"Matrix-binding vascular endothelial growth factor (VEGF) isoforms guide granule cell migration in the cerebellum via VEGF receptor Flk1"

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ABSTRACT

Vascular endothelial growth factor (VEGF) regulates angiogenesis, but also has important, yet poorly characterized roles in neuronal wiring. Using several genetic and in vitro approaches, we discovered a novel role for VEGF in the control of cerebellar granule cell (GC) migration from the external granule cell layer (EGL) toward the Purkinje cell layer (PCL). GCs express the VEGF receptor Flk1, and are chemoattracted by VEGF, whose levels are higher in the PCL than EGL. Lowering VEGF levels in mice in vivo or ectopic VEGF expression in the EGL ex vivo perturbs GC migration. Using GC-specific Flk1 knock-out mice, we provide for the first time in vivo evidence for a direct chemoattractive effect of VEGF on neurons via Flk1 signaling. Finally, using knock-in mice expressing single VEGF isoforms, we show that pericellular deposition of matrix-bound VEGF isoforms around PC dendrites is necessary for proper GC migration in vivo. These findings identify a previously unknown role for VEGF in...
Development/Plasticity/Repair

Matrix-Binding Vascular Endothelial Growth Factor (VEGF) Isoforms Guide Granule Cell Migration in the Cerebellum via VEGF Receptor Flk1

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Vascular endothelial growth factor (VEGF) regulates angiogenesis, but also has important, yet poorly characterized roles in neuronal wiring. Using several genetic and in vitro approaches, we discovered a novel role for VEGF in the control of cerebellar granule cell (GC) migration from the external granule cell layer (EGL) toward the Purkinje cell layer (PCL). GCs express the VEGF receptor Flk1, and are chemoattracted by VEGF, whose levels are higher in the PCL than EGL. Lowering VEGF levels in mice or ectopic VEGF expression in the EGL ex vivo perturbs GC migration. Using GC-specific Flk1 knock-out mice, we provide for the first time in vivo evidence for a direct chemoattractive effect of VEGF on neurons via Flk1 signaling. Finally, using knock-in mice expressing single VEGF isoforms, we show that pericellular deposition of matrix-bound VEGF isoforms around PC dendrites is necessary for proper GC migration in vivo. These findings identify a previously unknown role for VEGF in neuronal migration.

Introduction

Wiring of neuronal circuits relies on precise spatial positioning of neurons and axons (Rakic, 2002). The cerebellar cortex contains three layers in adult mammals: a molecular layer (ML), an intermediate Purkinje cell layer (PCL), and a deeper internal granular cell layer (IGL) (Rakic, 1990; Hatten, 1999; Sotelo, 2004). In development, an additional layer on the surface of the cerebellar cortex, termed the external granule cell layer (EGL), contains granule cell precursors (GCPs) from the rhombic lip. In rodents, GCPs continue to proliferate in the upper part of the EGL after birth and give rise to postmitotic granule cells (GCs), which migrate tangentially in the lower part of the EGL, while extending bipolar axons, which form parallel fibers (Solecki et al., 2006).

Neuronal migration and axon navigation are orchestrated by attractive and repulsive guidance cues (Dickson, 2002; Marin et
Diffusible molecules act as long-range cues, whereas matrix-associated or membrane-anchored molecules function as short-range signals (Park et al., 2002). In the cerebellum, GC migration involves neuronal–glial adhesion molecules (Hatten, 2002), stromal cell-derived factor (Ma et al., 1998; Zou et al., 1998; Klein et al., 2001), EphrinB (Lu et al., 2001), brain-derived neurotrophic factor (BDNF) (Rocamora et al., 1993; Borghesani et al., 2002; Zhou et al., 2007), and semaphorin 6A (Kerjan et al., 2005; Renaud et al., 2008), among other cues.

Vascular endothelial growth factor (VEGF) is best known for its role in angiogenesis (Carmeliet, 2003; Ferrara, 2004; Kerbel, 2008; Shibuya, 2008). A spatial gradient of VEGF isoforms, which differ in size and binding properties to heparin in the extracellular matrix, is critical for vessel patterning and morphogenesis (Carmeliet et al., 1999; Stalmans et al., 2003). In the nervous system, VEGF regulates proliferation, differentiation, and survival of neuronal cells (Ruiz de Almodovar et al., 2009). In addition, VEGF promotes tangential migration of motoneurons and migration of cranial neural crest cells (NCCs) via binding to neuropilin-1 (Npn-1), a coreceptor of the signaling receptor VEGFR-2 (Flk1) (Schwarz et al., 2004, 2008; McLennan et al., 2010; Schwarz and Ruhrberg, 2010). Since Npn-1 does not elicit signals in response to VEGF in neurons, the mechanism whereby VEGF attracts motoneurons and NCCs remains unknown. Furthermore, there is no conclusive evidence that VEGF regulates neuronal migration in vivo through direct activation of Flk1.

VEGF is expressed in the cerebellum (Acker et al., 2001); however, nothing is known about its effects on GC migration. Therefore, using various loss- and gain-of-function genetic and pharmacological approaches, we explored the role and underlying mechanisms of VEGF in GC migration. Our findings indicate that VEGF, via direct binding to its receptor Flk1 in postmitotic Gcs, regulates GC migration during cerebellar development. Moreover, we describe how matrix-bound VEGF isoforms are necessary for proper GC migration.

**Materials and Methods**

**Animals.** VEGF<sub>LacZ</sub> (Miquerol et al., 2000), VEGF<sub>164/165</sub> (Oosthuyse et al., 2001), VEGF<sub>188/188</sub> and VEGF<sub>200/200</sub> (Carmeliet et al., 1999; Stalmans et al., 2003) were previously generated. The transgenic MathlCreER and the Flk1-GFP and Flk1-LacZ mouse lines were kindly provided by Dr. J. Baker, St. Jude Children’s Research Hospital, Memphis, TN (Chow et al., 2006) and Dr. J. Rossant, University of Toronto, Toronto, ON, Canada (Ema et al., 2006), respectively. The Flk1<sub>LacZ</sub> mouse line was generated by crossing Flk1<sup>LacZ</sup> mice (Haigh et al., 2003) with Flk1<sup>LacZ</sup> mice. For each transgenic mouse line, littermates or proper wild-type (WT) control mice were used. Wistar rat pups (postnatal day 6 (P6) and P8) were also used for ELISA and for immunostainings, respectively. All animals were treated according to the guidelines approved by the French Ethical Committee (decreé 87-878) and the Animal Care Committee of the University of Leuven, Belgium.

**Histology, immunohistochemistry.** Mouse and rat pups (males and females), and adult mice (males and females), were perfused transcardially with saline followed by 4% paraformaldehyde (PFA). Serial cryosections were treated with 60 mg/kg pimonidazole hydrochloride (Millipore Bioscience Research Reagents) 1.5 h before brain dissection, and afterward subjected intraperitoneally with BrdU (50 mg/kg body weight). Sections were heated three times 5 min in 10x citrate buffer, pH 6.0, in a microwave, and subsequently double labeled for BrdU and VEGF, for BrdU/p27, Ki67/p27, Ki67/Pax6, or p27/Pax6, before being counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Roche). For the detection of hypoxic cells, mice were treated with 60 mg/kg pimonidazole hydrochloride (Millipore Bioscience Research Reagents) 1.5 h before brain dissection, and afterward staining for hydroxyprobe-1-Mab-1 was performed following manufacturer’s instructions. Immunostainings were examined using Imager and AxioP Zeiss microscopes equipped with epifluorescence illumination or confocal system (Zeiss CLSM510; 0.5–1.0 μm optical sections). VEGF immunostaining was analyzed by pseudocolor analysis, which attributed a specific color to defined intensity gray levels of the VEGF immunostaining, with red areas representing the highest intensity value, and the yellow, light green, dark green, blue, and dark purple areas representing decreasing intensity values, respectively.

**VEGF–alkaline phosphatase binding and staining.** VEGF<sub>164</sub>-AP and PIGF–AP fusion proteins were made by cloning their cDNA sequences into pETtag-5 vector (GenHunter). Proteins were expressed and secreted from HEK-293 cells, and the conditioned medium was used directly as described previously (Renaud et al., 2008). VEGF<sub>164</sub>-AP protein was quantified via ELISA. For competition experiments, recombinant VEGF<sub>164</sub> (R&D Systems; 493-MV) or VEGF–E (RELIATech 300-046S) was coincubated with VEGF<sub>164</sub>-AP-superant.

**Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining.** Each cryosection was fixed and permeabilized with 0.5% Triton X-100 in PBS followed by blocking with 10% fetal bovine serum (FBS) in PBS. Sections were stained with DAPI (0.5 μg/ml) for 20 min, and then incubated with a 1:500 solution of anti-biotin antibody (Vector; ABC200) for 1 h. Peroxidase was visualized with DAB (Sigma; D5637) for 5 min.

