Direct Stimulation of Adult Neural Stem Cells In Vitro and Neurogenesis In Vivo by Vascular Endothelial Growth Factor

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Hypoxia as well as global and focal ischemia are strong activators of neurogenesis in the adult mammalian central nervous system. Here we show that the hypoxia-inducible vascular endothelial growth factor (VEGF) and its receptor VEGFR-2/Flk-1 are expressed in clonally-derived adult rat neural stem cells in vitro. VEGF stimulated the expansion of neural stem cells whereas blockade of VEGFR-2/Flk-1-kinase activity reduced neural stem cell expansion. VEGF was also infused into the lateral ventricle to study changes in neurogenesis in the ventricle wall, olfactory bulb and hippocampus. Using a low dose (2.4 ng/d) to avoid endothelial proliferation and changes in vascular permeability, VEGF stimulated adult neurogenesis in vivo. After VEGF infusion, we observed reduced apoptosis but unaltered proliferation suggesting a survival promoting effect of VEGF in neural progenitor cells. Strong expression of VEGFR-2/Flk-1 was detected in the ventricle wall adjacent to the choroid plexus, a site of significant VEGF production, which suggests a paracrine function of endogenous VEGF on neural stem cells in vivo. We propose that VEGF acts as a trophic factor for neural stem cells in vitro and for sustained neurogenesis in the adult nervous system. These findings may have implications for the pathogenesis and therapy of neurodegenerative diseases.

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INTRODUCTION

Two regions of the adult mammalian brain continue to produce new neurons into old age, the lateral ventricle wall/olfactory bulb system and the hippocampus (32), where they are integrated into the normal brain circuitry (9, 68). It is also from these regions that adult neural stem cells were first isolated and propagated in vitro using protocols requiring FGF-2 and/or EGF as mitogens (56, 47).

In vivo neurogenesis is regulated by a variety of molecules such as hormones, growth factors and neurotransmitters, but also by pathological stimuli, such as global and focal cerebral ischemia (for review, see 34). It is speculated that hypoxia, the final condition of several neurological diseases, is a critical stimulus for neurogenesis, since reduced oxygen levels can promote survival, proliferation and differentiation of neural stem cells in vitro (65). However, the underlying mechanisms of neurogenesis and its up-regulation by hypoxia are not fully understood.

Vascular endothelial growth factor (VEGF), a key regulator of developmental, hypoxia-induced, and tumor-induced angiogenesis, is rapidly up-regulated in response to hypoxia (8, 39, 51, 62). Apart from its well-characterized effects on the vascular system, recent studies suggest that VEGF might act directly on neuronal cells. In the adult brain, VEGF is expressed in neurons and glial cells, and under normal conditions, the highest mRNA levels were found in the olfactory bulb and dentate gyrus (39), the 2 regions of adult neurogenesis. Under hypoxic conditions, neurons and glial cells throughout the brain express elevated levels of VEGF, while expression of its receptor VEGFR-2/Flk-1 (fetal liver kinase-1) is increased on neuronal cells (39, 45, 52) and endothelial cells (28). VEGF is capable of protecting cells isolated from the central and peripheral nervous system from apoptosis (3, 24, 25, 58). These data suggest a role for VEGF as a trophic factor for mature neurons. However, VEGFR-2/Flk-1 expression has been described on progenitor cells from the fetal retina and cortex (24, 72) as well as on hematopoietic stem cells (40), suggesting that VEGF could be involved in the regulation of neural stem cells as well.

For this reason, we propose that VEGF signaling may play a role in the generation of new neurons from neural stem cells in the adult brain. To test this hypothesis, we: *i)* analyzed expression of VEGF and its receptor VEGFR-2/Flk-1 in neural stem cell cultures from the adult rat brain, *ii)* determined effects of recombinant $VEGF_{165}$, the most prominent isoform of VEGF, and VEGFR-2/Flk-1 inhibitors on clonally-derived adult neural stem cells in vitro, *iii)* quantified in vivo neurogenesis after infusion of $VEGF_{165}$ into the lateral ventricle of adult rats, and *iv)* analyzed expression of VEGFR-2/Flk-1 in the lateral ventricle wall of adult rats and mice.

MATERIALS AND METHODS

Adult neural stem cell cultures. Adult female Fischer-344 rats (3-4 months; Charles River, Germany) were killed, the brains of each rat were removed and immersed in DPBS (PAN, Germany) with 4.5 g/L glucose (Merck, Germany) (DPBS/glu) at 4°C. The lateral wall of the lateral ventricle was dissected, mechanically dissociated, washed twice with DPBS/glu, resuspended in PPD-solution (0.01% Papain, Worthington Biochemicals), 0.1% dispase II (Roche), 0.01% DNase I (Worthington Biochemicals) and 12.4 mM $MgSO_4$ in HBSS (PAN, Germany) without Mg^{++}/

Ca^{++} (PAA, Germany) and digested for 30 to 40 minutes at 37°C with resuspension every 10 minutes. Dissociated cells were washed 3 times and resuspended in Neurobasal medium (Gibco BRL) supplemented with B27 (Gibco BRL), 2 mM L-glutamine (PAN), 0.1 g/L penicillin/streptomycin (PAN), 2 µg/ml heparin (Sigma), 20 ng/ml bFGF-2 and 20 ng/ml EGF (both R&D Systems). Cells were seeded in T-25 culture flasks and incubated at 37°C in a humidified atmosphere with 5% CO_2. Single cells began to form spheres within 5 to 7 days of suspension culture. The primary culture was passaged by treatment with Accutase (Innovative Cell Technologies Inc., United States) for 10 minutes at 37°C. Viable cells were counted by trypan blue exclusion assay. 10^4 cells/ml NB/B27 medium were plated in T75 culture flasks. Similarly, cells were further passaged every 5 to 7 days until used for analysis.

For subcloning, neurospheres from established cultures were dissociated by Accutase and the resulting single cell suspension was used for clonal analysis. Single cells were transferred to 96-well plates by plating at a density of 0.5 cells/well in 200 µl of growth medium. Medium was changed after 2 weeks and then on a weekly basis.

In vitro proliferation assay. 10^4 cells were grown in T25 flasks for 7 days and treated with $VEGF_{165}$ or VEGFR2/Flk-1 tyrosine kinase inhibitors SU1498 and PADQ (both Calbiochem). The inhibitors were used at their respective ED50 value (SU1498 at 700 nM or PADQ at 100 nM). Cells were labelled with BrdU (Sigma, 5 µM) and DNA was isolated using the DNeasy Tissue Kit (Qiagen) according to manufacturer's protocol. The genomic DNA was denaturated and degraded by treatment with 0.25 M NaOH for 30 minutes, 0.25 HCL and 0.2 M K_2HPO_4/KH_2PO_4 pH 7. The DNA (200 ng/ml) were added to DNA-binding ELISA plates (Costar) that had been precoated with 50 mM Na_2PO_4, pH 8.5, 1 mM EDTA. After an overnight incubation at 4°C, plates were washed 3 times with PBS and incubated with 3% BSA/PBS for 30 minutes. After 3 washing steps with PBS, plates were incubated with mouse anti-BrdU Antibody (Roche, 1:500) for one hour at room temperature. Following 3 washes in PBS, the secondary peroxidase-conjugated donkey anti-mouse antibody (Jackson Immuno Reearch, 1:1000) was added to the wells and incubated for one hour. After 3 washes in PBS, plates were incubated with o-Phenylenediamine (OPD) dihydrochloride (Sigma). The colour readout of the assay was allowed to develop for 60 minutes. Measurements were performed at 450 nm with a microplate reader (Molecular Devices).

In vitro expansion and apoptosis assays. 10^4 cells per well were seeded in 12-well plates in a volume of one ml of medium and stimulated with mouse recombinant $VEGF_{165}$ or VEGF receptor tyrosine kinase inhibitors (SU1498 at 700 nM or PADQ at 100 nM). After 7 days, cultures were dissociated by Accutase and viable cells were counted by trypan blue exclusion. Apoptosis was detected by measuring cytoplasmic histone-associated DNA fragments using a photometric enzyme immunoassay according to the protocol of the manufacturer (Cell Death Detection ELISA, Roche Diagnostics).

Immunocytochemistry. Neural stem cell cultures were grown on cover slips coated with poly-ornithine and laminin for 4 days, fixed with 4% paraformaldehyde pH 7.4 and processed for immunofluorescence. For intracellular antigens the cells were permeabilized with 1% Triton X-100 for 15 minutes. Cells were pre-incubated for a minimum of 30 minutes at room temperature in TBS (0.1M Tris-HCl pH 7.5, 0.15M NaCl) containing 3% donkey serum followed by overnight-incubation at 4°C with the primary antibodies and 2 hour incubation with secondary antibodies. In the case of anti-Flk-1 staining, the specificity was determined by pre-incubation of the antibody with the antigen (Flk-1 peptide, Calbiochem) at various concentrations (10 ng-100 µg/ml) prior to applying to the cells.

RT-PCR analysis. Total RNA from stem cells was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Two µg of total RNA was reverse transcribed using Retroscript™ RT-PCR Kit (Ambion Inc.) according to the instruction manual. PCR was performed with *Taq* PCR Master Mix (Qiagen) in an Eppendorf Master Cycler Gradient using one step 94°C, 2 minutes, 38 cycles (94°C, 45 seconds; 62°C, one minute; 72°C, 2 minutes) and one extension step (72°C, 8 minutes). Forward and reverse primers used were (5′-3′): Flk-1-for: GCCAA TGAAG GGGAA CTGAA GAC; Flk-1-rev: TCTGA CTGCT GGTGA TGCTG TC; VEGF-for: TGCAC CCACG ACAGA AGGGG A; VEGF-rev: TCACC GCCTT GGCTT GTCAC A; GAPDH-for: GGTCG GTGTG AACGG ATTTG and GAPDH-rev: GTGAG CCCCA GCCTT CTCCA T; β-Actin-for: GTGCC TTGAT AGTTC GCCA TGA; β-Actin-rev: GGTCA CCCAG GATAC TGACC TGG.

