posterior extent of the Cha in E16.5–E18.5 control mice (Figure 5A,C,E,I,N). In the Cha of the Fgf2 cKO embryos, by contrast, the numbers of Tnc+ cells were strongly reduced already at E16.5 (Figure 5B,D,F,J,M). Moreover, many Tnc+ expressing cells were ectopically positioned within the EGL of the mutant Cha (Figure 5B,D,F,J,N). Intensely Nissl-stained (glial) cells located within the forming PCL or migrating towards this layer showed an Ish signal for Fgf2 and Tnc in control embryos (Figure 5E,G,K,L). The Ish signal for Fgf2 was completely lost in the mutant Cha, and fewer intensely Nissl-stained and Tnc-expressing cells were detected within the forming PCL or en route to this layer in the Fgf2 cKO embryos (Figure 5F,H). These results suggested that it is in fact the cell-autonomous loss of FGFR2 function in glial cells (RG and BG) that causes the defective generation of BG cells and their abnormal positioning within the EGL in the mutant embryos.

We next assessed whether the PCs (expressing Calb1, [51]) and GCPs (expressing Pax6 and Cyclin D1 (Cndd1), [52–54]) had acquired their molecular identity and correct position within the developing Cha of the Fgf2 cKO embryos. At E18.5, Calb1+ PCs had not formed a monolayer underlying the most anterior (rostral) part of the Pax6+ EGL (Figure 6A-D), indicating a disrupted formation of the anterior PCL in the mutant embryos. Furthermore, Pax6+ GCPs were not aligned in a clearly delimited anterior EGL, as in control embryos, and the mutant anterior EGL appeared to be slightly distorted with single Pax6+ GCPs protruding into the Cha (Figure 6A-D). In line with these observations, the arrangement of cycling Cndd1+ GCPs within the outer EGL also appeared to be distorted in the anterior Cha of the Fgf2 cKO embryos (Figure 6E-J). Moreover, a reduced number of RG/BG precursors/cells expressing Cndd1 [54,55] was apparent in the mutant Cha at this stage, and some of these cells were ectopically located within the cerebellar VZ of the Fgf2 cKO embryos (Figure 6E-J). The glial high affinity glutamate transporter Glast (also known as Slc1a3 or Eaat1) is expressed in RG and BG cells and fibers [56]. Despite an apparently normal formation of the Glast+ RG fiber scaffold in the Cha of the mutant embryos, and concordantly with the decreased Cndd1+ cells in this region, the Glast signal appeared to be reduced in the mutant PCL (Figure 6K,L). Because the PC and GCP defects appeared only at around E17.5 (Figure S4 in File S1; see also Figure 7 for Math1 (Atoh1) detection in the E16.5 EGL), i.e., at least one day after the BG defects were detected in the Fgf2 cKO embryos, we concluded that the reduced numbers of RG/BG precursors/cells and the ectopic positioning of BG cells in the mutant EGL are the primary cerebellar defects in the Fgf2 cKO embryos.

Reduced cell survival in the anterior Cha of the Fgf2 cKO embryos

The reduced numbers of Sox2+/Blbp+ and Tnc+ RG/BG precursors/cells, and the disrupted formation of the presumptive anterior PCL, suggested that the proliferation of the BG and/or PC progenitors located in the VZ of the mutant Cha and/or their survival might also be affected in the absence of Fgf2. The latter possibility was more likely in the Fgf2 cKO embryos because Fgf2 is not expressed in the cerebellar VZ and Fgf1 expression was not altered in this region of the mutant Cha (Figure 3). Indeed, the numbers of mitotic (pH3+) cells in the VZ of the Cha were not significantly different between control and Fgf2 cKO embryos at E16.5 (Figure 7A,B,G), suggesting that the proliferation of BG and/or PC progenitors was not affected in the mutant embryos at this stage. The numbers of apoptotic (cCasp3+) cells, by contrast, were significantly increased by ~1.7-fold in the anterior Cha of the Fgf2 cKO embryos at E16.5 (Figure 7C,D,H), indicating that cell survival within the Cha was compromised in the mutant embryos. The increased apoptotic cell death in the anterior Cha of the Fgf2 cKO embryos coincided with a slight but not significant decrease of the total area of the mutant Cha by ~9% at E16.5 (control: 10.02×10^5±0.64×10^5 μm^2; Fgf2 cKO: 9.14×10^5±0.73×10^5 μm^2; P=0.40 Student’s t-test) and ~10% at E18.5 (control: 3.01×10^5±0.26×10^5 μm^2; Fgf2 cKO: 2.73×10^5±0.13×10^5 μm^2; P=0.40 Student’s t-test). Because the increased (~1.7-fold) number of apoptotic cells corresponded with the decreased (~1.5-fold) number of Tnc+ cells, we concluded that the reduced number of RG/BG precursors/cells in the mutant Cha might also be due to a reduced survival of these cells in the absence of FGFR2 signaling.