**DiI labeling.** To trace parallel fibers, we used the fluorescent carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Invitrogen) in 200-μm-thick fixed coronal vibratome slices of VEGF<sub>164/165</sub> and WT cerebella (P12). Sliced crystals (50 μm diam-
eter) were placed in the ML of brain slices of putative littersmates using a patch pipette and slices were kept in the dark at 37°C for 2–4 d to allow appropriate dye diffusion. Images were acquired using a Leica confocal system (0.5–1.0 μm optical sections).

In vitro chemotaxis assay. Purified GCs were resuspended in serum-free DMEM [supplemented with N2 (Invitrogen) and B27 (Invitrogen)]. In vitro migration of GCs was assessed using laminin (Sigma-Aldrich; 20 μg/ml)-coated-PTF (polyethylene) track-etched membranes with 8 μm pore size in modified Boyden chambers as previously described (Lu et al., 2001). Briefly, 200 μl of serum-free DMEM containing 1 × 10^5 GCs was placed in the upper chamber. The corresponding treatment was added in serum-free medium in the lower chamber. After 17 h at 37°C in 5% CO₂, the upper surface of membranes was scraped free of cells and debris, membranes were fixed in 4% PFA for 1 h at room temperature, stained for β3-tubulin and DAPI. Cells that had migrated through pores and adhered to the membrane were analyzed under high-power fluorescent microscopy and counted in 30 adjacent high-power fields. Experiments were performed in triplicate, and data are expressed as fold increase in number of cells present at the lower side of the filter with respect to the number of cells present at that side of the filter in control conditions. N values represent the number of independent experiments performed.

Growth cone turning assay. GCs that formed only a single thick leading process and were not migrating at the start or during recording, were selected for analysis. Concentration gradients of recombinant human VEGF, R&D Systems and recombinant human BDNF, R&D Systems were produced for 1 h by application of VEGF or BDNF solution through a glass pipette. The turning angle between the original direction of the neurite and its new direction at the end of the 1 h period was measured using Software Analysis (Soft Imaging System). For statistical analysis nonparametric Kruskal–Wallis and Mann–Whitney tests were used.

Cocultures of HEK-293 cells with GCs. We generated HEK-293 cells, stably expressing a single VEGF isoform (HEK-VEGF165; R&D Systems) and recombinant human BDNF (R&D Systems) were produced for 1 h by application of VEGF or BDNF solution through a glass pipette. The turning angle between the original direction of the neurite and its new direction at the end of the 1 h period was measured using Software Analysis (Soft Imaging System). For statistical analysis nonparametric Kruskal–Wallis and Mann–Whitney tests were used. N values indicate the total number of cells analyzed.

Cultures of HEK-293 cells with GCs. We generated HEK-293 cells, stably expressing a single VEGF isoform (HEK-VEGF188; R&D Systems) and mock control (HEK-Mock); despite intense screening, only clones with 10-fold lower VEGF188 levels than the other isoforms could be generated. These cells were plated on noncoated coverslips at sparse (50%) confluency, and cultured for 24 h in DMEM with 10% serum. Afterward, a fixed number of purified GCs was added to the cultures in serum-free DMEM [supplemented with the neuronal supplements N2 (Invitrogen) and B27 (Invitrogen)]. Seventeen hours later, cocultures were fixed with 4% PFA for 15 min at room temperature, permeabilized, and stained for Pax-6, β1-III-tubulin, actin, and DAPI, to visualize each cell type. Confocal images were taken from three independent experiments. Since purified GCs only survive if they adhere to these cellular substrates in this assay (they cannot adhere to the uncoated dish), we counted adhered GCs per HEK-293 cell (the conditions were selected such that with HEK-Mock cells, on average, one GC adhered per two HEK-293 cells).

Ex vivo cerebellar electroporation. Cerebellar dissected from P10 wild-type pups were injected with 8 μl of DNA of interest (3 μg/μl) (endoforce, GFP expression vector in 5:1 ratio) into the superficial layers of the cerebellum was subjected to five electric pulses of 85 V, 50 ms, and a 1.0 s treatment, slices were cultured 8 d. Brainbow-electroporated cerebellar slices from Math1CreER × Flk1lox/LacZ were treated for 2 d with 4-hydroxy-tamoxifen (Sigma-Aldrich) (100 nM) to activate Cre, and analyzed 2 d later.

Analysis of electroporated cerebellar slices. Confocal images were taken using the Zeiss CLSM510 mounted on an AxioVert100M and LSM software. To quantify radial migration of GFP + GCs in electroporated slices, the distance that each GFP + GC migrated radially from the EGL/ML border was measured (see Fig. 4F, distance b) using KS300 morphology software (Zeiss), and normalized for the total length of the cerebellar lobe (see Fig. 4F, distance a). The cerebellar cortex was then divided into five equally spaced areas. A comparable total number of migrating GCs was counted in control and treated groups; at least 1500 GFP + GC from at least three electroporated control and “treated” cerebella were analyzed in each group. Using binomial nonparametric statistics, the statistical differences between the observed and expected number of GCs in each area was determined, with the expected number of cells representing the number found in the control group (assuming that if the treatment would have no effect, a similar number of GCs would be expected in each of the areas). The difference between the observed versus expected number of cells in each area was expressed as a percentage of the expected number of cells.

Time-lapse imaging. Time-lapse movies were made (16 h) of ex vivo electroporated cerebellar slices after being 2 d in culture (for imaging tangential and radial migration, respectively). Images were obtained by using the Zeiss CLSM510 NLO META mounted on an AxioVert200M (Zeiss) inverted microscope. Multiphoton imaging of GFP + GCs was performed using 920 nm pulsed mode-locked laser emission from a tunable Ti:Sapphire Chameleon laser (Coherent). Time-lapse imaging was performed with minimal necessary laser power. Stacks of frame-averaged (five frames) confocal optical slices were collected digitally, at 20 min intervals for time-lapse sequences. Three-dimensional as well as four-dimensional reconstructions of image data were prepared using LSM software package. A semiautomatic macro, written in KS300 software, was used to analyze the tracks of migrating GFP + GCs and calculate the speed of tangential migration.

Induction of Cre activity in Math1CreER × R26R-stop-LacZ and in Math1CreER × Flk1lox/LacZ mice. Tamoxifen (Sigma-Aldrich) was dissolved in sunflower seed oil (Sigma-Aldrich) at 30 mg/ml. For all experiments, 4 mg of tamoxifen/35 g of body weight was administered by oral gavage to pregnant mice at embryonic day 17 (E17). After injection of BrdU at P6, BrdU− cells were counted 2 d later.

Quantification of VEGF and Flk1 by ELISA. Two defined regions, one comprising the EGL plus the upper ML, and a second one comprising the EGL only, were microdissected from 200 μm slices of rat pups or P6 mouse pups. The microdissected tissues from 6–10 pups were pooled and processed in one sample for additional analysis of VEGF protein concentration using the commercial Quantikine rat or mouse VEGF ELISA kit (R&D Systems; Quantikine RVR00 and MMV00, respectively). Flk1 expression was determined in lysates from purified GCs using the commercial mouse Flk1 ELISA kit (R&D Systems; Quantikine MVR00B). N values for this experiment indicate the number of independent experiments performed, each of them of pooled samples from 6–10 pups.

Quantitative real-time reverse transcription-PCR. Expression levels were quantified by real-time reverse transcription (RT)-PCR, relative to the expression level of β-actin or GAPDH, using the following forward (F) and reverse primers (R) and probes (P), labeled with fluorescent dye (FAM) and quencher (TAMRA). VEGF: F, 5′-AGTCCGATGACTGCAAGCTTGAAT-3′; R, 5′-ATCCGCGATGACGTGACGTAAGT-3′; P, 5′-FAM-TCGAGGAACTTATGACCTGAGTG-3′; Flk1: F, 5′-AACAAGGATTCCGTTCACAGT-3′; R, 5′-GAGAGGGAAATCGTGCGTGAC-3′; P, 5′-FAM-TGAGGATTCCGTTCACAGT-3′; Math1: F, 5′-AGTCCGATGACTGCAAGCTTGAAT-3′; R, 5′-GCCCGCGACCTTCCTTC-3′; P, 5′-FAM-CATCACTTGGACGCCCTTC-3′; β-actin: F, 5′-AGGAGGAAATGTCGCGTGAC-3′; R, 5′-AAATAGTGGAGATGCTGAGTGACATG-3′; P, 5′-FAM-ACACTGCGGATCCTCC-3′; GAPDH: F, 5′-ACCTGCGGCGTCATGAG-3′; R, 5′-
CCCTCAAGTGCTGCTCCA-3’; P', 5'-FAM-CAACCTTTTGTGTCATCATCACTGGCAGG-TAMRA-3’. For the different VEGF isoforms, a common forward primer and probe were used: F, 5'-TGAGGCTGCTG-TAACGGATG-3’; P, 5'-FAM-TGCTGCTTGGTGTGATCGTATCATTCCCATCCG-TAMRA-3’. The reverse primers specific for the VEGF isoforms were as follows: for VEGF164c;c, R, 5’-CCCTCAGGAATTCACCTATTTTCTC-3’; for VEGF164c;c, R, 5’-CTCAAGGATTTAAACCGGTT-3’; for VEGF164c;c, R, 5’-GAACAGGCTACAGTGATTCTTTC-3’; for Flk1, F, 5’-ACTCAGAGATGACGTTCATTTGC-3’; for VEGF120, R, 5’-TGAGGCTGCTG-TAACGGATG-3’; P, 5’-FAM-TGCTGCTTGGTGTGATCGTATCATTCCCATCCG-TAMRA-3’. N values indicate the number of independent experiments performed with different cultures of purified GCs.