Animals and surgery. All animal experiments were approved by the local government and were conducted according to European Union guidelines and German law. Two-month-old male Fischer-344 rats (n = 36) received intraventricular infusions via osmotic minipumps according to a previously established protocol (33). Surgical implantation of the minipumps was performed under deep anesthesia using intramuscular injections consisting of 62.5 mg/kg ketamine, 3.175 mg/kg xylazine, and 0.625 mg/kg of acepromazine maleate dissolved in 0.9% sterile saline. The animals received either recombinant mouse $VEGF\text{-}A_{165}$ (2.4 ng per day) or aCSF (artificial cerebrospinal fluid) at a flow rate of one µl per hour for 7 days. During the last 6 days of the pump period, animals received daily intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg, Sigma). After 7 days of infusion, 8 animals per group were transcardially perfused with 4% paraformaldehyde. The remaining animals (n = 10 per group) had the pumps removed and were perfused after an additional 4-week period without growth factor or vehicle infusion in order to analyze the amount of new cells that have differentiated into neurons. Brains were analyzed for cannula tract localization and animals with incorrect cannula placement were excluded from the analysis (n = 1-2 animals per group). Additional rats were used to provide fresh frozen brain tissue for localization of VEGF and VEGF receptors by situ hybridization and immunohistochemistry or to test for vascular permeability after $VEGF_{165}$ or aCSF-infusion (see below).

Histology. Immunohistochemistry, in situ hybridization and TUNEL were per-

formed as previously described in detail (4, 13, 33, 71), but a brief description is given below. The following primary antibodies were used: Mouse anti-betaIII-tubulin 1: 500 (Promega), rat anti BrdU (Harlan-Seralab), mouse anti-GFAP (Chemicon), rabbit anti-GFAP (Dako), mouse anti-Nestin (Pharmingen), mouse anti-NeuN (Chemicon), mouse anti-Neuropilin-1 (gift from H. Fushisawa, Nagoya, Japan), rabbit anti-VEGF (Santa Cruz, Calif), rat anti-VEGFR-2/Flk-1 (gift from H. Kataoka, Department of Molecular Genetics, Kyoto University, Japan), rabbit anti VEGFR-2/Fk-1 (clone c20, Santa Cruz, Calif), rabbit anti-VEGFR-1/Flt-1 (Santa Cruz, Calif), rabbit anti-S100β (Swant). For detection the following secondary antibodies were applied: anti-rabbit Alexa 488, anti-mouse Alexa 488 (Molecular Probes), anti-rat FITC, anti-mouse rhodamineX and anti-rabbit-CY5 (JacksonImmuno).

BrdU detection. Free floating sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS: 0.15M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 minutes. For detection of BrdU-labeled nuclei the following *DNA denaturation* steps preceded the incubation with α-BrdU antibody: 2 hours incubation in 50% formamide/2xSSC (2xSSC: 0.3 M NaCl, 0.03 M sodium citrate) at 65°C, 5-minute rinse in 2xSSC, 30-minute incubation in 2M HCl at 37°C, and 10-minute rinse in 0.1M boric acid, pH 8.5. Thereafter, incubation in TBS/0.25% Triton-X100/3% normal donkey serum for 30 minutes was followed by incubation with primary mouse α-BrdU antibody in TBS/donkey serum overnight at 4°C. Sections were incubated for one hour with biotinylated secondary antibodies directed against mouse. Following intermittent rinses in TBS, avidin-biotin-peroxidase complex was applied for one hour, then peroxidase detection for 10 minutes was performed (25 mg/ml diaminobenzidin [DAB], 0.01% H_2O_2 , 0.04% NiCl).

Immunofluorescence. In case of BrdU detection, free-floating sections were treated for DNA denaturation as described above. Sections were washed in TBS and incubated with primary antibodies in TBS-donkey serum for 48 hours at 4°C. Donkey α-rat-FITC, donkey α-mouse rhodamine X, and donkey α-rabbit-Cy5 were used as secondary antibodies (JacksonImmuno, all 1:500) for 2 hours. After mounting on slides, fluorescence was detected using a confocal scanning laser microscope (Leica TCS-NT, Bensheim, Germany).

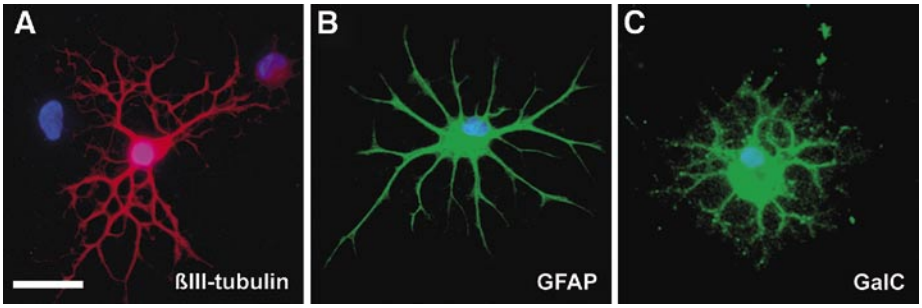

Figure 1. Individual clones derived from neurosphere cultures of the adult rat subventricular zone were tested for multipotency. Cells were differentiated on a poly-ornithin/laminin matrix in Neurobasal medium supplemented with B27 and 1% FCS for 7 days and immunostained for the presence of neurons with βIII-tubulin (**A**), astrocytes with GFAP (**B**) or oligodendrocytes with GalC (**C**). Subcloned neural stem cell lines that were able to produce neuronal as well as glial cells were used for further analysis. Scale bar = 25 µm.

TUNEL assay. The TUNEL assay was performed using the Apoptag In Situ Cell Death Detection Kit (Intergene, Purchase, NY) and a modified procedure for free floating sections. In brief, after rinsing sections in TBS for 10 minutes, an ascending isopropanol series (H_20, 70%, 90%; 2 minutes each) was followed by incubation in 100% isopropanol for 10 minutes and a descending isopropanol series (90%, 70%, H20; 2 minutes each). After 3 rinses in PBS, sections were incubated with *equilibration buffer* for one to 5 minutes at room temperature followed by TdT-Reaction solution for one hour at 37°C and a *stop buffer* for 10 minutes at room temperature (see TUNEL-Kit for solutions). To reduce background labeling the TdT-Reaction solution was diluted 1:1 with TUNEL Dilution Buffer (Roche Diagnostics). After rinsing in TBS and blocking in 3% donkey serum/0.1% Triton-X100/TBS (TBS++) for 2 hours the digoxigenin-labeled DNA was detected using an anti-digoxigenin-FITC antibody over night at 5°C in TBS++. After several rinses in TBS, a brief fixation in 4% paraformaldehyde (15 minutes) and rinses in phosphate buffer, the sections were mounted and dried on glass slides. Following incubation with propidium iodide (100 ng/ml) for 10 minutes and rinses in TBS, the sections were coverslipped using an antifade mounting medium.

VEGFR-2:lacZ mice. To study VEGFR-2/Flk-1 expression independent from

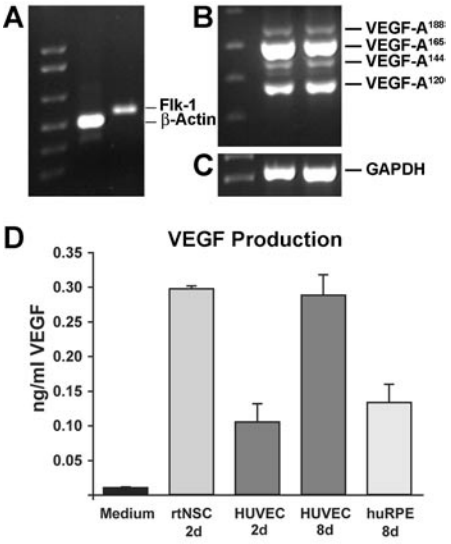

Figure 2. *Expression of VEGFR-2/Flk-1 and VEGF in neural stem cell cultures.* Cells were seeded at 10 000 cells/ml, grown for 7 days in culture. **A**. Using RT-PCR, Flk-1 was detectable in neurosphere cultures from adult rat ventricle wall (passage 4, β-actin as control). **B**. All major isoforms of VEGF-A were expressed in neural stem cells in non-passaged cells (lane 2) and at passage 4 (lane 3). Lane 1 in **A** and **B**, molecular weight marker. **C.** RT-PCR of the housekeeping gene GAPDH was used to control for mRNA content in (B). **D.** VEGF secretion into the defined culture medium as measured by ELISA. rtNSC—rat neural stem cells, HUVEC—human umbilical vascular endothelial cells, huRPE—human retinal pigment epithelial cells; 2d—2 days of medium conditioning, 8d—8 days of medium conditioning.

immunohistochemistry we made use of reporter mice, which express β-galactosidase under the endogenous VEGFR-2/Flk-1 promoter. These mice were generated by inserting the lacZ gene into the VEGFR-2 locus, by which one VEGFR-2 allele was disrupted (60). Hetereozygote knock-in animals were described to be without phenotype and β-galactosidase staining closely resembled the Flk-1 expression