The generation of the EGL and GCPs was not affected in the Fgf2 cKO embryos, as determined by the normal expression of Atoh1 (Math1), a transcription factor required for EGL and GCP development [57], in the developing mutant Cha at E16.5 and E18.5 (Figure 7E,F and data not shown). We also determined whether the proliferation and cell-cycle exit of GCPs in the anterior (rostral) EGL might have been affected in the Fgf2 cKO embryos. The density of Pax6+ GCPs and proliferating (EdU+) cells in the anterior EGL (Figure 7I–K), as well as the fraction of proliferating Pax6+ GCPs that had incorporated EdU after a single pulse given 24 h before (EdU+ and Pax6+ cells per total Pax6+ cells: control, 47.5±2.7%; Fgf2 cKO, 48.9±0.1%; P=0.65 in the Student’s t-test), were not significantly different between control and mutant embryos at E18.5, indicating that the numbers and the proliferation/cell-cycle exit of Pax6+ GCPs in the anterior EGL were not affected by the loss of Fgf2 expression in the Cha. Together, the previous results suggested that FGFR2-mediated signaling is also required for the proper survival of RG/BG precursors/cells and PCs within the Cha.
FGF target gene activation is almost completely abolished in the CbA of the Fgfr2 cKO embryos. To confirm that FGF signaling was in fact reduced or abolished in those regions of the mutant CbA where Fgfr2 is highly expressed (anterior CbA including the anterior PCL but excluding the VZ, see Figure 3), we determined the transcription of a known FGF target gene, Etv5 (Erm, [19,58]) in the developing CbA of control and Fgfr2 cKO embryos. At E16.5 and E18.5, Etv5 is strongly expressed in the cerebellar VZ, in scattered cells within the ChA, in the emerging PCL, and in the posterior EGL of control embryos (Figure 8A,I, [19]). By contrast, transcription of Etv5 was strongly reduced in almost the entire ChA (including the VZ and the PCL primordium but excluding the posterior EGL) of the mutant embryos at E16.5, and was almost completely abolished at E18.5 (Figure 8B,J). Notably, Etv5 was ectopically expressed in the anterior EGL of the Fgfr2 cKO embryos at both stages.
Figure 5. Reduced numbers and mispositioning of $Tnc^+$ BG cells in the EGL of the Fgfr2 cKO CbA. (A–J) Representative sagittal brightfield views of E16.5 (A–D, n = 4 embryos/genotype), E17.5 (E–H, n = 1 embryo/genotype) and E18.5 (I,J, n = 3 embryos/genotype) control (A,C,E,G,I) and Fgfr2 cKO (B,D,F,H,J) cerebella hybridized with radioactive Tnc (A,F,I) and Fgfr2 (G,H) riboprobes. (C,D) are higher magnifications of the boxed areas in (A,B). (E–H) are higher magnifications of the anterior CbA of the Fgfr2 views of E16.5 (A–D, n = 4 embryos/genotype), E17.5 (E–H, n = 1 embryo/genotype) and E18.5 (I,J, n = 3 embryos/genotype) and the mutant CbA. (M) ISH signals. (K,L) High magnification views of the EGL and PCL on adjacent sections from an E18.5 control (wild-type) embryo, hybridized with a radioactive riboprobe for Fgfr2 (red in K) or Tnc (black in L). Empty arrowheads point at larger, weakly Nissl-stained cells in the CbA of the Fgfr2 cKO embryo shown in (F,H), although some Fgfr2 $^+$ cells are detected in the (non-neural) mesenchyme overlying the mutant EGL. Red dotted line in (A,I) delimits the anterior area used for quantification. (K,L) Scale bars: 100 µm (L).