Nuclear magnetic resonance spectroscopy. P10 pups were anesthetized, and their cerebellum was rapidly dissected and snap frozen. After homogenization in 1 x HClO4, the extracts were centrifuged, the supernatants were neutralized, and the precipitated HClO4 was removed. Subsequently, samples were dried, resuspended in D2O, and adjusted to pH 7.0–7.5 before analysis. In vitro 1H nuclear magnetic resonance (NMR) spectroscopy (repetition time, 15 s; number of acquisitions, 64) analyses of lactate, glutamate, succinate, and NTP (ATP-NADH) content in cerebellar extracts were performed in a high-resolution AVANCEII (9.4 tesla) spectrometer (Bruker). Peak intensities were quantified by integration after baseline correction, using the spectrometer processing software. These values were related to total creatin levels. Mean peak intensities, relative to total creatin, of NTP (ATP-NADH), lactate, succinate, and glutamate after 1H NMR of P10 cerebellar extracts are summarized together with the SEM.

Electron paramagnetic resonance oximetry. Lithium phthalocyanine (LiPc) has a single, sharp electron paramagnetic resonance (EPR) line whose width is highly sensitive to pO2. Two small aggregates of LiPc crystals (80–100 μg) were implanted into brain tissue. EPR spectra were recorded using an EPR spectrometer (Magnetech) operating at 1.2 GHz with an extended loop resonator placed over the head of the mice. Clear signal-to-noise ratios were obtained in recorded spectra. The measurements reflect the average partial pressure of oxygen on the crystal surface.

Statistical analysis. All data represent mean ± SEM. Statistical significance for most analyses was performed using Student’s t tests, except otherwise stated.

Results
VEGF is expressed in a radial pattern from the PCL to the upper cerebellar layers

We first characterized the expression pattern of VEGF in the developing cerebellum of WT mice at different time points during inward GC migration (Komuro and Yacubova, 2003). We used a LacZ-VEGF mouse line (VEGFlacZ mice), in which an IRES-nsLacZ reporter cassette was knocked in, via homologous recombination, into the last exon of the Vegf gene (Miquerol et al., 2000). This line overexpresses VEGF from its endogenous locus by twofold (Miquerol et al., 2000), but the spatiotemporal expression pattern of β-galactosidase (β-Gal) reliably mimics that of the endogenous Vegf gene (Miquerol et al., 1999). Immunostaining revealed that β-Gal was expressed in Purkinje cells (PCs) at P13 (Fig. 1A, B). VEGF immunostaining at different time points during GC migration (P3, P5, P8, and P13) confirmed that VEGF was detectable in the PC soma and dendrites, even in dendritic extensions that reached the lower EGL (Fig. 1C; supplemental Fig. 1A–C, available at www.jneurosci.org as supplemental material). These data are consistent with reports that VEGF is detectable in the PCL from P1 to adulthood (Acker et al., 2001).

Semiquantitative pseudocolor conversion of VEGF immunostaining indicated that the VEGF-immunoreactive levels were higher in the PC somata than dendrites (see Fig. 3C); apotome microscopy confirmed these findings (data not shown). ELISA on microdissected P6 rat cerebella (used because of their larger size to facilitate reliable microdissection) also showed that VEGF protein levels were higher in the deeper than in the more superficial cerebellar layers (ng VEGF/mg protein: 77.7 ± 7.6 in P1 lower ML vs 25.4 ± 10.3 in EGL/upper ML; N = 4 samples of 10 pups each; p < 0.01); comparable results were obtained in mouse cerebella (supplemental Table 1, available at www.jneurosci.org as supplemental material), (for reasons of clarity, several of the data presented in the main text are overviewed again in the supplement). Using similar criteria as those used to judge the presence of a BDNF gradient (Borgesani et al., 2002; Zhou et al., 2007), these findings suggest that VEGF is deposited in a radial concentration gradient from the PCL to the upper layers. Measurements of extracellular VEGF would be needed to conclusively demonstrate the presence of such a radial VEGF concentration gradient.

VEGF was also detectable in Bergmann glia cells in VEGFlacZ mice (P13) when immunostained with an anti-β-Gal or anti-VEGF antibody (Fig. 1D, B, D), but in WT mice, we could not label these cells with anti-VEGF antibodies (Fig. 1C; supplemental Fig. 1A–C, available at www.jneurosci.org as supplemental material), suggesting that VEGF is expressed at lower levels in Bergmann glia than in PCs. Immunostaining with anti-β-Gal also revealed that VEGF is expressed in PCs, Bergmann glia, and in astrocytes in the IGL, but not in interneurons, in adult VEGFlacZ cerebellum (supplemental Fig. 1D–F, available at www.jneurosci.org as supplemental material).

VEGF receptor-2/Flk1 is expressed in granule cells

We next determined whether GCs express VEGF receptors. In purified GCs from P6 mice, both Flik1 mRNA (7.4 ± 1.3 mRNA copies per 10⁵ copies of β-actin mRNA; N = 4) (supplemental Table 1, available at www.jneurosci.org as supplemental material) and Flk1 protein (ng Flik1/mg protein: 0.53 ± 0.02; N = 3) (supplemental Table 1, available at www.jneurosci.org as supplemental material) were detectable; immunoblotting confirmed this finding (supplemental Fig. 1G, available at www.jneurosci.org as supplemental material). We also used a sensitive staining technique, based on the use of a VEGF164c alkaline phosphatase fusion protein (VEGF164c-AP), to study Flk1 expression in the cerebellum. At P10, VEGF164c-AP staining was strong in pial and parenchymal blood vessels, whereas a weaker but specific signal was detected in the deeper layer of the EGL (Fig. 1E; F; supplemental Fig. 1H, available at www.jneurosci.org as supplemental material). Control pharmacological experiments showed that VEGF164c-AP bound to Flk1 in the lower EGL (Fig. 1G; supplemental Fig. 1I, J, available at www.jneurosci.org as supplemental material). A similar Flk1 expression pattern was observed in the postnatal cerebellum of P10 Flk1-GFP knock-in mice (Ema et al., 2006), immunostained for GFP (Fig. 1H), or in P8 rat cerebellum, immunostained with an anti-Flk1 antibody, previously used to identify Flk1+ neurons (Marko and Damon, 2008) (supplemental Fig. 1N, available at www.jneurosci.org as supplemental material). Double immunostaining for GFP and Sema6A further revealed that Flk1 was expressed by Sema6A+ postmitotic GCs (supplemental Fig. 1K–M, available at www.jneurosci.org as supplemental material). In addition, Flk1 was also expressed on the cell surface of postmitotic GCs and their radial leading process in in vitro cocultures of GCs and Bergmann glia fibers (Fig. 1I).