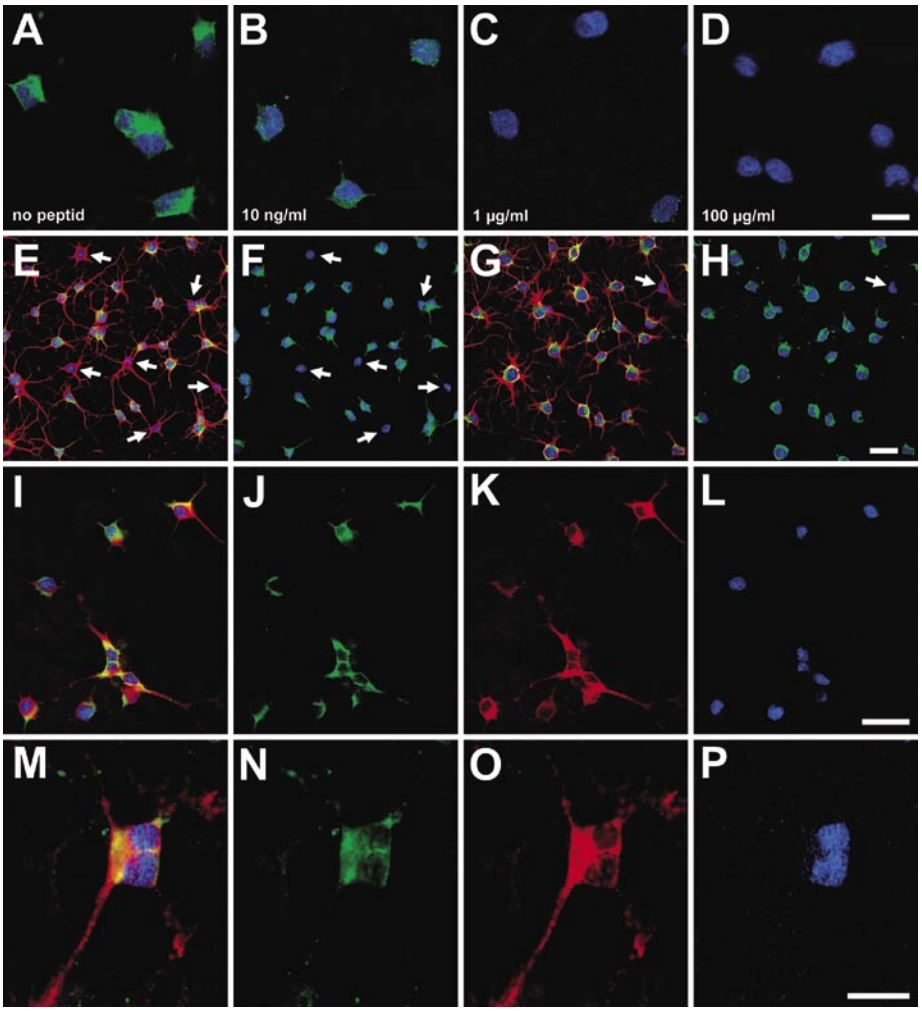

Figure 3. *VEGFR-2/Flk-1 immunofluorescence in vitro.* **A-D.** Specificity of Flk-1 immunofluorescence is controlled by pre-incubation of anti-Flk-1 antibody with Flk-1 peptide. **A.** No pre-incubation. **B-D.** Pre-incubation with 10 ng/ml, one µg/ml and 100 µg/ml peptide, respectively. **E-H.** Distribution of Flk-1 immunoreactivity in neural stem cell cultures. Co-labeling of Flk-1 (green) and the ubiquitously expressed alpha-tubulin (red) revealed that the majority of cells are immunoreactive for Flk-1. **E, G.** Triple labeling of Flk-1 in green, alpha-tubulin in red and nuclear counterstain ToPro-3 in blue; **F, H.** same pictures as in (**E, G**), but without red signal (alpha-tubulin). In primary cultures (passage 4) about 20% of the cells were negative for Flk-1 (arrows in **E** and **F**); however after subcloning, the number of Flk-1 negative cells decreased to less than 5% (arrows in **G** and **H**). Scale bar, 25 µm. **I-P**. Clonally-derived neural stem cells co-express VEGFR-2/Flk-1 and nestin. **I, M.** Triple labeling; **J, N.** Flk-1 (green), **K, O.** Nestin (red), Nuclear counterstaining ToPro-3 (blue) . Scale bars: **D, P** = 10 µm; **H, L** = 25 µm.

pattern (60). For the current experiment adult heterozygote mice were anesthetized, transcardially perfused and processed for β-galactosidase staining as previously described (60).

Vascular permeability. To test whether $VEGF_{165}$ infusion altered the permeability of the blood-brain-barrier, we anesthetized animals and infused Evan's Blue dye (2% in 0.9% NaCl) intravenously (5 ml/kg body weight) on the last day of intraventricular growth factor infusion. Animals were kept under deep anesthesia for 60 minutes and were perfused transcardially with 0.9% NaCl for 15 minutes. Brains were removed and analyzed for Evan's Blue dye content in the brain parenchyma as an indicator for vascular leakage.

Stereological quantification. A systematic, random counting procedure (70) was used as previously described (13, 71). Only brains with correct cannula placement into the lateral ventricle were included in the analysis. Series of every sixth section (240-µm interval) of both hemispheres from each brain were analyzed. The volume of each structure was determined by tracing the areas using a semi-automatic stereology system (Microbrightfield). For the lateral ventricle wall, counting frame and sampling grid sizes of 20 × 20 µm and 50 × 100 µm and for the olfactory bulb 60 × 60 µm and 300 × 300 µm were used. In the dentate gyrus, BrdU- and TUNEL-labeled cells are comparatively rare, and therefore, no counting frames were used. Cells, which intersected the uppermost focal plane (exclusion plane) or the lateral exclusion boundaries of the counting frames, were not counted; otherwise the cells were counted exhaustively.

A series of every sixth section was stained for triple immunofluorescence using antibodies against BrdU, NeuN and S100β. For each brain and neurogenic region 50 BrdU-positive cells were randomly selected and analyzed for double staining using confocal microscopy. The resulting percentage for each group was multiplied with the number of BrdU-positive cells to calculate the number of newly generated neurons (BrdU/NeuN-positive) and non-neuronal cells (BrdU-positive/NeuN-negative). For quantification of TUNEL in the dentate gyrus and the SVZ/RMS/olfactory bulb system, we analyzed every sixth section from each animals.

Statistics. Statistical analysis was performed using the unpaired, 2-sided t-test (Student's t-test) between VEGF165 and aCSF groups. Significant differences were assumed at a level of $p<0.05$. The data are expressed as mean values ± standard error of mean.

RESULTS

Expression of VEGF and VEGFR-2/Flk-1 in adult neural stem cells in vitro. To analyze VEGF and its receptor VEGFR-2 in adult neural stem cells, we isolated and propagated rat ventricle wall cells as spheroid cultures using defined medium with FGF-2 and EGF as mitogens (69). After 4 to 5 passages neural stem cells were subcloned, by growing spheroid cultures from single cells. Only clonally-derived cell lines were used, which were determined to be multipotent for neuronal, astrocyte and oligodendrocyte lineages (Figure 1). Analyzing the expression of the VEGFR-2/Flk-1 in vitro, we found that Flk-1 mRNA is expressed by neural stem cells as determined by RT-PCR (Figure 2A). At the protein level, labeling with an antibody against VEGFR-2/Flk-1 revealed a widespread

immunoreactivity in neural stem cell cultures (Figure 3). Specificity was determined by pre-incubation with the immunization peptide (Figure 3A-D). Co-labeling of Flk-1 with antibodies against ubiquitous markers such as alpha-tubulin and the stem cell marker nestin indicates that the majority of cells in vitro expressed Flk-1 (Figure 3E-P). When comparing clonally-derived cultures with primary cultures, we found an increased percentage of Flk-1 expressing cells after subcloning (95% versus 80%, Figure 3). These data indicate that Flk-1 is strongly and widely expressed in neural stem cell cultures, which points towards a role of Flk-1 during stem cell expansion in vitro.

As determined by RT-PCR, all major isoforms of VEGF mRNA are expressed in neural stem cell cultures - the most prominent isoform being VEGF-A_{165} (Figure 2B). Using ELISA, we also detected that significant amounts of VEGF protein are released into the culture medium (Figure 2D). VEGF levels from neural stem cells were higher than in conditioned media from retinal pigment epithelial cells and human umbilical vein endothelial cells (HUVEC), known sources of VEGF (1, 67). The production and release of VEGF by neural stem cells and the presence of the VEGFR-2/Flk-1 indicate an autocrine stimulatory mechanism for VEGF signaling during neural stem cell expansion. Therefore, in a second set of experiments, we used recombinant VEGF and VEGF inhibitors to study the effects on neural stem cell cultures.