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(Figure 8C,D,K,L). The ectopic $Etv5$-expressing cells clearly outnumbered the ectopically positioned $Tnc^+$ cells in the anterior EGL of the mutant embryos (Figure 8C–F,K–N). Moreover, the ectopic $Etv5$ cells were largely confined to the outer EGL at E18.5 (Figure 8L,P), whereas the ectopic $Tnc^+$ cells were predominantly located in the inner EGL at this stage (Figure 8N,P), indicating that FGF signaling was ectopically activated in cells other than the ectopically positioned $Tnc^+$ BG cells in this region of the mutant CbA. These results showed that FGF signaling and target gene activation were in fact strongly reduced or even abolished within the CbA, in the cerebellar VZ and in the forming PCL of the Fgfr2 cKO embryos, thus including regions where Fgfr2 is not or only weakly expressed (the VZ and posterior CbA/PCL, see Figure 3). As this correlated with an apparent reduction of $Fgf1$ expression in the mutant CbA (including the emerging PCL, Figure 3), we concluded that the lack of FGF2-mediated signaling makes a major contribution to the BG and PC defects in the Fgfr2 cKO embryos, but might additionally be reinforced by a reduction of FGFR1-mediated signaling in these embryos.

We next determined whether the relatively subtle PCL and EGL defects in the Fgfr2 cKO embryos might be due to an altered expression of $Shh$ in PCs or a defective $SHH$ signal transduction in GCPs. The transcription of $Shh$ and the $SHH$ target gene $Pch1$ was not changed in the developing CbA of the mutant embryos compared with control embryos, except for a notable lack of the $Shh^+$ PCL underlying the most anterior (rostral) EGL in the Fgfr2 cKO embryos at E18.5 (Figure S5 in File S1). The latter observation is most likely due to the lack of Calb1 $^+$ PCs in the most anterior PCL of the mutant embryos (see Figure 6). Together, these results suggested that the expression of $Shh$ in migrating and stationary PCs and its target gene $Pch1$ in GCPs is not altered by the reduced or even abolished FGF signaling in the mutant CbA,
and is therefore unlikely to contribute to the PCL and EGL defects in the Fgfr2 cKO embryos.

FGF9/FGFR signaling inhibits the migration of RG/BG precursors/cells in cerebellar microexplants in vitro

