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# VEGF overexpression induces post-ischaemic neuroprotection, but facilitates haemodynamic steal phenomena

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## Summary

**Therapeutic angiogenesis with vascular endothelial growth factor (VEGF) is a clinically promising strategy in ischaemic disease. The pathophysiological consequences of enhanced vessel formation, however, are poorly understood. We established mice overexpressing human $VEGF_{165}$ under a neuron-specific promoter, which exhibited an increased density of brain vessels under physiological conditions and enhanced angiogenesis after brain ischaemia. Following transient intraluminal middle cerebral artery (MCA) occlusions, VEGF overexpression significantly alleviated neurological deficits and infarct volume, and reduced disseminated neuronal injury and caspase-3 activity, confirming earlier observations that VEGF has neuroprotective properties. Brain swelling was not influenced in VEGF-overexpressing animals, while sodium fluorescein extravasation was moderately increased, suggesting that VEGF induces a mild blood–brain barrier leakage. To elucidate whether enhanced angiogenesis improves regional cerebral blood flow in the ischaemic brain, [$^{14}$C]iodoantipyrine autoradiography was performed. Autoradiographies revealed that VEGF induces haemodynamic steal phenomena with reduced blood flow in ischaemic areas and increased flow values only outside the MCA territory. Our data demonstrate that VEGF protects neurons from ischaemic cell death by a direct action rather than by promoting angiogenesis, and suggest that strategies aiming at increasing vascular density in the whole brain, e.g. by VEGF overexpression, may worsen rather than improve cerebral haemodynamics after stroke.**

**Keywords**: vascular endothelial growth factor; angiogenesis; neuroprotection; stroke; vascular permeability

**Abbreviations**: ACA = anterior cerebral artery; BBB = blood–brain barrier; CBF = cerebral blood flow; LDF = laser Doppler flow; MCA = middle cerebral artery; NMDAR = *N*-methyl-D-aspartate receptor; NSE = neuron-specific enolase; TUNEL = terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling; VEGF = vascular endothelial growth factor

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## Introduction

Vascular endothelial growth factor-A (VEGF-A or VEGF) is a homodimeric angiogenic growth factor existing in at least five isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$), which differ in their capability to bind heparin-rich matrix components and receptors (Robinson and Stringer, 2001). VEGF is ubiquitously expressed in the brain mainly by choroid plexus epithelial cells, but also by astrocytes and neurons (Monacci *et al*., 1993; Marti and Risau, 1998). In brain hypoxia and ischaemia, VEGF expression is induced through transcriptional activation via hypoxia-inducible factor (HIF)-1 and HIF-2 (Forsythe *et al*., 1996; Ema *et al*., 1997; Marti and Risau, 1998; Marti *et al*., 2000).

Therapeutic angiogenesis with VEGF appears to be promising in limb ischaemia, coronary heart disease and ischaemic stroke (Folkman, 1998; Isner and Asahara, 1999; Marti and Risau, 1999). Initial clinical trials have been encouraging

(Baumgartner *et al*., 1998; Hendel *et al*., 2000) and led to the 'vascular endothelial growth factor in ischemia for vascular angiogenesis' (VIVA) trial, a double blind, placebo-controlled study investigating the efficacy of VEGF in patients with chronic myocardial ischaemia (Henry *et al*., 2003). Although there was no evidence for an amelioration of myocardial perfusion within 60 days treatment, patients receiving high-dose VEGF had a significant improvement in their angina class by day 120 (Henry *et al*., 2003).

A number of reasons encourage VEGF treatment after stroke. First, VEGF is naturally expressed in the brain and upregulated by hypoxia (Marti and Risau, 1998; Marti *et al*., 2000) and, thus, is part of an endogenous adaptive system to protect the brain from ischaemia (Marti *et al*., 2000). Secondly, VEGF delivery increases capillary density in the ischaemic brain (Zhang *et al*., 2000; Sun *et al*., 2003) and thereby might improve cerebral blood flow (CBF) in haemodynamically compromised brain areas. Thirdly, numerous *in vitro* studies have demonstrated that VEGF is a potent neurotrophic factor which also confers protection to injured neurons (Silverman *et al*., 1999; Sondell *et al*., 1999; Jin *et al*., 2000; Marti, 2002). As a consequence, local intracerebroventricular delivery of VEGF recently has been shown to decrease brain injury after transient focal ischaemia (Sun *et al*., 2003).