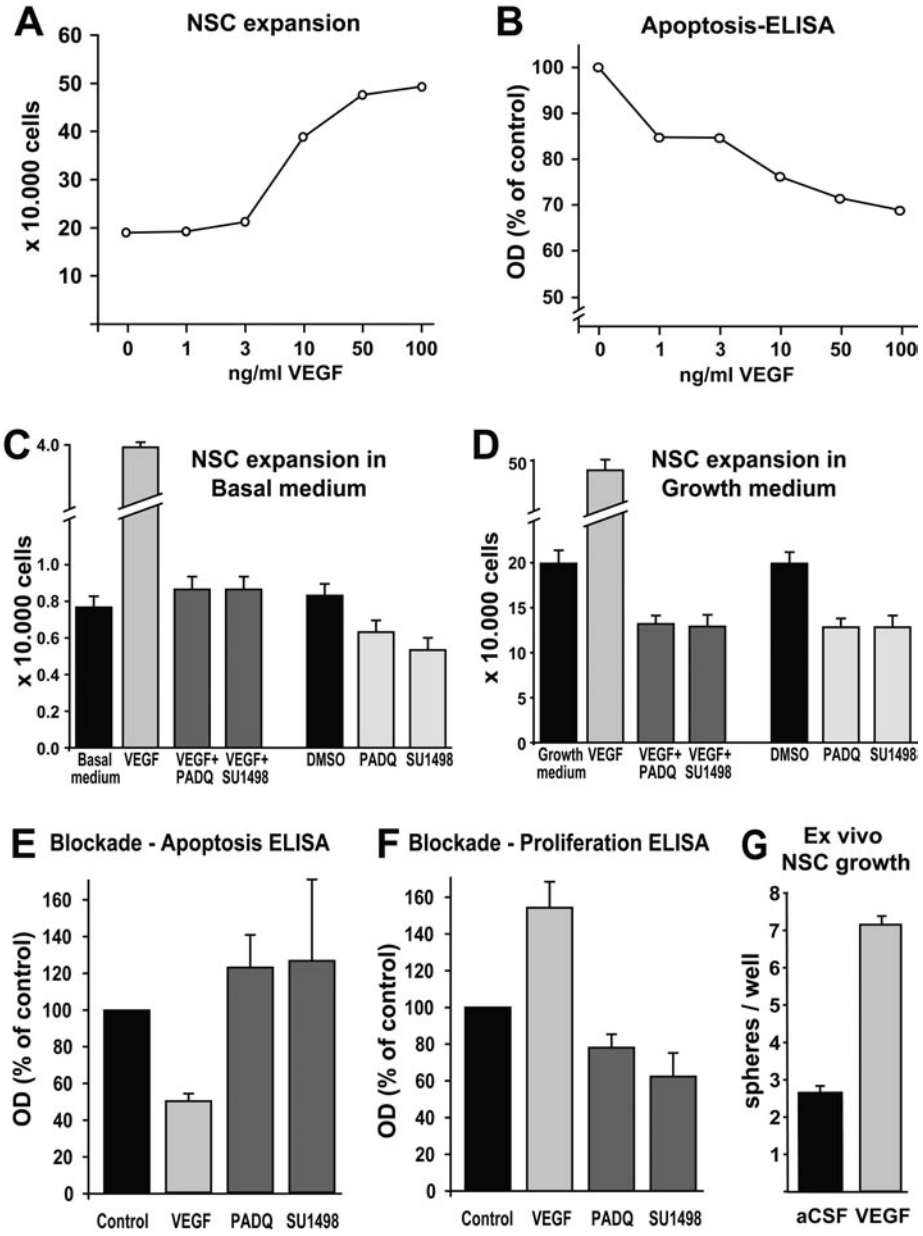

Figure 4. *In vitro analysis.* **A.** VEGF dose-response. 10 000 rtNSCs/well were incubated in growth medium for 7 days. On Day 1, 3 and 5 cells received different concentrations of $VEGF_{165}$. Maximum growth rate was reached at 50 ng/ml $VEGF_{165}$. **B.** Dose-dependent decrease in apoptosis. Cells were incubated under identical conditions as in **C**. DNA was isolated on Day 7 and histone-associated DNA fragmentation was measured by ELISA. Data are presented as mean percentage of optical density compared to the unstimulated condition (0 ng/ml $VEGF_{165}$). **C, D.** Neural stem cell culture expansion under VEGF receptor blockade. In basal medium (**C**) as well as growth medium (**D**) VEGF increased the expansion of neural stem cells. Two specific inhibitors of VEGFR-2/Flk-1 receptor tyrosine kinase activity (PADQ and SU1498) reversed the $VEGF_{165}$-induced increase. PADQ and SU1498 also reduced the growth rate under standard condition without addition of $VEGF_{165}$. **E, F.** Reciprocal effects of VEGF stimulation and VEGFR-2/Flk-1 receptor tyrosine kinase blockade on apoptosis and proliferation. Apoptosis and proliferation were measured 7 days after stimulation using ELISA. Data are presented as mean percentage of optical density of the unstimulated condition (control). **G.** Increase in the number of spheres formed in vitro from lateral ventricle tissue of animals, which received $VEGF_{165}$ infusion in comparison to aCSF infusion. Data are presented as the mean number of spheres formed per well (± standard deviation, n = 3 animals per group).

Stimulation of adult neural stem cell expansion by VEGF and blockade by VEGFR-2/Flk-1 inhibitors. Adult neural stem cells were grown as spheroid cultures using defined medium containing EGF and FGF-2 (69). When $VEGF_{165}$ was added to this growth medium, a dose-dependent increase (up to 2.5 fold) in the number of cells was observed after 7 days (Figure 4A) with an ED50 in the range of 10 ng/ml and maximal stimulation at 50 ng/ml $VEGF_{165}$. When $VEGF_{165}$ was added to the basal medium (without EGF and FGF-2), a 5-fold increase in the number of cells was detected after 7 days (Figure 4C). In order to block VEGF signaling, 2 different inhibitors of the VEGFR-2/Flk-1 tyrosine kinase were used. Both inhibitors were added to the medium at their respective ED50 concentration, which assured non-toxicity and high specificity for Flk-1 over other tyrosine kinase receptors (22, 64). Both inhibitors succeeded in blocking the effect of added $VEGF_{165}$ in basal medium (Figure 4C) and growth medium containing EGF and FGF-2 (Figure 4D). Under culture condition without exogenous $VEGF_{165}$, Flk-1 kinase inhibitor also decreased stem

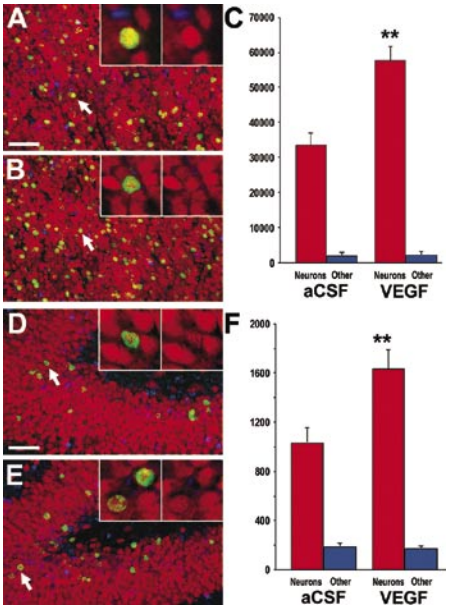

Figure 5. *In vivo neurogenesis.* **A-C.** Increased neurogenesis in the olfactory bulb of (**A**) adult aCSF-infused animals compared to (**B**) $VEGF_{165}$-infused animals. At 4 weeks after growth factor infusion/BrdU labeling, new neurons were detected with colabeling of BrdU (green) and the neuronal marker NeuN (red) using confocal analysis. Double-labeled cells appear yellow. Inserts show high magnification of cells marked with arrow in the overviews in order to demonstrate the overlay of green BrdU signal and red NeuN positive signal. **C.** The total number of newly generated neurons was estimated by multiplying the stereologically counted number of BrdU-positive cells with the percentage of neurons among the BrdU positive cells. Data are represented as group means ± standard error. ** indicates statistically significant differences at the 1% level (t-test). **D-F.** Increased neurogenesis in the dentate gyrus of (**D**) adult aCSF-infused animals compared to (**E**) $VEGF_{165}$-infused animals. **F**. Quantitative analysis - for details see **C**. Scale bar **A, D** = 50 µm.

	aCSF	VEGF	P-Value
BrdU - Last day of infusion			
Lateral ventricle wall	25 300 ± 2200	19 600 ± 1990	n.s.
Dentate gyrus - Granule cell layer	2200 ± 270	1940 ± 190	n.s.
Dentate gyrus - Molecular layer	560 ± 140	810 ± 210	n.s.
Dentate gyrus - Hilus	350 ± 70	510 ± 110	n.s.
BrdU - 4 weeks after infusion			
Olfactory bulb - New neurons	33 800 ± 3090	56 400 ± 3840	<0.001**
Olfactory bulb - New non-neuronal cells	2400 ± 700	2230 ± 670	n.s.
Dentate gyrus - GCL - New neurons	950 ± 80	1710 ± 140	<0.001**
Dentate gyrus - GCL - New non-neuronal cells	180 ± 50	180 ± 40	n.s.
Dentate gyrus - Molecular Layer	680 ± 100	710 ± 120	n.s.
Dentate gyrus - Hilus	310 ± 60	470 ± 100	n.s.
TUNEL - Last day of infusion			
SVZ/RMS/OB	6000 ± 450	4940 ± 380	<0.05*
Dentate gyrus	73 ± 11	51 ± 7	n.s.
TUNEL - 4 weeks after infusion			
SVZ/RMS/OB	3260 ± 260	2960 ± 320	n.s.
Dentate gyrus	53 ± 13	35 ± 9	n.s.

Table 1. *$VEGF_{165}$-infusion - Quantitative morphological changes.* Data are presented as total estimated number of cells per structure (± SEM) determined by stereological counting procedures.

cell expansion compared to basal medium (Figure 4C) and growth medium (Figure 4D), indicating that endogenous VEGF released into the culture medium acts as an autocrine factor in neural stem cell cultures.

VEGF is a known proliferative factor for endothelial cells (27, 37) and has recently been described to stimulate proliferation in fetal cortical progenitor cells (24, 74). However, other hypoxia-inducible growth factors such as erythropoietin have been shown to exert their effects on the nervous system via anti-apoptotic activity (61, 73). In order to determine whether VEGF's action on neural stem cultures involves anti-apoptotic or proliferative effects we directly compared proliferation and apoptosis of neural stem cells under VEGF stimulation and Flk-1 blockade. $VEGF_{165}$ increased BrdU incorporation, whereas VEGF receptor tyrosine kinase inhibitors decreased proliferation (Figure 4F). On the other hand, neural stem cells grown in the presence of $VEGF_{165}$ also revealed less apoptosis, as determined by a cell death detection ELISA, whereas the addition of VEGFR-2/Flk-1 inhibitors increased apoptosis (Figure 4B,E).

ICV infusion of VEGF increases the number of neurospheres generated in vitro from the lateral ventricle wall and adult neurogenesis in vivo. The expression and stimulatory function of VEGF and its receptor Flk-1 in adult neural stem cells in vitro suggests a possible role for VEGF during neurogenesis in the adult brain. To determine if VEGF can influence stem cells in vivo in a similar fashion, adult rats were infused with $VEGF_{165}$ into the lateral ventricle for 7 days (2.4 ng/d).

In a first experiment, we determined whether VEGF application in vivo could increase the efficiency to generate neural stem cell cultures. After infusion for 7 days with VEGF or aCSF (n = 3 animals per group), cells from the lateral ventricle wall were isolated and grown in defined medium in the presence of EGF and FGF-2. When quantifying the number of spheres that formed in vitro from $VEGF_{165}$-treated animals after 7 days a more than 2.5 fold increase over aCSF controls was detected (Figure 4G).

These ex vivo results prompted us to examine whether VEGF infusion could also lead in the adult brain to a higher number of newly generated neurons. Recombinant mouse $VEGF_{165}$, dissolved in artificial cerebrospinal fluid (aCSF), or aCSF alone were infused into the lateral ventricle of adult rats for one week. To label dividing cells bromodeoxyuridine (BrdU) was injected daily during the VEGF infusion period (50 mg/kg i.p.). Animals were perfused either at the last day of infusion or 4 weeks later in order to analyze the amount of new cells that have differentiated into neurons.

At 4 weeks post-infusion, a highly significant difference in neurogenesis between the 2 groups was observed. Using stereological counting procedures $VEGF_{165}$-infused rats had 80% more BrdU-labeled cells in the olfactory bulb (Table 1). Combining these data with confocal analysis of co-labeling for BrdU and the neuronal marker NeuN, we quantitatively determined the neuronal phenotype of the newly generated cells. We found that on average 56 000 new neurons per olfactory bulb were generated in $VEGF_{165}$-treated animals, compared with 34 000 new neurons in aCSF controls (Figure 5C).