In addition to the loss of RG/BG precursors/cells, the radial migration of Sox2+/Bhp3+ and Fca+ BG cells within the CbA did not stop at the level of the PCL, leading to the ectopic positioning of BG cells within the prenatal EGL or adult ML, respectively, of the Fgfr2 cKO mice (Figures 2, 4, 5). This suggested that FGFS secreted from the EGL and/or PCL inhibit the further migration of RG/BG precursors/cells, thereby controlling their correct alignment within the developing PCL. To test this hypothesis, we prepared Cbh microexplant cultures from E16.5 wild-type (Ct1) embryos and treated them for 36 h with control medium or medium containing recombinant human FGF9 or SU5402 (Figure 9A). FGF9 is an FGFR expressed by GCPs and PCs, and required for the proper differentiation and alignment of BG cells in the mouse cerebellum [19,20], whereas SU5402 is a known inhibitor of FGFR signaling [59]. After immunocytochemical staining for Pax6, a marker for GCPs and migrating GCs as well as other cells located within the CbA (Figures 6A, 9A, S4 in File S1; [52]), and Ccnd1 (Cyclin D1), which is expressed in proliferating GCPs from the outer EGL and in RG/BG precursors/cells within the CbA and forming PCL but not in VZ progenitors (Figures 6E, 9A, S4 in File S1; [53–55]), we determined the distance migrated by each cycling Pax6+/Ccnd1+ GCP and Pax6+/Ccnd1+ RG/BG precursor/cell or postmitotic Pax6+/Ccnd1+ GC from the border of the microexplant (Figure 9A). The average distance migrated by Pax6+/Ccnd1+ GCPs did not show any notable differences between control-, FGF9-, and SU5402-treated cultures (Figure 9B–E), whereas the average distance migrated by Pax6+/Ccnd1+ RG/BG precursors/cells was reduced after FGF9 treatment and slightly increased after SU5402 treatment relative to the control-treated cultures, although it did not reach statistical significance because of the high variance of migrated distances within and between experiments (Figure 9B–E). Because this already hinted at a migration-inhibiting effect of FGF9 and migration-promoting effect of the FGFR inhibitor SU5402 on RG/BG precursors/cells but not on GCPs, we analyzed the migratory behavior of RG/BG precursors/cells under these conditions in more detail. FGF9 treatment reduced significantly the proportion of Pax6+/Ccnd1+ RG/BG precursors/cells among the total number of Ccnd1 and Pax6 single- and double-positive cells (including Pax6+/Ccnd1+ RG/BG, Pax6+/Ccnd1+ GCPs and Pax6+/Ccnd1+ GCs, Figures 6A, 6E, 9A) that had migrated from the core of the microexplant (regardless of distance) relative to the control- and SU5402-treated explants (Figure 9F), indicating that FGF9 in fact inhibited the outward migration of RG/BG precursors/cells in these cultures. Next, we assessed the proportion of Pax6+/Ccnd1+ RG/BG precursors/cells among all Ccnd1 and Pax6 single- and double-positive cells in 50-μm bins from the border of the microexplants (Figure 9A,G; Table S2 in File S1). We noted that under control conditions, the average proportion of RG/BG precursors/cells in each bin corresponded well with a normal distribution according to the distance migrated, with fewer or no cells in the more proximal and distal bins, respectively, relative to the border of the microexplants (Figure 9B,G). After FGF9 treatment, however, the average proportion of RG/BG precursors/cells in each bin was strongly decreased and these cells were only detected up to a distance of 200–250 μm from the border of the microexplants (Figure 9C,G), indicating that the inhibition of FGFR signaling augmented the distance migrated by the RG/BG precursors/cells in vivo in a similar manner to what was observed in the Fgfr2 cKO embryos in vitro (Figures 4,5). Together, these data showed that FGF9 inhibits the outward migration of RG/BG precursors/cells whereas
blocking FGFR signal transduction has the opposite effect and promotes the outward migration of these cells for longer distances from the microexplant, and thus strongly suggested that during cerebellar development, FGF9/FGFR2-mediated signaling in migrating RG/BG precursors/cells inhibits their further migration beyond their proper position within the PCL.

**Discussion**

We show here that the conditional inactivation of Fgf2 in neural progenitors results in specific cellular and layering defects in particular in the anterior (rostral) cerebellum of adult Fgf2 cKO mice, where Fgf2 is highly expressed during normal CbA development. The developmental deficits in these mice include a reduced generation and ectopic positioning of BG cells within the EGL, the misalignment and lack of PCs in the most anterior PCL, and a reduced cell survival in the developing CbA. We also show that FGF9/FGFR2-mediated signaling inhibits the outward migration of RG/BG precursors/cells in vitro, and might thereby control the correct positioning of BG cells within the PCL in vivo.
FGFR2-mediated signaling promotes the generation of BG cells and cell survival in the developing CbA

Adult Fgf2 cKO mice displayed similar but generally weaker cerebellar defects than previously described in Fgf11−/−;Fgf2 flox/flox [20] and hGFAP-Cre;Fgf2 flox/flox; Fgf2O/O [21] mice. Therefore, we determined the relative contribution of the lack of Fgfr2 transcription to these phenotypes. Our results indicated that Fgf2 transcription in the developing CbA is highest in cells located anterior to the EGL, and that it is highest in cells located anterior to PCL, and that it is highest in cells located anterior to the cerebellar VZ. Thus, we propose that the restricted high expression of Fgfr2 in the anterior CbA imposes a stronger need of a balanced FGF signaling for the proper development of this region. Nevertheless, the strongly reduced or even abolished transcription of the FGF target gene Etv5 in the entire CbA (except the EGL) of the Fgfr2 single mutant mice (see comments below).