Potentially detrimental effects, on the other hand, might compromise beneficial actions of VEGF. For example, VEGF has been involved in the development of vascular leakage after brain hypoxia as causative factor (Schoch *et al*., 2002). In fact, early post-ischaemic systemic delivery of VEGF increased blood–brain barrier (BBB) leakage and tissue damage after stroke, while VEGF antagonization with the high-affinity VEGF-binding protein mFlt(1–3)-IgG improved neurological outcome as well as reduced brain oedema and infarct size (van Bruggen *et al*., 1999; Zhang *et al*., 2000).

At present, it is unknown whether VEGF-induced angiogenesis indeed improves cerebral haemodynamics after stroke. The latter assumption, however, is critical for the concept of therapeutic angiogenesis. To elucidate the consequences of increased vessel formation, we have established transgenic mice overexpressing VEGF in the brain, using a cDNA encoding the human $VEGF_{165}$ isoform under control of two neuron-specific promoters. These mice developed an increased global density of morphologically and functionally normal capillaries, without concomitant vascular leakage under physiological conditions (Vogel *et al*., 2004). We now submitted these mice to focal cerebral ischaemia, using intraluminal middle cerebral artery (MCA) occlusions of two different durations (90 or 30 min), and examined the effects of brain-selective VEGF overexpression on injury development, BBB dysfunction, angiogenesis and cerebral haemodynamics.

## Materials and methods

### *Construction of plasmids*

The neuron-specific enolase (NSE)-$VEGF_{165}$ plasmid was constructed by insertion of a 1794 bp *Ecl*136II–*Hin*dIII rat NSE promoter fragment (Forss-Petter *et al*., 1990) into the *Hin*cII- and *Hin*dIII-digested plasmid pVRBpA, containing a 587 bp human $VEGF_{165}$ cDNA and the 281 bp bovine growth hormone poly(A) signal sequence. The plasmid pVRBpA was constructed by excision of the human $VEGF_{165}$ cDNA from the plasmid CMV-hVEGF by *Eco*RI and *Eco*RV digestion and insertion into the plasmid pPGKneobpA which contained, after digestion with *Xba*I, filling in overhanging ends and digestion with *Eco*RI, the bovine growth hormone poly(A) signal sequence. Subsequently, a 2700 bp fragment, containing the rat NSE promoter, the human $VEGF_{165}$ cDNA and the poly(A) signal sequence (Fig. 1A), was cleaved from the plasmid backbone by digestion with *Acc*65I and *Not*I, and the gel-isolated fragment was purified by Qiaex (Qiagen, Hilden, Germany). The *N*-methyl-D-aspartate receptor (NMDAR)-$VEGF_{165}$ plasmid was constructed by inserting a 870 bp fragment containing the human $VEGF_{165}$ cDNA and the bovine growth hormone poly(A) signal sequence, which was prepared by digesting the plasmid pVRBpA with *Eco*RI, filling in overhanging ends and digestion with *Not*I, into the plasmid pMK40. The plasmid pMK40, which contains a 5.5 kb genomic fragment of the $\varepsilon_2$ subunit of the mouse NMDAR spanning from the promoter to intron 3 (Klein *et al*., 1998), was digested with *Kas*I and, after filling in overhanging ends, further digested with *Not*I, leaving a 2300 bp fragment spanning from the promoter to the end of exon 3. Subsequently, a 3100 bp fragment, containing the NMDAR $\varepsilon_2$ genomic fragment, the human $VEGF_{165}$ cDNA and the poly(A) signal sequence (Fig. 1A), was cleaved from the plasmid backbone by digestion with *Xho*I, and purified as described above.