Of all VEGF receptors, only Flk1 was detected in postmitotic GCs. Indeed, staining of cerebellar sections (from P10 WT mice) using a fusion protein of placental growth factor (PIGF) (a VEGF homolog that binds to another VEGF receptor Flt1) and alkaline phosphatase (PIGF-AP) or immunostaining for neuropilin-1
Granule cells and their growth cones are attracted by VEGF

To assess whether VEGF is a chemoattractant factor for postmitotic GCs, we first analyzed GC migration in a modified Boyden chamber assay (Lu et al., 2001). GCs that migrated to the lower side of the filter expressed postmitotic differentiation markers (data not shown). When VEGF (50 ng/ml) was added to the lower chamber, GC migration from the upper to the lower compartment was increased, comparably with the effect induced by BDNF, a known GC chemoattractant (Zhou et al., 2007). Indeed, relative to control solution (PBS) did not induce any effect on growth cone orientation (Fig. 2A,B). In contrast, topical application of VEGF induced a clear turning response toward VEGF (Fig. 2C,D). In the control situation, a comparable number of growth cones turned toward positive (turning) or away (negative turning) from the medium, or failed to turn at all (no turning) (Fig. 2E,G). In contrast, when exposed to the VEGF gradient, all but one growth cone positively turned toward VEGF (Fig. 2F,G). The effect of VEGF was comparable with that of BDNF, a known chemoattractant factor for GCs (Borgohesani et al., 2002; Li et al., 2005) (Fig. 2F,G). When measuring the turning angle, growth cones turned by $-4.1 \pm 5.5^\circ$ in control conditions versus $+35.8 \pm 16.8^\circ$ when exposed to VEGF ($N = 12; p = 0.001$ vs control) (Fig. 2H; supplemental Table 2, available at www.jneurosci.org as supplemental material). In this assay, VEGF was equipotent to BDNF ($+24.7 \pm 10.3^\circ; N = 8; p < 0.05$, vs control) (Fig. 2H; supplemental Table 2, available at www.jneurosci.org as supplemental material). Incubation with an anti-Fk1 blocking antibody ($\alpha$Fk1) inhibited the VEGF-induced chemoattraction of GC growth cones (Fig. 2E,G,H), indicating that VEGF attracts growth cones of GCs via Fk1.

Reduced VEGF levels delay granule cell migration in VEGF$^{+/0}$ mice

We then performed a number of loss- and gain-of-function experiments to provide genetic evidence that endogenous VEGF
VEGF protein levels were also lower in P10 mice (supplemental Table 3, available at www.jneurosci.org as supplemental material). Cerebellar VEGF levels were reduced in cerebella of mice in which deletion of a regulatory element in the VEGF promoter reduced VEGF levels (Oosthuyse et al., 2001). RT-PCR analysis revealed that VEGF mRNA levels were reduced in cerebella of P10 mice (copies VEGF mRNA per 10^5 copies β-actin mRNA: 19 ± 2 in WT vs 10 ± 2 in VEGF^−/−; N = 5; p < 0.05) (supplemental Table 3, available at www.jneurosci.org as supplemental material). Consistent with a defect in GC migration, significantly more p27^+ cells accumulated in the EGL of VEGF^−/− mice than in WT mice (Table 1). Morphometry confirmed that the EGL was thicker in P12 VEGF^−/− mice (EGL area, percentage of total cerebellar cortex area in lobe IX: 4.8 ± 1.2% in WT vs 8.3 ± 1.1% in VEGF^−/−; N = 5; p < 0.05) (supplemental Table 3, available at www.jneurosci.org as supplemental material). Additional analysis showed that proliferating Ki67^+ GCs were only found in the upper EGL, whereas postmitotic p27^+ GCs were only present in the lower EGL, indicating that the upper and lower EGL are properly segregated (supplemental Fig. 2A,B, available at www.jneurosci.org as supplemental material). Also, there were no genotypic differences in the density of Pax6^+ cells (Table 1), or in the number of Ki67^+ cells in the EGL (Table 1), indicating normal formation of the upper EGL and proliferation of GCPs. Normal proliferation rate of GCPs in P12 VEGF^−/− mice was confirmed by BrdU labeling and phosphohistone 3^+ labeling (supplemental Fig. 2C,D, available at www.jneurosci.org as supplemental material; Table 1). We could also not detect developmental defects of PCs or Bergmann glia fibers (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material; Table 1). The shape and patterning of the folia, and overall cerebellar structure were also

regulates GC migration in vivo. We therefore used the hypomorphic VEGF<sup>α/α</sup> mouse line, in which deletion of a regulatory cis-element in the VEGF promoter reduced VEGF levels (Oosthuyse et al., 2001). RT-PCR analysis revealed that VEGF mRNA levels were reduced in cerebella of VEGF<sup>α/α</sup> mice at P12 (copies VEGF mRNA per 10<sup>5</sup> copies β-actin mRNA: 19 ± 2 in WT vs 10 ± 2 in VEGF<sup>α/α</sup>; N = 5; p < 0.05) (supplemental Table 3, available at www.jneurosci.org as supplemental material). Consistent with a defect in GC migration, significantly more p27<sup>+</sup> cells accumulated in the EGL of VEGF<sup>α/α</sup> mice than in WT mice (Table 1). Morphometry confirmed that the EGL was thicker in P12 VEGF<sup>α/α</sup> mice (EGL area, percentage of total cerebellar cortex area in lobe IX: 4.8 ± 0.6% in WT vs 8.3 ± 1.1% in VEGF<sup>α/α</sup>; N = 5; p < 0.05) (supplemental Table 3, available at www.jneurosci.org as supplemental material). Additional analysis showed that proliferating Ki67<sup>+</sup> GCs were only found in the upper EGL, whereas postmitotic p27<sup>+</sup> GCs were only present in the lower EGL, indicating that the upper and lower EGL are properly segregated (supplemental Fig. 2E,F, available at www.jneurosci.org as supplemental material). Also, there were no genotypic differences in the density of Pax6<sup>+</sup> cells (Table 1), or in the number of Ki67<sup>+</sup> cells in the EGL (Table 1), indicating normal formation of the upper EGL and proliferation of GCPs. Normal proliferation rate of GCPs in VEGF<sup>α/α</sup> mice was further confirmed by BrdU<sup>+</sup> labeling and phosphohistone 3<sup>+</sup> immunostaining (supplemental Fig. 2A,B, available at www.jneurosci.org as supplemental material; Table 1). The reduced GC migration in VEGF<sup>α/α</sup> mice was also not attributable to differences in GC apoptosis (Table 1), or GC differentiation (Fig. 3I,J; supplemental Fig. 2E,F,I,J,M,N, available at www.jneurosci.org as supplemental material; Table 1). We could also not detect developmental defects of PCs or Bergmann glia fibers (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material; Table 1). The shape and patterning of the folia, and overall cerebellar structure were also

Figure 2. VEGF chemoattracts GC growth cones. A–D, Representative GC growth cones at the onset (A) and 1 h (B) after application of control solution (PBS), or at the onset (C) and 1 h (D) after induction of VEGF gradient. The white arrows represent the orientation of VEGF or PBS application. E, F, Superimposed traces of growth cone trajectories in response to topical application of PBS (E, black curves), to a BDNF gradient (F, black traces), to a VEGF gradient (F, red traces) or to a VEGF gradient in the presence of αFlk1 (E, red traces). The black arrows denote the orientation of the PBS, VEGF, or BDNF gradient application. The tick marks along the x- and y-axis represent 2 μm. G, Scatterplots illustrating the turning responses of growth cones to topical application of PBS (black dots, left panel), to a BDNF gradient (black dots, right panel), to a VEGF gradient (red dots, left panel) or to a VEGF gradient in the presence of αFlk1 (red dots, left panel). H, Histogram representing the average turning angle of growth cones when exposed to PBS, a BDNF gradient, or a VEGF gradient with and without αFlk1. Note the chemotactic response induced by VEGF (N = 8–11; ***p < 0.001). Error bars indicate SEM. Scale bar: A–D, 5 μm.
Figure 3. GC migration defects in VEGF−/− mice. A, B. VEGF immunostaining showing reduced VEGF expression in Purkinje cell bodies (asterisks) and dendrites (arrows) in VEGF−/− mice (B) compared with WT mice (A). C, D. Pseudocolor images of VEGF immunostaining indicating an overall reduction of the VEGF-immunoreactive signal in Purkinje cell bodies (asterisk) and dendrites (arrow) in VEGF−/− mice (D) compared with WT mice (C). E, F. Double immunolabeling for BrdU (green) and VEGF (red), combined with DAPI counterstaining (blue), in cerebella of a P12 WT (E) and VEGF−/− (F) mice, revealing impaired migration of BrdU+ GCs in the VEGF−/− mouse (F) compared with the WT mouse (E). Note that more BrdU+ cells accumulated at the interface between the EGL and the ML (arrows) in the VEGF−/− (F) compared with the WT (E) mouse. G, H. Double immunostaining for BrdU (green) and p27 (red), labeling postmitotic neurons in the inner two-thirds of the EGL (marked by dotted lines), revealing an enlarged p27+ area in the EGL of VEGF−/− (H) compared with WT (G) mice. Note the more numerous double-labeled BrdU+/p27+ cells (yellow) in the EGL of VEGF−/− mice (H). I, J. Dil labeling of parallel fibers in adult WT (I) and VEGF−/− (J) revealing a similar fiber pattern throughout the ML in both genotypes. K, L. Immunostaining for CD31 (red) revealing a comparable microvasculature in the cerebellar cortex of a WT (K) and VEGF−/− (L) mouse. Scale bars: A–D, 50 μm; E, F, 65 μm; G, H, 20 μm; I, J, 50 μm; K, L, 320 μm.