The most notable defect in the developing CbA of the Fgf2 cKO embryos was a strong reduction of Sox2+, Blbp- and Tnc-expressing BG precursors and cells that became apparent already at E16.5, i.e. less than two days after the failed induction of Fgf2 expression in the corresponding neural progenitors. Sox2+ neural progenitors indeed appeared to accumulate over time in the cerebellar VZ of the Fgf2 cKO embryos, suggesting that these...
Figure 9. FGF9/FGFR signaling inhibits the migration of RG/BG precursors/cells in cerebellar microexplants in vitro. (A) Migration assays were performed with similarly sized (black dotted bars) CbA microexplants from E16.5 wild-type (CD-1) mice, containing Pax6+/Ccnd1+ GCPs in the outer EGL (yellow), Ccnd1+ RG/BG precursors and cells (green), Pax6+ postmitotic GCs and inner CbA cells (pink), and DAPI+ PCs (blue). ChPl, choroid plexus; EGL, external granular layer; IC, inferior colliculus; VZ, cerebellar ventricular zone. CbA microexplants were treated with control medium or medium containing 100 ng/ml FGF9 or 20 μM SU5402. The distance (d) migrated by each Pax6+/Ccnd1+ (green), Pax6+/Ccnd1+ (yellow) and Pax6+/Ccnd1+ (pink) cell from the border of the microexplant was measured after 36 h of incubation. (B–D) Representative confocal overviews of Ccnd1+ (green) and/or Pax6+ (red) cells (double-positive cells appear in yellow), counterstained with DAPI (blue) (overlays with single-positive cells appear in light green and pink, respectively), that migrated from the border of the CbA microexplant (white line) in control (B), FGF9- (C) or SU5402- (D) containing medium. Red arrowheads point at the front-most Ccnd1+/Pax6+ (green) cells. (E) Quantification of the average distance migrated by RG/BG precursors/cells (green bars) and GCPs (yellow bars) in control-, FGF9- or SU5402-treated microexplant cultures (Distance (μm): RG/BG precursors/cells, control, 86.2 ± 16.8; +FGF9, 52.6 ± 20.9; +SU5402, 89.3 ± 33.2; GCPs, control, 111.7 ± 15.3; +FGF9, 97.7 ± 16.8; +SU5402, 100.2 ± 15.6; one-way ANOVA). (F) Quantification of the proportion of Ccnd1+/Pax6+ RG/BG precursors/cells among the total number of migrating Ccnd1+ and/or Pax6+ cells in control-, FGF9- or SU5402-treated microexplant cultures (% Ccnd1+/Pax6+ RG/BG precursors/cells: control, 3.66, 95% confidence interval [2.95, 4.48] (8 experiments for controls with 128–589 migrated cells, among them 2–29 RG/BG cells); +FGF9, 1.31, 95% confidence interval [0.84, 1.92] (8 experiments for FGF9 with 79–501 migrated cells, among them 0–9 RG/BG cells); +SU5402, 2.89, 95% confidence interval [1.99, 4.04] (6 experiments for SU5402 with 78–371 migrated cells, among them 0–15 RG/BG cells); P-values from contrasts of a logistic model). (G) Average proportions of Ccnd1+/Pax6+ RG/BG precursors/cells among the total number of migrating Ccnd1+ and/or Pax6+ cells in each 50-μm bin in control- (grey bars), FGF9- (red bars) or SU5402- (green bars) treated microexplant cultures were estimated with a logistic model. Values are given in Table S2 in File S1. Scale bar (D): 100 μm.

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cells failed to generate the proper amount of migrating Blbp+ and Tnc+ BG precursors/cells in the absence of Fgfr2. A similar albeit much stronger BG phenotype was recently described in conditional mouse mutants for the protein tyrosine phosphatase, non-receptor type 11 gene (Ptpn11), also known as Sbp2, an intracellular effector of the FGF/FGFR signaling pathway (E1/;Ptpn11cko mice, [47]). The generation of BG is completely abolished in these mice, apparently because RG fails to transform into BG in the absence of Ptpn11, which subsequently leads to foliation defects in the mutant cerebellum [47]. Our results thus suggest that FGFR2 is primarily involved in the transduction of FGF signals required for the proper transformation and/or differentiation of RG precursors into BG cells.