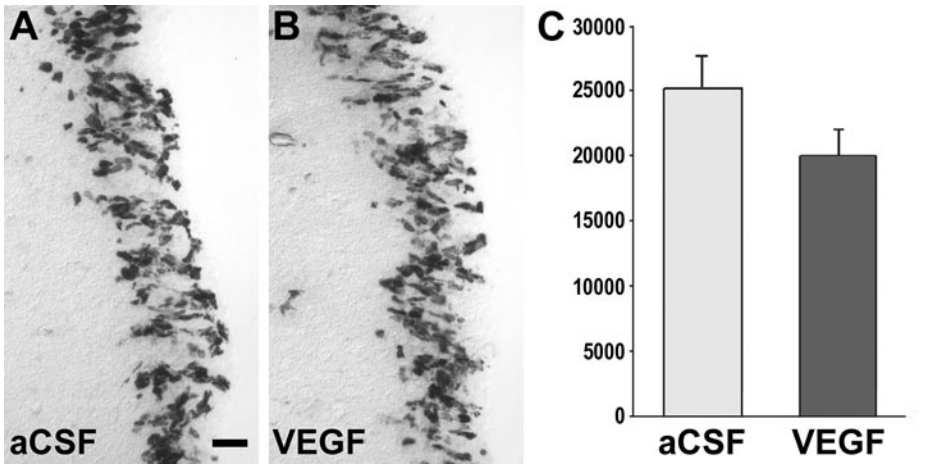

Figure 6. *Proliferation in the lateral ventricle wall.* Cell division was visualized by BrdU immunohistochemistry on the last day of intraventricular infusion in (**A**) aCSF infused animals compared to (**B**) $VEGF_{165}$-infused animals. **C.** Stereological BrdU cell counts are represented as the total number of BrdU-positive cells per subventricular zone. No alteration in proliferative activity was detected (t-test, no significance). Interference contrast optics. Scale bar **A** = 25 µm.

Surprisingly, analysis of BrdU-positive cells on the last day of growth factor infusion, which reflects the proliferative activity of neural stem cells and progenitors, revealed no significant difference in the lateral ventricle wall between $VEGF_{165}$-infused and aCSF-infused animals (Figure 6).

Since it was previously shown that apoptotic elimination is an important mechanism by which neural stem cell and progenitor activity is controlled in vivo (5, 6, 7, 71), we hypothesize that VEGF may act as a survival factor for neural stem/progenitor cells in vivo. We performed TUNEL labeling in the lateral ventricle wall/olfactory bulb system of $VEGF_{165}$-treated animals and counted apoptotic cells in this region. Cell death was significantly decreased immediately after infusion, whereas 4 weeks after $VEGF_{165}$ infusion no significant difference was observed (Figure 7; Table 1).

Similar to the lateral ventricle wall/olfactory bulb system, $VEGF_{165}$ infusion stimulated neurogenesis in the dentate gyrus (Figure 5D-F; Table 1). On average 1710 new neurons were labeled in the dentate gyrus under VEGF compared to 950 BrdU/NeuN double-positive cells in aCSF infused animals. The proliferative accumulation of hippocampal progenitor cells on the last day of infusion was not affected by $VEGF_{165}$ (Table 1) suggesting that ventricular and hippocampal progenitor cells are equally affected. Also in paralleling the results from the lateral ventricle wall/olfactory bulb system, a trend towards less TUNEL-positive cells in the VEGF-treated dentate gyrus was detected one day and 4 weeks after infusion (Figure 7G, H; Table 1). However, the intact dentate gyrus contains a very low number of TUNEL-positive cells (about one cell per section), which led to a higher variability and no significance differences.

ICV infusion of VEGF does not alter genesis of non-neuronal cells and vascularization of neurogenic regions. Our data demonstrate an effect of $VEGF_{165}$ on neurogenesis in the adult brain; however, VEGF is most prominently known for its effects on the vascular system and therefore, our effects of intraventricular infusion of $VEGF_{165}$ on neurogenesis could be mediated through signaling from the vasculature. Although we infused a dose of VEGF, which has previously been determined to leave the brain vasculature unaffected, we determined whether angiogenesis and vascular permeability, the 2 principle vascular responses to VEGF, were altered. At the infused dose of 2.4 ng/d, $VEGF_{165}$ did not induce neovascularization adjacent to the ventricle wall or in the dentate gyrus as determined by quantitative immunohistochemistry using the rat endothelial cell marker RECA (Figure 8). Moreover, the number of non-neuronal BrdU-positive cells, which have previously been determined to mainly consist of astrocytes and endothelial cells (48), were unchanged in the granule cell layers of the olfactory bulb and dentate gyrus, as well as the dentate gyrus molecular layer and hilar region after VEGF infusion (Table 1). Finally, we injected Evans blue dye intravenously into a separate set of animals on the last day of intraventricular infusion and observed no leakage in the blood-brain-barrier of $VEGF_{165}$-treated animals as compared to aCSF-infused controls (Figure 9). We also did not detect changes of brain volume or any clinical signs of increased intracranial pressure, which would imply enhanced vascular permeability (data not shown).

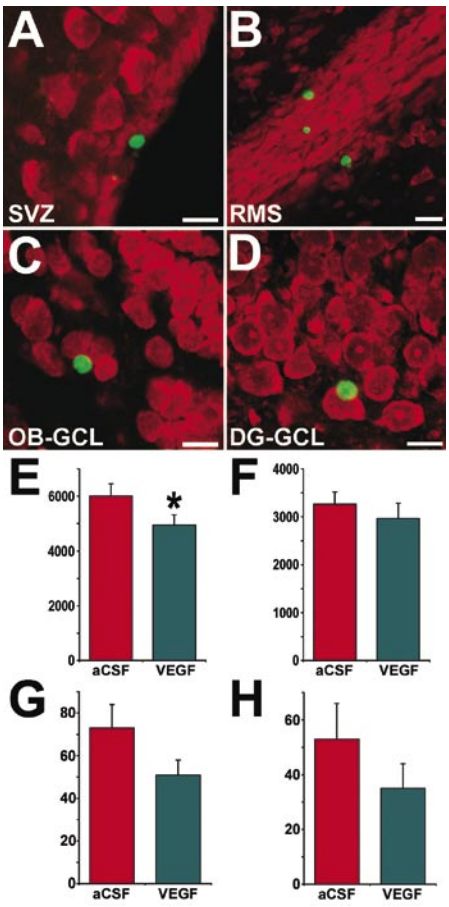

Figure 7. *Cell death in neurogenic brain regions.* **A-D.** Cells with nuclear DNA fragmentation, a hallmark of programmed cell death, were consistently detected throughout the subventricular zone (SVZ), rostral migratory stream (RMS) olfactory bulb granule cell layer (OB-GCL) and dentate gyrus granule cell layer (DG-GCL) using TUNEL labeling (green). Red counterstaining: propidium iodide. **E, F.** Quantification of TUNEL-positive cells in aCSF and VEGF infused animals was performed by stereological cell counts on the last day of intraventricular infusion (**E**) and 4 weeks after infusion (**F**). Since cell death occurs continually throughout the development olfactory bulb granule cells, the SVZ, RMS and OB-GCL were analyzed as a single structure. **G, H.** Quantification of TUNEL-positive cells in the dentate gyrus on the last day of intraventricular infusion (**G**) and 4 weeks after infusion (**H**). * indicates statistically significant differences at the 5% level (t-test). Scale bars **A, C, D** = 10 µm; **B** = 25 µm.

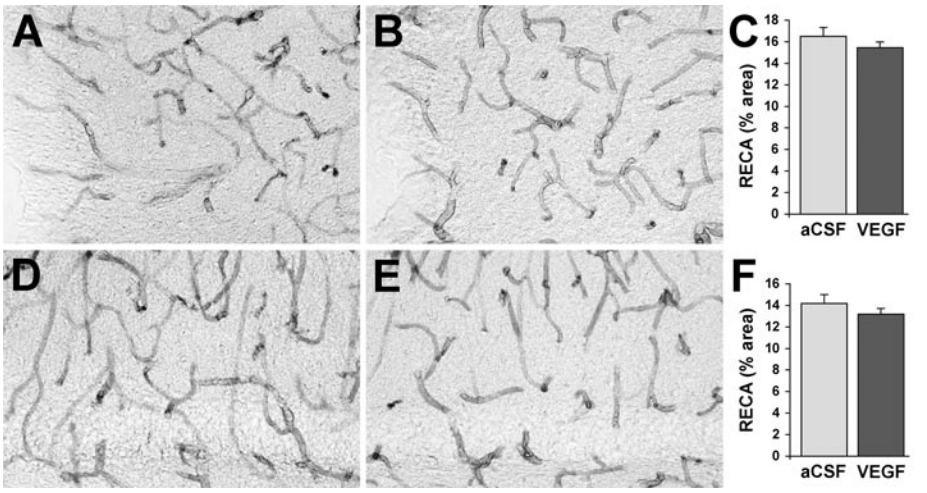

Figure 8. *Vascular density after VEGF$_{165}$ infusion.* An antibody against rat endothelial cell antigen (RECA) was used to determine whether vascular density was altered in tissue adjacent to the lateral ventricle wall (**A-C**) and in the dentate gyrus (**D-F**) aCSF infused animals (**A, D**) and VEGF$_{165}$ infused animals (**B, E**). Immunohistochemical stainings were quantified by determining the percentage of area covered by RECA staining on 4 sections per animal. No alteration in vascular density was detected (t-test, no significance).