Table 1. Normal GC proliferation, differentiation, cell death, and blood vessel density in VEGF−/− and VEGF188/188 mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>VEGF−/−</th>
<th>VEGF188/188</th>
<th>p value</th>
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<tbody>
<tr>
<td>P27+ GCs—lower EGL</td>
<td>38.5 ± 3.0</td>
<td>55.3 ± 5.6</td>
<td>p &lt; 0.05</td>
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<tr>
<td>Pax6 density (P10)</td>
<td>26.1 ± 4.1</td>
<td>27.6 ± 3.4</td>
<td>p = NS</td>
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<tr>
<td>GCP proliferation—upper</td>
<td>33.6 ± 3.0</td>
<td>37.8 ± 1.5</td>
<td>p = NS</td>
<td></td>
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<tr>
<td>GCP proliferation (P10)</td>
<td>26.5 ± 1.5</td>
<td>23.3 ± 1.3</td>
<td>p = NS</td>
<td></td>
</tr>
<tr>
<td>GCP proliferation (P10)</td>
<td>13.2 ± 1.4</td>
<td>14.5 ± 2.2</td>
<td>p = NS</td>
<td></td>
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<tr>
<td>GC apoptosis (P10)</td>
<td>10.9 ± 0.6</td>
<td>10.9 ± 0.9</td>
<td>p = NS</td>
<td></td>
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<tr>
<td>Blood vessel density (P12)</td>
<td>5.7 ± 0.8</td>
<td>4.8 ± 0.8</td>
<td>p = NS</td>
<td></td>
</tr>
<tr>
<td>P27+ GCs—lower EGL</td>
<td>26.2 ± 1.6</td>
<td>17.6 ± 2.0</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Pax6 density (P10)</td>
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<td>27.1 ± 1.3</td>
<td>p = NS</td>
<td></td>
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<tr>
<td>GCP proliferation—upper</td>
<td>12.0 ± 1.4</td>
<td>12.0 ± 1.0</td>
<td>p = NS</td>
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<tr>
<td>GCP proliferation (P10)</td>
<td>19.4 ± 0.5</td>
<td>19.3 ± 1.7</td>
<td>p = NS</td>
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<tr>
<td>GC proliferation (P10)</td>
<td>17.8 ± 2.5</td>
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<td>p = NS</td>
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<tr>
<td>GC apoptosis (P10)</td>
<td>10.6 ± 0.8</td>
<td>10.6 ± 0.7</td>
<td>p = NS</td>
<td></td>
</tr>
<tr>
<td>Blood vessel density (P12)</td>
<td>4.2 ± 0.6</td>
<td>4.1 ± 0.6</td>
<td>p = NS</td>
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comparable (supplemental Table 3, available at www.jneurosci.org as supplemental material) (data not shown). Also, staining for the endothelial marker CD31 revealed no angiogenic defects at P12 (Fig. 3K, L; Table 1). Additional vascular and metabolic studies showed no overt signs of ischemia (data not shown) or metabolic insufficiency in VEGF−/− cerebella (supplemental Fig. 3G, supplemental Table 4, available at www.jneurosci.org as supplemental material).
Analysis in older and adult VEGF
mice revealed that GCs eventually resumed migration (supplemental Table 3, available at www.jneurosci.org as supplemental material); the transient nature of this migratory defect is likely attributable to the moderate reduction of VEGF protein levels in the cerebellum of these hypomorph mice and to a possible compensation by other cues. A transient impairment of GC migration has been also reported in BDNF
mice (Schwartz et al., 1998; Borghesani et al., 2002).

Ectopic VEGF expression in the EGL perturbs granule cell migration
To further examine the chemotropic role of VEGF in GC migration, we performed VEGF gain-of-function experiments. In normal cerebellar development, VEGF is produced by PC bodies and dendrites, but not by GCs in the EGL. VEGF levels are also higher in the PCL/ML than in the EGL/ML (see above). If this spatial expression pattern is critical for GC migration, then ectopic VEGF expression in the EGL would be expected to perturb this process. We therefore overexpressed VEGF in GCs in the EGL by electroporating a VEGF expression plasmid in P10 cerebella [using conditions known to transflect primarily GCs in the EGL (Savill et al., 2005; Renaud et al., 2008)] and cultured slices of electroporated cerebella ex vivo for 4 d. These organotypic cultures, in which the cytoarchitecture of the developing cerebellum is preserved, are an established model to study GC migration (Komuro and Rakic, 1998; Soleciki et al., 2004; Savill et al., 2005). To identify electroporated GCs, a GFP expression plasmid was also electroporated. Double immunostaining for GFP and markers of various cerebellar cell types confirmed that >95% of GFP
GCs were GCs (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Another advantage of this model is that the role of VEGF in GC migration can be studied independently of its vascular effects, as vessels degenerate in these cultures (Fig. 4A–D) (Rosenstein et al., 1998; Moser et al., 2003). Electroporation of the VEGF expression plasmid upregulated VEGF mRNA levels by 250% in these slices (Fig. 4E).

To quantify the migration of GFP
GCs in electroporated slices, the cerebellar cortex was divided in five equally spaced areas, and the number of GFP
GCs that had reached each of these areas was counted in control cerebellar slices, in slices electroporated with the VEGF expression vector and a GFP expression vector, or in slices electroporated with a GFP expression vector and treated with a Flk1 antibody or the VEGF receptor inhibitor PTK. The observed number of GCs in each area in the treated slices was then statistically compared, using binomial nonparametric statistics, with the expected number of GCs, as detected in control slices. The difference between the observed versus expected number of cells in each area was expressed as a percentage of the expected number of cells. Data of PTK and a Flk1 treatment in area 5 could not be determined (ND) because of the low amount of cells present in this area.

![Image](image-url)

**Figure 4.** Ex vivo overexpression of VEGF in the EGL induces GC migration defects. A–D, Immunostaining of cerebellar slices for CD31, revealing progressive regression and disintegration of blood vessels at day 1 (A), day 2 (B), day 3 (C), or day 4 (D) of culture. E, Quantitative RT-PCR analysis, revealing a 250% increase of VEGF mRNA levels in cerebellar slices electroporated with a plasmid encoding VEGF compared with slices electroporated with the corresponding control plasmid (N = 5; *p < 0.05). Results are normalized to the expression levels in the control. Error bars indicate SEM. F, Scheme illustrating the methods used to quantify GC migration in the electroporated cerebellar slices. The cerebellar cortex from the EGL to IGL (a) was divided in five equally spaced areas, and the number of GFP
GCs that reached each of these areas and had migrated a specific distance from the EGL/ML border (b), was counted. G, H, Confocal microscopy of electroporated cerebellar slices after 4 d of culture, revealing that a substantial fraction of GFP
GCs had already migrated into the PCL/IGL in control slices (electroporated with empty vector, G); by contrast, in slices electroporated with a VEGF expression plasmid (which results in ectopic expression of VEGF in the EGL), a larger fraction of GFP
GCs stalled at the EGL/ML border (H). Scale bars: A–D, G, H, 100 μm.

<table>
<thead>
<tr>
<th>Area (Ectopic VEGF overexpression)</th>
<th>αFlk1</th>
<th>PTK</th>
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<tbody>
<tr>
<td>1 (+14.0%)**</td>
<td>+16.2%**</td>
<td>+30.0%**</td>
</tr>
<tr>
<td>2 (−4.0%)</td>
<td>+2.7%</td>
<td>−6.0%</td>
</tr>
<tr>
<td>3 (−18.0%)</td>
<td>−19.31%**</td>
<td>−48.0%**</td>
</tr>
<tr>
<td>4 (−22.0%)</td>
<td>−33.78%**</td>
<td>−66.0%**</td>
</tr>
<tr>
<td>5 (−26.0%)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Area 1–5 represent equally divided areas of the cerebellar cortex, from the EGL/ML border to the IGL (Fig. 4F), and the number of GFP
GCs that migrated into each of these areas was counted in control cerebellar slices, in slices electroporated with the VEGF expression vector and a GFP expression vector, or in slices electroporated with a GFP expression vector and treated with αFlk1 antibody or the VEGF receptor inhibitor PTK. The observed number of GCs in each area in the treated slices was then statistically compared, using binomial nonparametric statistics, with the expected number of GCs, as detected in control slices. The difference between the observed versus expected number of cells in each area was expressed as a percentage of the expected number of cells. Data of PTK and a Flk1 treatment in area 5 could not be determined (ND) because of the low amount of cells present in this area.