### *Generation and characterization of VEGF-overexpressing mice*

The purified linearized DNA was microinjected into the pronuclei of fertilized mouse oocytes, isolated from superovulated (C57BL/6 × C3H/He)$F_1$ hybrid mice. Microinjected oocytes were then implanted into pseudopregnant females of the same hybrid mouse strain. After screening DNA samples of the litters, we bred transgenic founders with C57BL/6 wild-type mice (Harlan, Borchen, Germany) to establish the transgenic lines TgN(NSEVEGF)1651–1653 (V1–V3) and TgN(NMDARVEGF)1654–1658 (V4–V8). Detection of the human VEGF transgene was performed by *in situ* hybridization and enzyme-linked immunosorbent assay (ELISA).

### *Polymerase chain reaction (PCR)-based screening for VEGF transgene*

Genomic DNA was prepared from mouse tail biopsies, and genotyping was performed by PCR analysis on a Perkin Elmer Thermal Cycler using *Taq* polymerase (Promega) and the primer pair HVBPA405 (5′-AGGAGAGATGAGCTTCCTACAG-3′) and RV1B (5′-GATGGCTGGCAACTAGAAGGCAC-3′). Reaction conditions were as follows: 94°C for 1 min for initial denaturation; 30 cycles of: 94°C for 1 min, 65°C for 1 min, 72°C for 2 min, followed by a final elongation at 72°C for 6 min. The product of 277 bp was visualized on the agarose gel with ethidium bromide.

### In situ *hybridization*

*In situ* hybridizations were performed using [$^{35}$S]UTP-labelled RNA probes for mouse VEGF (cross-reacting with human VEGF) as described (Breier *et al*., 1992; Marti and Risau, 1998).