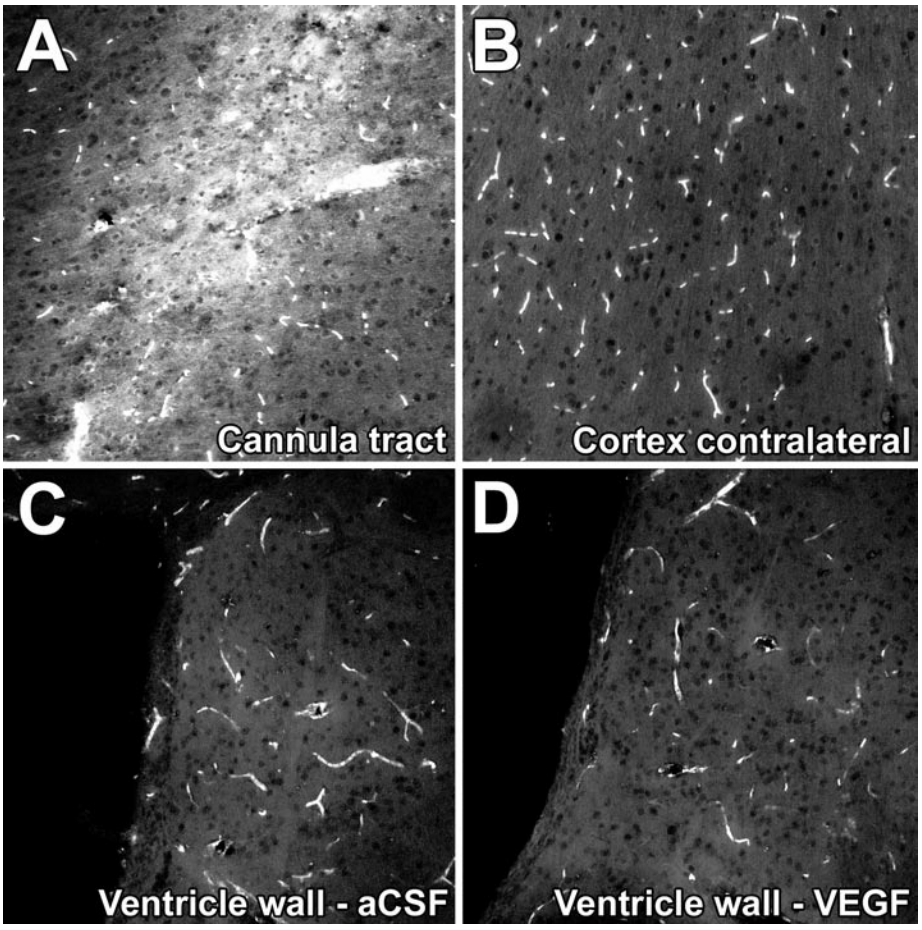

Figure 9. *Vascular permeability.* The presence of Evan's Blue dye in the brain parenchyma after intravenous injection acts as an indicator for vascular leakage. **A.** Evan's Blue dye penetrated into the tissue around the cannula tract (diffuse fluorescent signal) and served as a positive control for altered blood brain barrier function. **B.** In comparison, on the contralateral side no extravascular Evan's blue signal was detected. **C, D.** No extravasation of Evan's Blue was detected in the parenchyma close to the ventricle in (**B**) CSF-treated or (**C**) VEGF$_{165}$-treated animals (n = 3 animals per group).

Expression of VEGF and VEGFR-2/Flk-1 in vivo. VEGF and its receptor VEGFR-2/Flk-1 are expressed not only in the brain vasculature but also by glial cells and neurons especially during development and under hypoxic conditions (30, 44, 52). In addition, in the dentate gyrus subgranular zone, a close spatial interaction between proliferating neural progenitors and dividing endothelial cells, which express VEGFR-2/Flk-1 has been described (48). However, little is known on VEGF and VEGFR-2/Flk-1 expression with regard to the ventricular neural stem cell compartment and we therefore, analyzed these regions with antibodies against Flk-1 and VEGF.

For VEGFR-2/Flk-1 a strong immunoreactivity was detected in the ependymal cell layer throughout the lateral ventricles (Figure 10). Ependymal cells lining the lateral and medial side of the lateral ventricles, demonstrated an intense, cell membrane-specific staining. As shown by double immunofluorescence analysis, VEGFR-2/Flk-1 expression is restricted to the ependymal cells, which are negative for GFAP (Figure 10C) and the progenitor marker nestin (Figure 10A). Subventricular cells and cells in the rostral migratory stream (RMS) showed strong nestin expression with only single cells within the RMS demonstrating low Flk-1 expression (Figure 10B). Comparable results were obtained in reporter mice, in which one Flk-1 allele was partially replaced with the lacZ gene (60). Similar to the Flk-1 immunoreactivity, β-galactosidase expression in these mice was also limited to the ependymal lining of the ventricle (Figure 10D). Ependymal cells did not express other VEGF receptors, such as VEGFR-2/Flt-1 and neuropilin-1; however, neuropilin-1 expression was detectable in cells residing in the subventricular zone and the RMS (Figure 10I-K).

The choroid plexus plays an important role in supporting neuronal function by secreting growth factors such as FGF-2, IGF-1, and TGF-β into the cerebrospinal fluid (12, 63). In situ hybridization and immunohistochemistry confirmed that VEGF mRNA and protein were expressed by the epithelial cells of the choroid plexus (Figure 10E,H). Using ELISA, VEGF levels in the cerebrospinal fluid of adult rats were found to be 2.98 ng/ml (± 0.37, n = 3). Previous reports on VEGF expression in the choroids plexus suggested a local autocrine function in CSF production such as altering the permeability of the blood-liquor-barrier (8); however, the high levels of VEGF in the cerebrospinal fluid of adult rats may also serve additional paracrine functions in the brain,

such as stimulating the survival of neural stem cells, progenitor cells and neurons in the adjacent parenchyma.

DISCUSSION

VEGF has initially been identified as a vascular permeability factor (59) and a mitogen for endothelial cells in vitro (16, 37). The essential role of VEGF and its receptors -1 and -2 for the development of the vascular system has been demonstrated in knock-out mice (10, 17, 18, 60). More recently, a role for VEGF in brain angiogenesis has been unraveled in Nestin-cre/VEGF$^{lox/lox}$ mice (19). In addition to its well-known function in the vascular system neuroprotective and neurogenic effects of VEGF have been described in several studies (for review, see 11). However, previous reports on neuroprotective and neurogenic effects of VEGF in vivo have not ruled out the possibility that angiogenic responses could account for the increased number of new neurons after VEGF infusion. We therefore, designed in vivo and in vitro experiments to study more directly the effects VEGF on neural stem cells and neurogenesis. We used neural stem cell cultures from the lateral ventricle wall of adult rats, but in contrast to other studies (24, 41, 74), we studied VEGF effects in clonally derived multipotent stem cell cultures, which are free from any possible contamination of endothelial cells. We demonstrate that VEGF is produced by these cells and released into the culture medium and its receptor VEGFR-2/Flk-1 is expressed by the vast majority of stem cells, indicating an autocrine function of VEGF. In these cells, VEGF dose-dependently stimulated the expansion of stem cell cultures in the presence as well as in the absence of the mitogens EGF and FGF-2. In contrast to a previous study (24), we used 2 receptor tyrosine kinase antagonists at concentrations that are specific for blocking the VEGFR-2/Flk-1 receptor, and at these doses the VEGF-induced expansion of neural stem cells was blocked. We observed a decreased apoptosis in the neurosphere cultures under VEGF exposure and increase apoptosis under Flk-1 blockade, indicating that a survival-promoting effect could be responsible for the increased number of dividing cells under VEGF treatment. These data argue against a previous view, that VEGF exerts no survival enhancing but exclusively proliferative effects on neural progenitor cells (24). Palmer and colleagues have shown that VEGF has a stronger effect on the selective accumulation/survival of proliferative neuroblasts rather than on proliferation itself (15). These data strongly suggest a separate role for VEGF signaling in neural