We also observed a slightly increased number of apoptotic cells within the CbA of the Fgfr2 cKO embryos. The reduced cell survival most likely includes migrating and stationary PC and RG/BG precursors/cells that are born at earlier developmental stages in the cerebellar VZ, and might contribute to the reduced numbers of Sox2+/Blbp+/Tnc+/S100b+ RG/BG precursors/cells and to the lack of Calb1+ PCs in the anterior PCL of the embryonic and adult Fgfr2 cKO cerebellum. BG cells are also decreased in the hGFAP-Cre;Fgfr1f/f;Fgfr2f/f cerebellum, although apoptotic cell numbers do not appear to be changed in these mice [21]. In contrast to cell survival, the proliferation of cerebellar VZ progenitors was not affected in the Fgfr2 cKO embryos. Although we cannot exclude that FGFR2 might control the proliferation of migrating RG/BG precursors, including those generating the prospective BG cells [13], FGFR2-mediated signaling is unlikely to control the proliferation of cerebellar VZ progenitors was not affected in the Fgfr2 cKO embryos.

In contrast to cell survival, the proliferation of cerebellar VZ progenitors was not affected in the Fgfr2 cKO embryos. Although we cannot exclude that FGFR2 might control the proliferation of migrating RG/BG precursors, including those generating the prospective BG cells [13], FGFR2-mediated signaling is unlikely to control the proliferation of cerebellar VZ progenitors for several reasons: 1) Fgfr2 is not transcribed at detectable levels in the cerebellar VZ throughout embryonic development; 2) PCs are born at E10-13 in the mouse [1,60], long before Fgf2 expression initiates in the CbA (after E14.5); 3) The onset of Fgfr2 transcription in the CbA coincides with the peak of BG radial migration toward the PCL at E15 in the mouse [13].

In contrast to the hGFAP-Cre,Fgfr1f/f;Fgfr2f/f [21] and Nestin-Cre,Fgfr2fl/+ mice [20] in which we did not detect any defects in GCP numbers and proliferation in our Fgfr2 cKO mice. This is consistent with the lack of Fgfr2 transcription in the EGL of the wild-type embryo, and coincides with an ectopic activation of FGF signaling (assessed by Ets3 expression) in the anterior EGL of the Fgfr2 cKO embryos. Notably, the ectopic Ets3-expressing cells did not overlap with the ectopically positioned Tnc+ BG cells in this region of the mutant CbA. This finding suggests either that the ectopic Ets3-expressing cells derived from the Ets3+ posterior EGL and failed to downregulate the expression of Ets3 during their tangential migration towards the anterior (rostral) EGL, or that FGF signaling was ectopically activated in these cells by an unknown, non-cell-autonomous mechanism in the absence of Fgfr2. Furthermore, PCs were ectopically positioned within the GL and GCS “protruded” into the PCL in the anterior lobules of the adult Fgfr2 cKO cerebellum. The apparently normal alignment of Glast+ RG fibers in the CbA of the mutant embryos suggests that these are most likely secondary phenotypes appearing during postnatal cerebellar development in the Fgfr2 cKO mice. The disruption of the Blbp+ and Gfap+ BG fiber scaffold in the mutant cerebellum might thus lead to an aberrant alignment of single PCs within the PCL, and to the blocked migration of GCS along these fibers through the PCL into the GL.

Incomplete penetrance and Fgfr1 as a genetic modifier of the Fgfr2 cKO cerebellar phenotype

The cerebellar defects of the Fgfr2 cKO mice are not completely penetrant and may have been missed inadvertently in previous analyses of Fgfr2 single mutant mice [20,21]. Because cerebellar phenotypes are particularly sensitive to genetic backgrounds [61], it is very likely that the incomplete penetrance of the Fgfr2 cKO cerebellar phenotype is due to genetic modifiers in the mixed genetic background of our mice [62]. Indeed, the transcription of Fgfr1 also appeared to be partially decreased in the CbA of the affected Fgfr2 cKO embryos. As this was also true for regions within the CbA where Fgfr2 is not expressed at high levels or in many cells (such as the posterior CbA/PCL), the reduced expression of Fgfr1 might be one genetic modifier in these mice. Alternatively, the loss of FGF2 function might affect the transcription of Fgfr1 cell-autonomously or non-cell-autonomously by yet unknown mechanism(s) in the developing CbA. Another reason for the different cerebellar phenotypic outcomes of our Fgfr2 cKO and the previously generated conditional Fgfr2 mutant mice might be the different gene targeting strategies used for generating these mice [27,63], although they should all result in the absence of a functional FGFR2 receptor in the developing CbA.