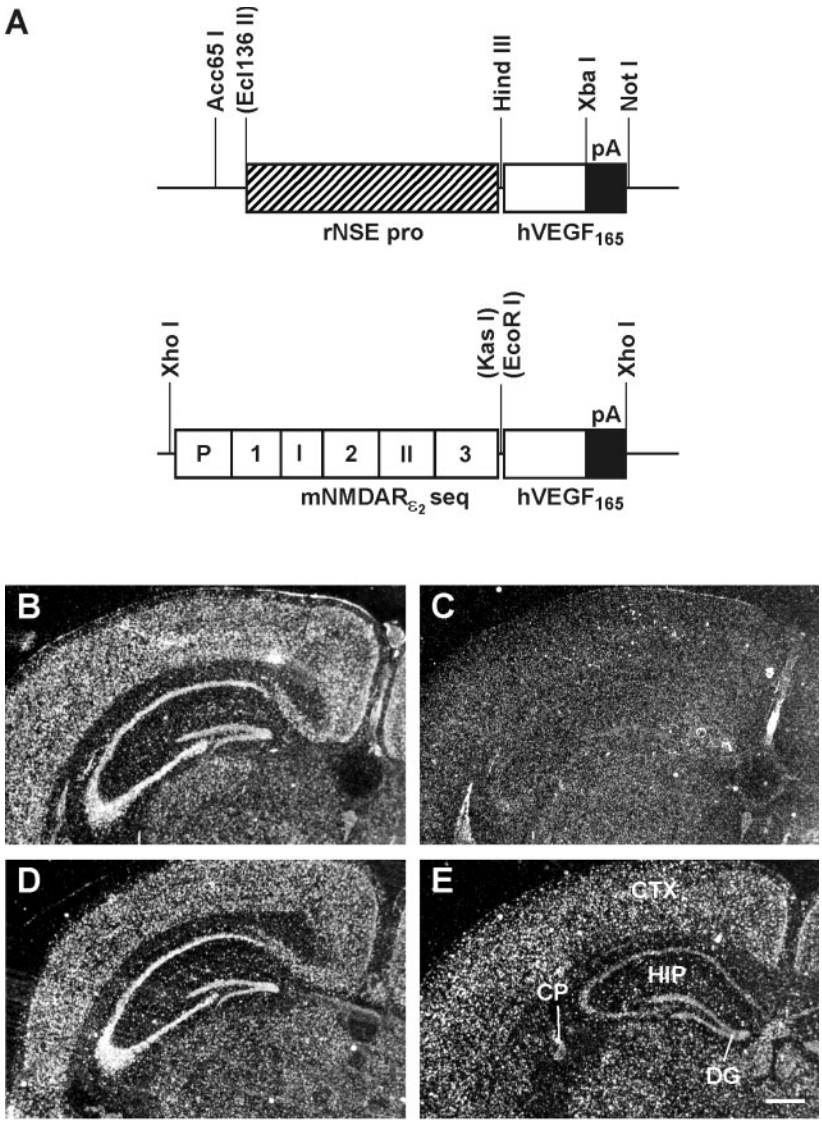


**Fig. 1** Generation and initial characterization of VEGF transgenic mice. (**A**) Brain-specific transcription of human $VEGF_{165}$ is controlled by the rat NSE promoter or regulatory sequences spanning from the promoter (P), to exon 1 and 2 (1, 2), the first two introns (I, II) up to exon 3 (3) of the mouse NMDAR $\varepsilon_2$ gene in the transgenic constructs. (**B–E**) Brain-specific expression was checked by *in situ* hybridization analysis in brains from the NSE-VEGF transgenic line V1 (**B**, **C** and **E**) and the NMDAR-VEGF line V6 (**D**) with a $^{35}$S-labelled riboprobe recognizing both mouse and human VEGF mRNA. Specific hybridization signals are present in dentate gyrus (DG), hippocampus (HIP) and cortex (CTX) of transgenic animals (**B** and **D**), but not of non-transgenic littermates (V1, **E**), where endogenous mouse VEGF expression in epithelial cells of the choroid plexus (CP) and in astrocytes is shown. No specific signal is detectable with a VEGF sense probe (V1, **C**). Bar = 500 μm.

### *Preparation of brain extracts and measurement of VEGF protein*

Tissue lysates from brain hemispheres were prepared as detailed before (Marti *et al*., 2000). Mouse and human VEGF was quantified using commercially available immunoassay kits specific for mouse (Quantikine M, R&D Systems) and human (Quantikine, R&D Systems) VEGF.

### *Induction of MCA occlusions*

Animal experiments were carried out with governmental approval according to NIH guidelines for care and use of laboratory animals. Adult VEGF-overexpressing mice (transgenic; derived from the V1 line) and their non-transgenic littermates (21–28 g) were anaesthetized with 1% halothane (30% $O_2$, remainder $N_2O$) [$n$ = 11/group (90 min ischaemia) and $n$ = 7–8/group (30 min MCA occlusion)]. Rectal temperature was maintained between 36.5 and 37.0°C using a feedback-controlled heating system. Focal ischaemia was induced using an intraluminal technique as previously described (Hata *et al*., 2000; Hermann *et al*., 2001*a*), using a 8-0 silicon-coated (Xantopren: Bayer Dental, Osaka, Japan) nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany). During the experiments, laser Doppler flow (LDF) was monitored using a flexible 0.5 mm fibreoptic probe (Perimed, Stockholm, Sweden) attached to the intact skull overlying the MCA territory. LDF changes were measured during ischaemia

and up to 30 min after reperfusion onset. At that time, anaesthesia was discontinued. After 24 (90 min MCA occlusion) or 72 h (30 min ischaemia) of reperfusion, neurological deficits were evaluated using a 5-point neurological deficit score ranging from 0 = normal function to 4 = absence of spontaneous motor activity (Hata *et al*., 1998).

### *Sodium fluorescein extravasation*

Immediately thereafter, one subset of animals [$n$ = 6/group (90 min MCA occlusion) and $n$ = 7–8/group (30 min ischaemia)] was re-anaesthetized with halothane. Vascular permeability was assessed with sodium fluorescein, as described (Schoch *et al*., 2002), except that fluorescence of the supernatant was measured at 485 nm at an excitation wavelength of 330 nm using a fluoroscope (GENios Pro, TECAN's advanced, multi-functional injector reader). Measured values are shown as relative fluorescence units (r.f.u.) per mg of brain tissue.