*p ≤ 0.001 versus corresponding controls.
Movies 2, 3, available at www.jneurosci.org as supplemental material. In some cases, the radial leading process of GCs even turned back toward the EGL shortly after initiation of radial migration (supplemental Movie 2, available at www.jneurosci.org as supplemental material), a process that was never observed in control slices (supplemental Movie 1, available at www.jneurosci.org as supplemental material). Analysis of GC proliferation and cell death revealed no differences in VEGF-overexpressing slices when compared with control slices (data not shown). Although technical reasons do not allow us to formally exclude that ectopic expression of VEGF in GCs might influence GC differentiation, our results suggest that ectopic VEGF expression in the EGL perturbs GC migration.

To start exploring the underlying mechanisms of VEGF’s chemotactic activity, we addressed two questions: first, are matrix-binding VEGF isoforms involved, and second, is Flk1 directly mediating VEGF signaling?

A matrix-binding VEGF isoforms gradient is present in the developing cerebellum

Alternative splicing generates various VEGF isoforms, which differ in molecular weight, receptor binding specificity, and affinity for heparin-rich extracellular matrix proteins (Ferrara et al., 2003). The short VEGF$_{120}$ isoform is freely diffusible, whereas the long VEGF$_{188}$ isoform sticks to heparin-rich matrix, and the VEGF$_{164}$ isoform exhibits an intermediate profile (Ferrara et al., 2003). All three isoforms bind to Flk1. Previous studies using knock-in mice, selectively expressing a single VEGF isoform, showed that a pericellular VEGF gradient of these isoforms is critical for normal vessel branching morphogenesis (Stalmans et al., 2002, 2003; Ruiz de Almodovar et al., 2009). In contrast to VEGF$_{120}$, VEGF$_{188}$ promotes endothelial navigation along matrix scaffolds (Stalmans et al., 2002, 2003). We therefore explored whether a similar VEGF isoforms gradient also exists in the cerebellum.

To address this question, we first determined the expression of the VEGF isoforms in cerebella of P12 WT mice. Quantitative RT-PCR analysis revealed that VEGF$_{164}$ was more abundant than the other isoforms (copies VEGF isoform mRNA per 10$^6$ copies $\beta$-actin mRNA: 28 $\pm$ 2, 143 $\pm$ 10, and 7.0 $\pm$ 0.3 for VEGF$_{120}$ VEGF$_{164}$ and VEGF$_{188}$ respectively; $N = 5$) (supplemental Table 1, available at www.jneurosci.org as supplemental material). As none of the available anti-VEGF antibodies discriminates between the distinct isoforms, we used VEGF$_{120/120}$, VEGF$_{164/164}$, and VEGF$_{188/188}$ knock-in mice (which express selectively the indicated isoform) and a pan-anti-VEGF antibody that recognizes all isoforms to visualize the spatial expression pattern of the soluble and matrix-bound VEGF isoforms. Immunostaining revealed that the pattern of VEGF expression in VEGF$_{164/164}$ mice was similar as in WT mice (data not shown), whereas PC bodies and individual dendritic branches in VEGF$_{188/188}$ mice were strongly labeled, even up to their very extremities (Fig. 5 B, D). In contrast, in VEGF$_{120/120}$ mice (see further), only PC bodies but not dendrites were labeled, and some diffuse labeling distant from the PC bodies was observed (supplemental Fig. 5B, available at www.jneurosci.org as supplemental material). This staining pattern suggested higher pericellular VEGF levels (and likely also a steeper pericellular VEGF gradient) around PC dendrites and individual dendritic branches in VEGF$_{188/188}$ mice, compared with a soluble VEGF isoform, a matrix-bound VEGF isoform is expected to create a steeper radial VEGF gradient from the deeper to upper cerebellar layers, as unrestricted diffusion of VEGF would ultimately abrogate such a gradient. We therefore...
used these strains to evaluate the importance of pericellular matrix-bound VEGF deposits in GC migration.

Matrix-binding VEGF isoforms are required for normal GC migration

We first analyzed GC migration in VEGF<sup>164/164</sup> mice using the above-mentioned BrdU-labeling protocol and found that GC migration was normal. Indeed, counting of BrdU<sup>+</sup> GCs in the IGL revealed no differences in GC migration (BrdU<sup>+</sup> cells in IGL, percentage of total BrdU<sup>+</sup> cells in EGL, ML, and IGL: 22.6 ± 0.5% in WT vs 20.9 ± 0.8% in VEGF<sup>164/164</sup>; N = 6–5; p = NS) (supplemental Table 5, available at www.jneurosci.org as supplemental material). The microvascular density in the cerebellum of these mice was also normal (CD31<sup>+</sup> area, expressed as percentage of total cerebellar area: 4.2 ± 0.6% in WT vs 4.1 ± 0.8% in VEGF<sup>164/164</sup>; N = 5; p = NS) (supplemental Table 5, available at www.jneurosci.org as supplemental material). The normal GC migration in VEGF<sup>164/164</sup> mice is not surprising, when considering that VEGF<sup>164</sup> has intermediate matrix-bound properties and is the most abundant isoform in WT mice.

We then analyzed GC migration at P12 in VEGF<sup>188/188</sup> knock-in mice, engineered to express only the matrix-binding short-range acting VEGF<sup>188</sup> isoform. GC migration was enhanced in these mice, as 35% more BrdU<sup>+</sup> GCs reached their final position (BrdU<sup>+</sup> cells in IGL, percentage of total BrdU<sup>+</sup> cells in EGL, ML, and IGL: 22.6 ± 0.5% in WT vs 30.7 ± 1.5% in VEGF<sup>188/188</sup>; N = 6–5; p < 0.001) (Fig. 5C,D; supplemental Table 6, available at www.jneurosci.org as supplemental material). Also, fewer BrdU<sup>+</sup> GCs stalled in the p27<sup>+</sup> area (BrdU<sup>+</sup> cells in p27<sup>+</sup> area, percentage of total BrdU<sup>+</sup> cells in the EGL: 45.9 ± 0.6% in WT vs 39.6 ± 0.9% in VEGF<sup>188/188</sup>; N = 5–3; p < 0.01) (Fig. 5E,F; supplemental Table 6, available at www.jneurosci.org as supplemental material) and the EGL area was thinner (area of EGL, percentage of total area of the total cerebellar cortex in lobe IX: 6.9 ± 0.3% in WT vs 5.1 ± 0.5% in VEGF<sup>188/188</sup>; N = 5; p < 0.05) (supplemental Table 6, available at www.jneurosci.org as supplemental material). Consistent with an enhanced GC migration, there were fewer p27<sup>+</sup> cells in the EGL in VEGF<sup>188/188</sup> mice compared with WT mice (Table 1). Additional analysis showed that proliferating Ki67<sup>+</sup> GCs were only found in the upper EGL, whereas postmitotic p27<sup>+</sup> GCs were only found in the lower EGL, indicating that the upper and lower EGL are properly segregated in these mice (supplemental Fig. 2G,H, available at www.jneurosci.org as supplemental material). Also, there were no genotypic differences in the density of Pax6<sup>+</sup> cells (Table 1), or in the number of Ki67<sup>+</sup> cells in the EGL (Table 1), indicating normal formation of the upper EGL and proliferation of GCPs. Normal proliferation rate of GCPs in VEGF<sup>188/188</sup> mice was further confirmed by BrdU<sup>+</sup> labeling and phosphohistone 3 (PH<sup>3</sup>) immunostaining (supplemental Fig. 2C,D, available at www.jneurosci.org as supplemental material; Table 1). This phenotype was not attributable to changes in GC apoptosis (Table 1) or differentiation (supplemental Fig. 2G,H,K,L,O,P, available at www.jneurosci.org as supplemental material; Table 1), neither to defects in Bergmann glia fibers or PCs (supplemental Fig. 5C,D, available at www.jneurosci.org as supplemental material). VEGF<sup>188/188</sup> cerebella also had normal vascular densities (Fig. 5G,H; Table 1), consistent with previous findings (Stalmans et al., 2003); oxygenation and metabolic parameters were also normal (supplemental Fig. 3G, supplemental Table 4, available at www.jneurosci.org as supplemental material). Thus, a matrix-bound VEGF isoform, which increases pericellular VEGF levels (and therefore likely also steepens the pericellular and radial VEGF concentration gradient), facilitates/enhances GC migration.