FGF9/FGFR2-mediated signaling might act as a positioning cue for migrating BG cells

BG cells were located ectopically in the anterior EGL/ML of the Fgfr2 cKO cerebella, indicating that FGFR2 signaling is necessary for their proper positioning within the PCL. The radial migration of BG precursors and cells from the VZ toward the PCL starts at ~E14 and reaches a peak between E15–16 in the mouse [13,36], the time interval when Fgf2 transcription initiates in the developing CbA. SHH secreted from PCs is a potent chemotactrant for BG cells that strongly promotes their migration [15]. The normal transcription of Shh in PCs of the mutant CbA suggests that this guidance cue is not affected in the Fgfr2 cKO embryos. The migration of BG cells, however, must be inhibited once these cells have reached their final destination in the PCL to prevent their ectopic positioning beyond this layer [13]. We therefore hypothesized that an FGF signal emitted from the EGL and/or PCL might provide such a “stop signal” to migrating BG cells. One potential candidate was FGF9 expressed in GCPs and PCs and required for the proper positioning of BG cells in the PCL [19,20], although other FGFs expressed within the EGL (Fgf1/10) or PCL (FGF4/15) [19] might have a similar function. The outward migration of RG/BG precursors/cells from CbA microexplants was indeed inhibited after FGF9 treatment of these explants, whereas FGF2 blockade promoted the outward migration of RG/BG precursors/cells for longer distances from the explants. These results strongly suggest that RG/BG precursors/cells fail to detect the probably concentration-dependent FGF9 “stop signal” from the EGL/PCs in the absence of FGF2-mediated signaling, and therefore migrate beyond their normal position within the PCL. Altogether, our findings thus reveal the specific pro-differentiation, anti-apoptotic and cell positioning functions of FGF2-mediated signaling in RG/BG precursors/cells during cerebellar development in the mouse, and might provide new mechanistic insights to the pathogenesis of cerebellar ataxias.