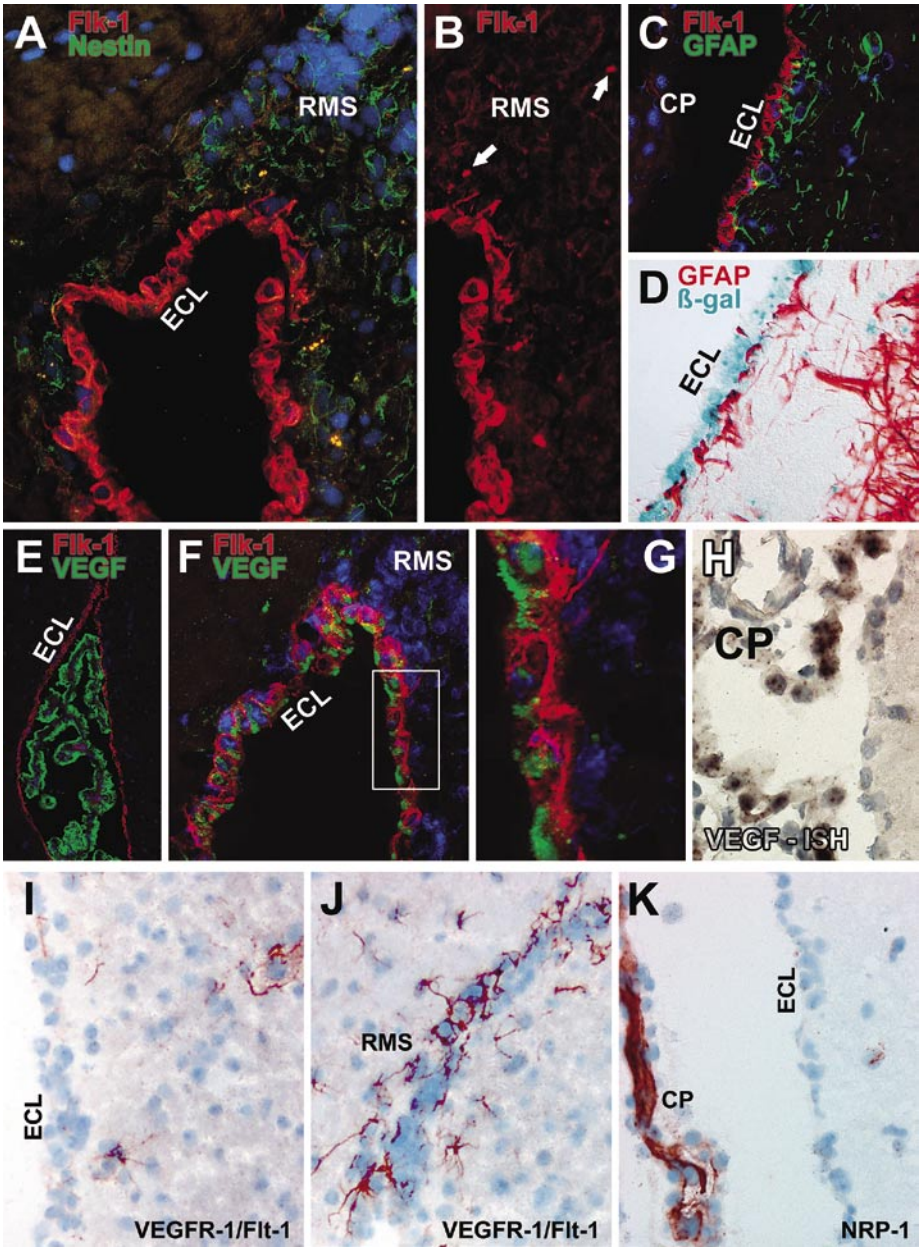

Figure 10. *VEGFR-2/Flk-1 and VEGF localization in vivo.* **A-C**, **E-F**. Intense VEGFR-2/Flk-1 immunoreactivity (red) was seen in ependymal cells, which showed no co-expression of nestin (green in **A**) or GFAP (green in **C**). **B.** Low level immunoreactivity against VEGFR-2/Flk-1 can be detected in the RMS (same area as right half of **A**, but red channel only). Arrows point to higher intensity stainings. (**D**) Ependymal VEGFR-2/Flk-1 expression was confirmed by β-galactosidase staining in ependymal cells of reporter mice, in which part of one VEGFR-2/Flk-1 allele was replaced by the lacZ gene (60). No co-expression with GFAP (red) was observed. **E.** Strong VEGF protein expression (green) in choroid plexus in close proximity to ependymal cells. **F.** Asymmetrical, predominantly luminal VEGF protein staining (green) in ependymal cells. **G.** Enlargement of framed area in (**F**). **H.** Little or no VEGF mRNA (black-brown) was detectable in ependymal cells by in situ hybridization in comparison to strong VEGF mRNA expression of choroid plexus cells. **I-K.** VEGFR-1/Flt-1 and neuropilin-1 immunoreactivity. Compared to VEGFR-2/Flk-1 staining (see Figure 1), VEGFR-1/Flt-1 (**I**) and neuropilin-1 (**K**) expression is largely absent from the ependymal cell layer. **I, J.** However, VEGFR-1/Flt-1 is readily visible in the rostral migratory stream (**I**), whereas neuropilin-1 (**J**) is present in the choroid plexus (CP). Vector red was used as a substrate for immunohistochemical detection of Flt-1 and neuropilin-1. Counterstaining: **A, C, E-G**—DAPI; **H-K**—Hematoxylin. CP: choroid plexus, ECL: ependymal cell layer, RMS: rostral migratory stream.

stem cells, independent from effects on endothelial cells.

Recent studies have demonstrated that VEGF has the potential to stimulate the survival of mature neurons after ischemia and neurogenesis (24, 66). The ICV-infusion experiment in the current study was designed to exclude endothelial involvement in adult neurogenesis by choosing a very low dose of VEGF (2.4 ng/d). At the previously used 100-fold higher doses (24, 66) that stimulate neurogenesis, changes in vascular permeabilization, angiogenesis and edema have been observed (20, 31). At our selected low dose, we demonstrate that no alterations in angiogenesis or vascular permeability in the neurogenic regions occurred; however, neurogenesis was still significantly increased in the ventricle wall/olfactory bulb system and the dentate gyrus, again arguing for an effect of VEGF on neural progenitors/stem cells that is independent from vascular signals.

Increased generation of new neurons after VEGF infusion was not mediated through proliferation of neural stem cells and progenitors but through increased survival of progenitor cells and young neurons, as indicated by the significant reduction of apoptosis in the SVZ/RMS/olfactory bulb system. Similar to our in vitro data, these findings stand in contrast to previously reported data by Greenberg and colleagues, which propose exclusively a proliferative effect of VEGF on in vivo neurogenesis (24). Differences to the earlier study are the lower concentration of VEGF, no alterations in vascular function and the use of stereological counting criteria to assess differences in cell number between VEGF-infused and aCSF-infused animals.

Previous studies have shown that apoptotic death of progenitors is an important regulator of neurogenesis in the embryonic and adult brain (6, 14, 43, 54, 71). Therefore, we propose a new role for VEGF to provide trophic support to the progenitor cells and stem cells within the neurogenic regions. A similar survival promoting effect of VEGF has been proposed for mature neurons under hypoxic stress (21, 25, 49).

The intraventricular infusion of VEGF and the consecutive enhanced growth of neurosphere cultures from the stimulated ventricle wall, suggests that neural stem cells residing in the lateral ventricle wall are able to respond to elevated VEGF levels with increased readiness to proliferate under in vitro conditions. It is also intriguing to note the strong and uniform expression of VEGFR-2/Flk-1 in neural stem cells in vitro and in ependymal cells in vivo, in particular, since ependymal cells have been suggested to possess properties of neural stem cells (26). However, ependymal cells are not the only cells responding to VEGF, since GFAP-positive cells have also been reported to express Flk-1. Moreover, since GFAP/Nestin co-expressing cells are currently favored to represent the neural stem cell population of the adult brain (for review, see 2), it is likely that several cell types of the lateral ventricle wall respond to the infused VEGF and the endogenous production of VEGF by the choroid plexus.

Although ependymal cells and GFAP-positive cells express VEGFR-2/Flk-1 in vivo, we observed no changes in BrdU-positive cells in the ventricle wall immediately after VEGF infusion. Rather, the VEGF effects became apparent in the apoptotic cell counts of the SVZ/RMS/OB at an early stage and in the number of newly generated neurons at 4 weeks after infusion. One explanation is that the stem cells of the brain are relatively quiescent cells and therefore, changes in the survival rate could, early on, be masked by the large pool of rapidly dividing neuronal progenitor cells. But small changes in the stem cell pool could translate later into a significant increase in neurogenesis once the cells have undergone exponential expansion and neuronal differentiation.

With respect to other stem cell systems, it is important to note that VEGFR-2/Flk-1/KDR is expressed in retinal progenitor cells (72) and that it represents a defining marker for hematopoietic stem cells (50, 75). Flk-1 activation stimulates recruitment of hematopoietic stem cells for reconstitution of hematopoiesis (55). Similar to our experiments, this effect was mediated by an increased cell survival with minimal effect on proliferation of hematopoietic stem cells (35).

For mature neurons a survival-promoting role of VEGF was also suggested, because VEGF is upregulated in neuronal and glial cells after ischemia (36, 52) and increased VEGF levels protect neurons against ischemic damage in vivo and in vitro. Deletion of the hypoxia-responsive element in the VEGF promoter leads to early motor neuron degeneration, suggesting that VEGF is necessary for neuronal survival in the adult CNS (46). The survival-promoting function, whether in neuronal or in hematopoietic cells, appears to be mediated through the VEGFR-2/Flk-1 receptor via the Akt/PI3 kinase pathway, whereas proliferation, at least in endothelial cells, is mediated by the MAP kinase pathway (29).

An interaction between the vascular system and neurogenic activity in the adult brain has recently been suggested based on anatomical findings (42, 48). VEGF was shown to mediate hormone-induced stimulation of neurogenesis in adult songbirds by stimulating the release BDNF from the vasculature, which in turn induced neurogenesis (38). These finding suggest an indirect mechanism for VEGF; our data, however, indicate that neural stem cells directly respond to VEGF via activation of the VEGF receptor Flk-1, and that VEGF can regulate neurogenesis in vivo in the absence of angiogenic effects. Moreover, the production and release of VEGF from the choroid plexus and the constitutive expression of VEGFR-2 in nearby ependymal cells suggests that in the adult brain physiological neurogenesis could be driven by VEGF released into the CSF by choroids plexus epithelial cells. In summary, we conclude that $VEGF_{165}$ is a potent stimulus for neurogenesis in the adult brain. Our data strongly suggest that $VEGF_{165}$ affects neurogenesis rather independently from the vascular system. However, as discussed above, other cell types within the brain such as astrocytes cannot definitely be ruled out as mediators of VEGF stimulation.

Our findings may have several implications for the pathogenesis and treatment of neurodegenerative diseases. Although the nature of most neurodegenerative diseases is still unknown, hypoxia as well as oxidative stress have been implicated in their pathogenesis (23, 53). VEGF expression attenuates with old age (57). Lowered VEGF levels in response to chronic hypoxic injuries could thus contribute to neurodegenerative diseases by lowering the neuroprotective threshold and in addition by decreasing the amount of cell replacement in the CNS. Finally, our results suggest that VEGF could be useful as a therapeutic agent for replacing lost neurons in the adult brain.

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REFERENCES

1. Adamis AP, Shima DT, Yeo KT, Yeo TK, Brown LF, Berse B, D'Amore PA, Folkman J (1993) Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. *Biochem Biophys Res Commun* 193:631-638.

2. Alvarez-Buylla A, Seri B, Doetsch F (2002) Identification of neural stem cells in the adult vertebrate brain. *Brain Res Bull* 57:751-758.

3. Bagnard D, Vaillant C, Khuth ST, Dufay N, Lohrum M, Puschel AW, Belin MF, Bolz J, Thomasset N (2001) Semaphorin 3A-vascular endothelial growth factor-165 balance mediates migration and apoptosis of neural progenitor cells by the recruitment of shared receptor. *J Neurosci* 21: 3332-3341.

4. Beck H, Acker T, Wiessner C, Allegrini PR, Plate KH (2000) Expression of angiopoietin-1, angiopoietin-2, and tie receptors after middle cerebral artery occlusion in the rat. Am J Pathol 157:1473-1483.

5. Bernier PJ, Vinet J, Cossette M, Parent A (2000) Characterization of the subventricular zone of the adult human brain: evidence for the involvement of Bcl-2. *Neurosci Res* 37:67-78.

6. Biebl M, Cooper CM, Winkler J, Kuhn HG (2000) Analysis of neurogenesis and programmed cell death reveals a self- renewing capacity in the adult rat brain. *Neurosci Lett* 291:17-20.