To explore whether long-range acting VEGF isoforms are necessary for GC migration, we analyzed VEGF<sup>120/120</sup> knock-in mice, expressing only VEGF<sub>120</sub>. As most homozygous VEGF<sup>120/120</sup> mice die within a few days after birth (Carmeliet et al., 1999), we could use only a few VEGF<sup>120/120</sup> mice (of hundreds of litters), which survived long enough for the analysis of GC migration. Since not a single VEGF<sup>120/120</sup> mouse survived up to P12, we injected BrdU at P7 and counted BrdU<sup>+</sup> GCs at P9. This analysis revealed that 42% fewer postmitotic GCs reached the IGL in VEGF<sup>120/120</sup> mice (BrdU<sup>+</sup> cells in IGL, expressed as percentage of total BrdU<sup>+</sup> cells in EGL, ML, and IGL: 23.5 ± 1.3% in WT vs 11.5 ± 2.1% in VEGF<sup>120/120</sup>; N = 3; p = 0.008) (supplemental Fig. 5C,D, supplemental Table 7, available at www.jneurosci.org as supplemental material). Consistent with previous findings in other organs (Carmeliet et al., 1999; Mattot et al., 2002; Maes et al., 2004), fewer vessels developed in VEGF<sup>120/120</sup> cerebella (CD31<sup>+</sup> area, percentage of total area: 2.8 ± 0.4% in WT vs 1.3 ± 0.3% in VEGF<sup>120/120</sup>; N = 3; p < 0.05) (supplemental Fig. 5E,F, supplemental Table 7, available at www.jneurosci.org as supplemental material). Double immunolabeling for GFAP and calbindin revealed a normal number, radial organization, and location of Bergmann glia fibers and PCs (supplemental Fig. 3E,F, available at www.jneurosci.org as supplemental material). Thus, the presence of only a diffusible VEGF isoform, which results in lower pericellular VEGF (and, therefore, likely also in a more shallow pericellular and radial VEGF concentration gradient), impairs GC migration and indicates that matrix-bound VEGF isoforms are required for proper GC migration.

VEGF matrix-binding isoforms facilitate GC adhesion

We finally explored whether matrix-binding VEGF isoforms could facilitate the positioning of postmitotic GCs onto Bergmann glia fibers, along which they slide inwardly, by promoting their attachment. However, since recombinant VEGF<sub>188</sub> cannot be purified because of its matrix-binding properties, and GCs require a matrix substrate to adhere to culture dishes, we tested whether GCs would attach preferentially to cells, expressing the matrix-binding VEGF isoforms. HEK-293 cells, stably expressing a single VEGF isoform (HEK-VEGF<sub>188</sub>, HEK-VEGF<sub>164</sub>, HEK-VEGF<sub>120</sub>, or mock control (HEK-Mock), were plated onto uncoated dishes at sparse confluency. We then added a fixed number of purified GCs with the various types of HEK-293 cells overnight. Since purified GCs only survive if they adhere to these cellular substrates in this assay (they cannot adhere to the uncoated dish), we counted the number of adhered GCs per HEK-293 cell (the conditions were selected such that, on average, three GCs could adhere to one HEK-293 cell). This analysis revealed that more GCs attached to HEK-VEGF<sub>188</sub> or HEK-VEGF<sub>164</sub> cells than to HEK-VEGF<sub>120</sub> or HEK-Mock cells (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Thus, adhesion of postmitotic GCs is facilitated onto cells expressing matrix-binding VEGF isoforms.

Together, our results suggest that matrix-binding VEGF isoforms (VEGF<sub>188</sub> and VEGF<sub>164</sub>), expressed by PC dendrites, located in close vicinity to Bergmann glia fibers, and possibly by Bergmann glia, function as a local chemoattractant and/or adhensive cue for postmitotic GCs toward the glia fibers and thereby control their migration.
Inhibition of Flk1 impedes granule cell migration

As described above, Flk1 is expressed in GCs. We thus assessed whether Flk1 mediates the chemotropic effect of VEGF by supplementing αFlk1 or the VEGF receptor tyrosine kinase inhibitor PTK787 (PTK) (Thomas et al., 2005) to the aforementioned ex vivo model of electroporated cerebellar slices. Both αFlk1 and PTK impaired GFP + GC migration when electroporated slices were incubated in their presence during 4 d (Fig. 6A,B; Table 1). For instance, in PTK-treated slices, area 1 (close to the EGL/ML border) contained 30% more GCs, whereas 66% fewer GCs reached area 4 in the deeper layers of the IGL (Table 2). Notably, treatment of slices with αFlk1 for a longer period (8 d) caused a persisting GC migration defect (area 1 contained 27% more GCs, whereas 28% fewer GCs reached area 4). Thus, inhibition of Flk1 in cerebellar slices impaired GC migration.

To determine whether the impaired GC migration could be attributable to primary defects in GC tangential migration, we quantified the speed of GC tangential migration in cerebellar slices treated with αFlk1. We therefore electroporated GCs with a GFP expression vector, cultured them for 48 h in the presence or absence of αFlk1, and performed time-lapse analysis. Quantification of the tangential migration speed revealed no differences between control and αFlk1-treated slices (4.57 ± 0.17 μm/h in control vs 4.68 ± 0.20 μm/h in αFlk1-treated slices; p = NS; N = 246–242). Thus, inhibition of Flk1 does not impair tangential migration of GCs in cerebellar slices.

Impaired granule cell migration in GC-specific Flk1 knock-out mice

To underscore that VEGF regulates GC migration via a direct effect on postmitotic GCs, we generated mice with selective loss of Flk1 in these cells. We therefore used a Cre-driver line, expressing a tamoxifen-inducible form of Cre (CreER; abbreviated as CreER) under the control of the Math1 promoter (Math1CreER mice) (Chow et al., 2006). To analyze the expression pattern of CreER-mediated recombination did not occur in all GCs (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). Nonetheless, 28% fewer BrdU + GCs reached the IGL in Flk1CreER +/− mice than in Flk1CreER −/− mice (termed Flk1 CreER +/− (25.9 ± 1.3%) in Flk1CreER −/− mice (35.9 ± 1.0%) in Flk1CreER −/− mice; N = 9–5; p < 0.0001) (Fig. 6L; supplemental Table 8, available at www.jneurosci.org as supplemental material; BrdU injected at P8 and analyzed at P8). Thus, despite the mosaic activation of CreER, GC-specific inactivation of Flk1 impaired GC migration. Analysis of older (P21) Flk1CreER +/− mice revealed that GCs eventually reached the IGL, their numbers were too low to allow robust statistical analysis. Error bars indicate SEM; *p < 0.05. Scale bars: A, B, 100 μm; C–E, 200 μm; F–H, 50 μm, I, J, 100 μm.
age (supplemental Table 8, available at www.jneurosci.org as supplemental material). Moreover, cerebella size and foliation were also similar between these genotypes (supplemental Table 8, available at www.jneurosci.org as supplemental material) (data not shown).

In a final effort to circumvent the problem of the mosaic expression of Cre and to analyze selectively the migration of those GCs, in which CreER was active, we electroporated ex vivo the Brainbow1.0 plasmid in cerebella of [Math1CreER + × Flk1lox/lacZ] mice that had not received tamoxifen yet. The Brainbow1.0 plasmid is a construct in which the cDNA of red (RFP), yellow (YFP), and blue (M-CFP) fluorescent proteins are flanked by incompatible sets of lox sites, such that RFP is expressed in baseline control, we also electroporated Brainbow1.0 in cerebella of [Math1CreER + × Flk1lox/lacZ] cerebellar slices, electroporated with Brainbow1.0, CreER-activated GCs expressed YFP and could thus be distinguished from GCs that did not express CreER; these YFP+ GCs were assumed to have the flk1 floxed allele excised and termed YFP+/Flk1<sup>Cre</sup>; these YFP+GCs retained their RFP color; these RFP+/Flk1<sup>Cre</sup> GCs were termed RFP+/Flk1<sup>Cre</sup>GC<sub>wt</sub>/LacZ and served as controls. As another control, we also electroporated Brainbow1.0 in cerebella of Flk<sup>1</sup><sup>lox+</sup> mice without observable vessel or oxygenation defects of the VEGF isoforms, highlights that matrix-binding VEGF isoforms promote GC migration. Indeed, replacement of soluble VEGF isoforms with the matrix-bound VEGF<sub>164</sub> isoform in VEGF<sup>188/188</sup> mice facilitated/enhanced migration of GCs, whereas the opposite phenotype was found when mice expressed solely a soluble VEGF<sub>120</sub> isoform in VEGF<sup>120/120</sup> mice. Such a role for the matrix-binding VEGF<sub>188</sub> isoform in neuronal guidance has not been documented yet. Another study reported that the guidance effects of the VEGF<sub>164</sub> isoform for the somata of motoneurons relied on its selective binding to Npn-1, but in this study, a spatial VEGF isoform concentration gradient was not invoked as a possible underlying mechanism (Schwarz et al., 2004). In addition, navigation of axon growth cones of these motoneurons occurred independently of VEGF (Schwarz et al., 2004). Furthermore, a recent study challenged that only the VEGF<sub>164</sub> isoform is capable of binding to Npn-1 and documented similar binding capacity for VEGF<sub>120</sub> (Pan et al., 2007). Together, the precise mechanisms of how VEGF controls neuron migration are still ill defined. Since GCs do not express Npn-1 in vivo (Solowska et al., 2002; this study), the VEGF isoforms must regulate GC migration via a distinct mechanism in the cerebellum. Also, contrary to the effects of VEGF on motoneuron migration, GCs and their growth cones were chemoattracted by VEGF in vitro and ex vivo, implying distinct mechanisms of GC migration. We therefore favor the interpretation that the VEGF isoforms are important for establishing a spatial VEGF concentration gradient that promotes GC chemoattraction.