Supporting Information

File S1 Table S1. Locomotor behaviors of control and Fgfr2 cKO mice. 12 weeks old male Fgfr2lox/lox (control, n = 15) and Nestin-Cre;Fgfr2fl/fl (Fgfr2 cKO, n = 12) mice were tested in the modified hole board (mHB) for horizontal and vertical locomotor abilities. Motor coordination and balance was assessed with the rotating rod apparatus (Rotarod). All values given are mean ± s.e.m. Table S2. Average proportion of Ccnd1+/−/−/−.
**Pax6**-RG/BG precursors/cells among the total number of migrating Cnd1+ and/or Pax6+ cells in each 50-μm bin in control, FGFR2- or SU5402-treated microexplant cultures. Values represent the average proportion of Cnd1+ /Pax6+ RG/BG precursors/cells among the total number of migrating Pax6+ and/or Pax6+ cells in each 50-μm bin (distance migrated from the border of the microexplant) and for each treatment, and the 95% confidence interval estimated with a logistic model (in 8 bins and 3 treatments: total cells migrated: 1168, among them RG/BG precursors/cells: 146). **Figure S1.** Correlation of locomotor and cerebellar phenotypes in adult Fgfr2 cKO mice. (A) Rotarod performance (latencies to fall) of 15 control and 12 Fgfr2 cKO males. Highlighted in red are the control male with the shortest latency to fall (16 sec, ID 30064116), and two Fgfr2 cKO males with the longest (140 sec, ID 30064154) and shortest (25 sec, ID 30064164) latencies to fall. (B–G) Sagittal cerebellar sections from the adult males highlighted in red in (A), counterstained with DAPI (B,D,F) or immunostained for cycling GCPs and RG/BG precursors/cells (green in A–D; a marker for GCPs) and Calb1 (red in A–D; a marker for RG/BG fibers), and counterstained with DAPI (blue in A–F), KI (a nuclear marker). (C,D) are close-up views of the boxed areas in (A,B). (G–I) are single color channel views of (E,F), respectively. Yellow arrowheads in (D) delimit the anterior outer EGL in the mutant embryos. **Figure S2.** The ventral mid-/hindbrain region is not affected in Fgfr2 cKO mice. (A–F) Sagittal views of the ventral MH from adult control (A,C,E,G) and Fgfr2 cKO mice (B,D,F), hybridized with riboprobes for Tyrosine hydroxylase (Th; the rate-limiting enzyme for dopamine and noradrenergic synthesis) (A,B), the Serotonin transporter (Sert, also known as Slc6a4, expressed on serotonergic neurons) (C,D) and the vesicular Acetylcholine transporter (VAChT, also known as Slc18a3, expressed in cholinergic neurons) (E,F). Gross anatomical alterations of these neuronal populations were not detected in the Fgfr2 cKO mice. DR, dorsal raphe nucleus; LC, locus ceruleus; LDTg, laterodorsal tegmental nucleus; RF, reticular formation (brainstem); SNc, substantia nigra pars compacta; VTA, ventral tegmental area. Scale bar (A): 500 μm. **Figure S3.** Fgfr expression in the developing murine mid-/hindbrain region. (A–R) Representative brightfield (A–C,G–L,O–R) and darkfield (D–F,J,L–P) views of the mid- /hindbrain region on cresyl-violet-stained midsagittal sections from wild-type (CD-1) mouse embryos at E14.5 (A–F; n = 5 embryos), E16.5 (G–L; n = 5 embryos), and E18.5 (M–R; n = 6 embryos), hybridized with radioactive Fgfr1 (A,D,G,J,M,P), Fgfr2 (B,E,H,K,N,Q) and Fgfr3 (C,F,I,L,O,R) riboprobes. CbA, cerebellar anlage; ChPl, choroid plexus; EGL, external granular layer; IC, inferior colliculus; PCL, Purkinje cell layer; rH, rostral hindbrain; rI, rhombic lip; Tg, tegmentum; VZ, cerebellar ventricular zone. Scale bar (C): 200 μm. **Figure S4.** Disruption of the anterior PCL but apparently normal RG scaffold in the E17.5 Fgfr2 cKO Cha. (A–L) Representative confocal overview (A,B,E–L) and close-up views (C,D) of the anterior Cha on sagittals sections from control (A,C,E,G,I,K) and Fgfr2 cKO (B,D,F,H,J,L) embryos at E17.5 (n = 1 embryo/genotype), immunostained for Pax6 (cyan/green in A–D; a marker for GCPs) and Calb1 (red in A–D; a marker for PCs), or Cnd1 (cyan/green in E–H; a marker for cycling GCPs and RG/BG precursors/cells) and Glast (red in E,F,L; a marker for RG/BG fibers), and counterstained with DAPI (blue in A–F,K,L; a nuclear marker). (C,D) are close-up views of the boxed areas in (A,B). (G–I) are single color channel views of (E,F), respectively. Yellow arrowheads in (D) delimit the anterior outer EGL in the mutant embryos. (H,J) delimit the distorted Cnd1+ anterior outer EGL in the mutant embryos. **Figure S5.** SHH signaling does not appear to be affected in the Cha of Fgfr2 cKO embryos. (A–H) Representative sagittal darkfield (A,B) and brightfield (C–H) views of the Cha in E16.5 (A–D; n = 5 embryos/genotype) and E18.5 (E–H; n = 4 embryos/genotype) control (A,C,E,G) and Fgfr2 cKO (B,D,F,H) embryos, hybridized with riboprobes for Shh (A,B,E,F) and Pax6 (C,D,G,H). Red arrowheads in (F) delimit the anterior outer EGL in the mutant embryos. EGL, external granular layer; PCL, Purkinje cell layer. Scale bar (A): 100 μm. (PDF)

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**Author Contributions**

Conceived and designed the experiments: FM SD SMH RK DMVW NP. Wrote the paper: FM FG SD NP.

**References**


gene expression pattern of a novel gene encoding brain-fatty acid binding protein 
mediates GFN-induced BDNF expression in nigrostriatal dopaminergic