7. Blaschke AJ, Weiner JA, Chun J (1998) Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system. *J Comp Neurol* 396:39-50.

8. Breier G, Albrecht U, Sterrer S, Risau W (1992) Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* 114:521-532.

9. Carlen M, Cassidy RM, Brismar H, Smith GA, Enquist LW, Frisen J (2002) Functional integration of adult-born neurons. *Curr Biol* 12:606-608.

10. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380:435-439.

11. Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. *Nat Rev Genet* 4:710-720.

12. Chodobski A, Szmydynger-Chodobska J (2001) Choroid plexus: target for polypeptides and site of their synthesis. *Microsc Res Tech* 52: 65-82.

13. Cooper-Kuhn CM, Vroemen M, Brown J, Ye H, Thompson MA, Winkler J, Kuhn HG (2002) Impaired adult neurogenesis in mice lacking the transcription factor E2F1. *Mol Cell Neurosci* 21:312-323.

14. Dayer AG, Ford AA, Cleaver KM, Yassaee M, Cameron HA (2003) Short-term and long-term survival of new neurons in the rat dentate gyrus. *J Comp Neurol* 460:563-572.

15. Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci* 18:2803-2812.

16. Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161:851-858.

17. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380:439-442.

18. Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376:66-70.

19. Haigh JJ, Morelli PI, Gerhardt H, Haigh K, Tsien J, Damert A, Miquerol L, Muhlner U, Klein R, Ferrara N, Wagner EF, Betsholtz C, Nagy A (2003) Cortical and retinal defects caused by dosage-dependent reductions in VEGF-A paracrine signaling. *Dev Biol* 262:225-241.

20. Harrigan MR, Ennis SR, Sullivan SE, Keep RF (2003) Effects of intraventricular infusion of vascular endothelial growth factor on cerebral blood flow, edema, and infarct volume. *Acta Neurochir (Wien)* 145:49-53.

21. Hayashi T, Abe K, Itoyama Y (1998) Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia. *J Cereb Blood Flow Metab* 18: 887-895.

22. Hennequin LF, Thomas AP, Johnstone C, Stokes ES, Pl inverted question marke PA, Lohmann JJ, Ogilvie DJ, Dukes M, Wedge SR, Curwen JO, Kendrew J, Lambert-van der Brempt C (1999) Design and structure-activity relationship of a new class of potent VEGF receptor tyrosine kinase inhibitors. *J Med Chem* 42:5369-5389.

23. Jellinger KA (2003) General aspects of neurodegeneration. *J Neural Transm* Suppl:101-144.

24. Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A* 99:11946-11950.

25. Jin KL, Mao XO, Greenberg DA (2000) Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proc Natl Acad Sci U S A* 97:10242-10247.

26. Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J (1999) Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 96:25-34.

27. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246:1309-1312.

28. Kremer C, Breier G, Risau W, Plate KH (1997) Up-regulation of flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system. *Cancer Res* 57:3852-3859.

29. Kroll J, Waltenberger J (1997) The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. *J Biol Chem* 272: 32521-32527.

30. Krum JM, Rosenstein JM (1998) VEGF mRNA and its receptor flt-1 are expressed in reactive astrocytes following neural grafting and tumor cell implantation in the adult CNS. *Exp Neurol* 154:57-65.

31. Krum JM, Mani N, Rosenstein JM (2002) Angiogenic and astroglial responses to vascular endothelial growth factor administration in adult rat brain. *Neuroscience* 110:589-604.

32. Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* 16:2027-2033.

33. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J Neurosci* 17:5820-5829.

34. Kuhn HG, Palmer TD, Fuchs E (2001) Adult neurogenesis: a compensatory mechanism for neuronal damage. *Eur Arch Psychiatry Clin Neurosci* 251:152-158.

35. Larrivee B, Lane DR, Pollet I, Olive PL, Humphries RK, Karsan A (2003) VEGFR-2 induces survival of hematopoietic progenitor cells. *J Biol Chem* 278:22006-22013.

36. Lennmyr F, Ata KA, Funa K, Olsson Y, Terent A (1998) Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. *J Neuropathol Exp Neurol* 57:874-882.

37. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246:1306-1309.

38. Louissaint A, Jr., Rao S, Leventhal C, Goldman SA (2002) Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. *Neuron* 34:945-960.

39. Marti HH, Risau W (1998) Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. *Proc Natl Acad Sci U S A* 95:15809-15814.

40. Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR (1991) A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c- kit. *Proc Natl Acad Sci U S A* 88:9026-9030.

41. Maurer MH, Tripps WK, Feldmann RE, Jr., Kuschinsky W (2003) Expression of vascular endothelial growth factor and its receptors in rat neural stem cells. *Neurosci Lett* 344:165-168.

42. Mercier F, Kitasako JT, Hatton GI (2002) Anatomy of the brain neurogenic zones revisited: fractones and the fibroblast/macrophage network. *J Comp Neurol* 451:170-188.

43. Naruse I, Keino H (1995) Apoptosis in the developing CNS. *Prog Neurobiol* 47:135-155.

44. Ogunshola OO, Stewart WB, Mihalcik V, Solli T, Madri JA, Ment LR (2000) Neuronal VEGF expression correlates with angiogenesis in postnatal developing rat brain. *Brain Res Dev Brain Res* 119: 139-153.

45. Ogunshola OO, Antic A, Donoghue MJ, Fan SY, Kim H, Stewart WB, Madri JA, Ment LR (2002) Paracrine and autocrine functions of neuronal VEGF in the CNS. *J Biol Chem* 277:11410-11415.

46. Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, et al (2001) Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet* 28:131-138.

47. Palmer TD, Ray J, Gage FH (1995) FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol Cell Neurosci* 6:474-486.

48. Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479-494.

49. Patt S, Danner S, Theallier-Janko A, Breier G, Hottenrott G, Plate KH, Cervos-Navarro J (1998) Upregulation of vascular endothelial growth factor in severe chronic brain hypoxia of the rat. *Neurosci Lett* 252:199-202.

50. Pelosi E, Valtieri M, Coppola S, Botta R, Gabbianelli M, Lulli V, Marziali G, Masella B, Muller R, Sgadari C, Testa U, Bonanno G, Peschle C (2002) Identification of the hemangioblast in postnatal life. *Blood* 100:3203-3208.

51. Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* 359:845-848.

52. Plate KH, Beck H, Danner S, Allegrini PR, Wiessner C (1999) Cell type specific upregulation of vascular endothelial growth factor in an MCA-occlusion model of cerebral infarct. J Neuropathol Exp Neurol 58:654-666.

53. Pong K (2003) Oxidative stress in neurodegenerative diseases: therapeutic implications for superoxide dismutase mimetics. *Expert Opin Biol Ther* 3:127-139.

54. Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* 262:695-700.

55. Rafii S, Avecilla S, Shmelkov S, Shido K, Tejada R, Moore MA, Heissig B, Hattori K (2003) Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment. *Ann N Y Acad Sci* 996:49-60.

56. Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255:1707-1710.

57. Rivard A, Berthou-Soulie L, Principe N, Kearney M, Curry C, Branellec D, Semenza GL, Isner JM (2000) Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. *J Biol Chem* 275:29643-29647.

58. Schratzberger P, Schratzberger G, Silver M, Curry C, Kearney M, Magner M, Alroy J, Adelman LS, Weinberg DH, Ropper AH, Isner JM (2000) Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy. *Nat Med* 6:405-413.

59. Senger DR, Perruzzi CA, Feder J, Dvorak HF (1986) A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* 46:5629-5632.

60. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62-66.

61. Shingo T, Sorokan ST, Shimazaki T, Weiss S (2001) Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *J Neurosci* 21:9733-9743.

62. Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843-845.

63. Stopa EG, Berzin TM, Kim S, Song P, Kuo-LeBlanc V, Rodriguez-Wolf M, Baird A, Johanson CE (2001) Human choroid plexus growth factors: What are the implications for CSF dynamics in Alzheimer's disease? *Exp Neurol* 167:40-47.

64. Strawn LM, McMahon G, App H, Schreck R, Kuchler WR, Longhi MP, Hui TH, Tang C, Levitzki A, Gazit A, Chen I, Keri G, Orfi L, Risau W, Flamme I, Ullrich A, Hirth KP, Shawver LK (1996) Flk-1 as a target for tumor growth inhibition. *Cancer Res* 56: 3540-3545.

65. Studer L, Csete M, Lee SH, Kabbani N, Walikonis J, Wold B, McKay R (2000) Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J Neurosci* 20:7377-7383.

66. Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA (2003) VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J Clin Invest* 111: 1843-1851.

67. Uchida K, Uchida S, Nitta K, Yumura W, Marumo F, Nihei H (1994) Glomerular endothelial cells in culture express and secrete vascular endothelial growth factor. *Am J Physiol* 266:F81-88.

68. van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. *Nature* 415: 1030-1034.

69. Wachs FP, Couillard-Despres S, Engelhardt M, Wilhelm D, Plötz S, Vroemen M, Klucken J, Karl C, Tebbing J, Svendsen C, Weidner N, Kuhn HG, Winkler J, Aigner L (2003) High efficacy of clonal growth and expansion of adult neural stem cells. *Lab Invest* 7:949-962.

70. Williams RW, Rakic P (1988) Three-dimensional counting: an accurate and direct method to estimate numbers of cells in sectioned material. *J Comp Neurol* 278:344-352.

71. Winner B, Cooper-Kuhn CM, Aigner R, Winkler J, Kuhn HG (2002) Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. *Eur J Neurosci* 16:1681-1689.

72. Yang K, Cepko CL (1996) Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. *J Neurosci* 16:6089-6099.

73. Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, Lin CS, Nikodem VM, Hempstead B, Flanders KC, Costantini F, Noguchi CT (2002) Erythropoietin receptor signalling is required for normal brain development. *Development* 129:505-516.

74. Zhu Y, Jin K, Mao XO, Greenberg DA (2003) Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. *Faseb J* 17:186-193.

75. Ziegler BL, Valtieri M, Porada GA, De Maria R, Muller R, Masella B, Gabbianelli M, Casella I, Pelosi E, Bock T, Zanjani ED, Peschle C (1999) KDR receptor: a key marker defining hematopoietic stem cells. *Science* 285:1553-1558.