Immunostaining for VEGF showed that the PC soma and dendrites were more strongly labeled and better identifiable as sharply demarcated extensions in VEGF<sup>188/188</sup> mice, whereas these structures were more vague and diffusely marked in VEGF<sup>120/120</sup> mice. These findings suggest that the matrix-bound VEGF<sub>188</sub> isoform is associated more closely around the cell surface of PCs, whereas the soluble VEGF<sub>120</sub> isoform diffuses further away from its producer cell. It is tempting to speculate that such a different type of pericellular VEGF isoform concentration gradient may help to explain the observed phenotypes. Indeed, not only the pericellular VEGF isoform concentration gradient locally around the PC dendrites and Bergman glia fibers, but also the VEGF concentration gradient in the radial direction from the deeper to upper cerebellar layers would be expected to be steeper in VEGF<sup>188/188</sup> mice and to be more shallow in VEGF<sup>120/120</sup> mice. Indeed, matrix-associated isoforms are instrumental in establishing and maintaining a spatial concentration gradient, whereas freely diffusible isoforms would spread out in a more scattered pattern and dilute out the gradient. Heparan sulfate proteoglycans (HSPGs) in the EGL (Rubin et al., 2003), can provide an additional level of regulation and NMDA-type glutamate receptor (NMDAR)-mediated Ca<sup>2+</sup> influx and currents via activation of Flk1, and that this effect can be blocked by αFlk1 or a VEGF receptor tyrosine kinase inhibitor.

Second, GC-specific deletion of Flk1 in vivo or pharmacological inhibition of Flk1 (signaling) ex vivo delayed GC migration, thereby providing additional supportive evidence that Flk1 on postmitotic GCs mediates the chemoattractive effects of VEGF. Third, GC migration defects were unlikely attributable to changes in cerebellar vascularization, as GC migration was impaired in VEGF<sup>188/188</sup> mice without observable vessel or oxygenation defects; also, postmitotic GCs migrated normally in cerebellar slices, even when blood vessels were not perfused and regressed.

The analysis of knock-in mice, expressing selectively a single VEGF isoform, highlights that matrix-binding VEGF isoforms promote GC migration. Indeed, replacement of soluble VEGF isoforms with the matrix-bound VEGF<sub>164</sub> isoform in VEGF<sup>188/188</sup> mice facilitated/enhanced migration of GCs, whereas the opposite phenotype was found when mice expressed solely a soluble VEGF<sub>120</sub> isoform in VEGF<sup>120/120</sup> mice. Such a role for the matrix-binding VEGF<sub>188</sub> isoform in neuronal guidance has not been documented yet. Another study reported that the guidance effects of the VEGF<sub>164</sub> isoform for the somata of motoneurons relied on its selective binding to Npn-1, but in this study, a spatial VEGF isoform concentration gradient was not invoked as a possible underlying mechanism (Schwarz et al., 2004). In addition, navigation of axon growth cones of these motoneurons occurred independently of VEGF (Schwarz et al., 2004). Furthermore, a recent study challenged that only the VEGF<sub>164</sub> isoform is capable of binding to Npn-1 and documented similar binding capacity for VEGF<sub>120</sub> (Pan et al., 2007). Together, the precise mechanisms of how VEGF controls neuron migration are still ill defined. Since GCs do not express Npn-1 in vivo (Solowska et al., 2002; this study), the VEGF isoforms must regulate GC migration via a distinct mechanism in the cerebellum. Also, contrary to the effects of VEGF on motoneuron migration, GCs and their growth cones were chemoattracted by VEGF in vitro and ex vivo, implying distinct mechanisms of GC migration. We therefore favor the interpretation that the VEGF isoforms are important for establishing a spatial VEGF concentration gradient that promotes GC chemoattraction.

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further fine-tune GC migration, as HSPG-bound VEGF induces distinct Flk1 signaling responses than unbound VEGF (Jakobs-
son et al., 2006; Chen et al., 2010). Whether HSPGs are present in spatiotemporal patterns/gradients in the cerebellar layers, which can favor GC migration, remains to be explored.

The differences in GC migration in the VEGF isoform-specific mice can be explained by several complementary, nonexclusive mechanisms via which matrix-binding VEGF isoforms modulate this process. First, VEGF might bind to Flk1 on postmitotic GCs in the EGL and modulate their tangential migration. Indeed, VEGF-producing PC dendrites are positioned in the ML all along the EGL/ML border, Flk1 is expressed in postmitotic GCs in the lower layer of the EGL, and postmitotic GC growth cones turn toward a VEGF source in vitro and in slices ex vivo (our unpublished data). However, pharmacological inhibition of Flk1 did not affect tangential migration speed of GCs in situ. Thus, even though tangential migration of GCs is responsive to VEGF in experimental conditions, VEGF is present in a radial—not tangential—gradient in the developing cerebellum, explaining why radial migration is more likely regulated by VEGF.

Second, given that postmitotic GCs migrate along Bergmann glia fibers and PC dendrites lie in close contact with these fibers (Stottmann and Rivas, 1998; Yamada et al., 2000; Lordkipanidze and Dunaevsky, 2005), pericellular VEGF around PC dendrites and Bergmann glia fibers could chemottract Flk1-expressing postmitotic GCs and facilitate their positioning relative to the glia fibers. A steeper VEGF concentration gradient around these structures would be expected to increase inward GC migration by promoting the positioning and/or alignment of the radial leading process of GCs along these fibers. In line with this hypothesis, more GCs adhered to HEK-293 cells expressing matrix binding VEGF isoforms than the VEGF120 isoform or control. Although an effect of VEGF on adhesion has been amply characterized for endothelial cells (Byzova et al., 2000; Hutchings et al., 2003; Vlahakis et al., 2007), such an activity of VEGF on neurons has not been documented yet. Finally, VEGF might also promote chemotraction of postmitotic GCs from the EGL toward the deeper cerebellar layers, since VEGF is expressed in a radial gradient, and ectopic VEGF expression in the EGL chemotactically radial leading processes and GC bodies back to the upper layers. When this radial VEGF gradient is steeper, it would also be expected to promote chemotraction of postmitotic GCs from the EGL toward the deeper cerebellar layers. Additional support for the latter model will have to await confirmation of the presence of a radial concentration gradient of extracellular VEGF, which cannot be readily resolved using available technology to date.

In VEGFknockout mice, GC migration was delayed but resumed at later time points, suggesting that other signals compensated for the reduced VEGF levels. It is nonetheless noteworthy that, unlike other signals (such as BDNF, SemalA, Barh1, PlexinB2, Jagged1, etc.) whose expression must be near completely elimi-
nated in vivo to impair GC migration (Borghesani et al., 2002; Li et al., 2004; Kerjan et al., 2005; Weller et al., 2006; Friedel et al., 2007), even a relatively small decrease in VEGF levels sufficed to perturb this process. Another consideration is that the spatial VEGF expression pattern leads us to conclude that migration of postmitotic GCs beyond the PCL into the IGL must involve other cues than VEGF. Furthermore, in addition to the aforementioned role of VEGF in GC migration, this molecule might exert additional activities in the cerebellum, which we did not characterize in this study. For instance, VEGF might have a neurotrophic activity or stimulate axon outgrowth, as reported for neurons in the peripheral nervous system (Sondell et al., 1999, 2000). However, no differences in GC survival were observed in VEGFknockout hypomorph mice. In a separate study, we document how VEGF cross talks with NMDARs to enhance calcium influx in GCs via enhancing NR2B phosphorylation in a Src family kinase-dependent manner (our unpublished data), a molecular mecha-
nism that may explain in part how VEGF modulates GC migration during cerebellar development.

In conclusion, although it is becoming increasingly evident that vessels coopted axon guidance signals to navigate (Carme-
liet, 2003; Carmeliet and Tessier-Lavigne, 2005), there is still little evidence that molecules involved in angiogenesis also guide mi-
grating neurons. Our findings shed new light on the emerging importance of the neurovascular link and may stimulate future interest to study the involvement of VEGF in additional guidance events in the nervous system.

References