### *CBF autoradiography*

At the same time, another subset of animals submitted to 90 min ischaemia ($n$ = 5/group) received intraperitoneal injections of 0.2 μCi 4-iodo-*N*-methyl-[$^{14}$C]antipyrine (IAP) (Amersham Life Sciences, Freiburg, Germany). Two minutes later, animals were decapitated, and arterial blood samples (50–100 μl) were taken from the animals' trunks, which subsequently were measured in a liquid scintillation counter. Brains were quickly removed and frozen with dry ice. Sections of 20 μm were prepared, dried on a hot plate and exposed to X-ray film (Amersham Hyperfilm) with calibrated $^{14}$C-labelled standards. Using an image processing system (ImageMG, NIH, Bethesda, MD), CBF was calculated, as previously described (Maeda *et al*., 2000; Kilic *et al*., 2001).

### *Cresyl violet staining*

Coronal brain sections from five equidistant rostrocaudal brain levels, 2 mm apart, were submitted to cresyl violet staining ($n$ = 11/group). Sections were digitized, and the border between infarcted and non-infarcted tissue was outlined using an image analysis system (Image J). On these sections, the areas of infarction, brain swelling and infarct volume were determined, as previously described (Kilic *et al*., 2001). Sections were also evaluated by measuring the distance of the infarct border from the midline at the level of the striatum and thalamus.

### *Terminal transferase-mediated dUTP nick end labelling (TUNEL)*

Brain sections were fixed for 20 min at 4°C with 4% paraformaldehyde/0.1 M phosphate-buffered saline (PBS). TUNEL staining was then performed, as previously described (Hermann *et al*., 2001*b*). Shortly after labelling with terminal deoxynucleotidyl transferase (TDT) mix, containing 12.5 mg/ml TDT (Boehringer-Mannheim, Mannheim, Germany) and 25 mg/ml biotinylated dUTP (Boehringer-Mannheim), sections were stained with fluorescein isothiocyanate (FITC)-conjugated streptavidin, counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and coverslipped. Sections were analysed by counting DNA fragmented cells in rectangular fields measuring 25 000 μm$^2$ in six regions of interest in the striatum as previously described (Hermann *et al*., 2001*b*). Mean values were calculated for these areas.

### *Immunohistochemistry*

Sections were fixed in ice-cold acetone (CD31, VEGF receptor-2) or 4% paraformaldehyde/0.1 M PBS (activated caspase-3), washed and immersed for 1 h in 0.1 M PBS containing 0.3% Triton (PBS-T)/10% normal goat serum. Sections were incubated overnight at 4°C with the following primary antibodies: (i) monoclonal rat anti-mouse CD31 (catalogue number 557355; BD Biosciences, diluted 1 : 100); (ii) monoclonal rat anti-mouse VEGF receptor-2 (Marti *et al*., 2000) (diluted 1 : 100); or (iii) polyclonal rabbit anti-mouse activated caspase-3 (Hermann *et al*., 2001*b*) (diluted 1 : 100). After washing, sections were incubated in secondary Cy-3 antibodies, counterstained with DAPI and coverslipped. Sections were evaluated by counting the number of CD31- and VEGF receptor-2-positive vessel profiles or caspase-3-positive cells in rectangular fields, measuring 500 000 and 25 000 μm$^2$, respectively.

### *Visualization of the anastomotic line between the MCA and anterior cerebral artery (ACA)*

Further animals ($n$ = 3/group) were anaesthetized with halothane and injected with a lethal dose of papaverine hydrochloride (50 mg/kg in sterile water) over the right femoral vein. The thoracic aorta was clipped and the ascending aorta cannulated with PE10 tubing. Warm (38.0°C) latex (Maeda *et al*., 1999) mixed with carbon black (10 μl/g) was injected through the cannula with slight pressure (0.3 ml/animal over 20 s). After 10 min, animals were decapitated and their brains removed. Photographs were taken of the dorsal brain surfaces, which allowed localization of the anastomotic lines between the MCA and ACA territories by tracing peripheral branches to the points at which vessels were connected, as previously described (Maeda *et al*., 1999). The distance from the midline to the line of anastomosis was measured at coronal planes 4 and 6 mm from the frontal pole, respectively. These levels corresponded closely to the striatal and thalamic levels, at which infarct areas had been evaluated.

### *Statistics*

All measurements were performed by two independent investigators blinded to the experimental conditions. For statistical analyses, a standard software package (SPSS for Windows 10.1) was used. Values are given as means ± SD. Differences between groups were compared by using two-tailed $t$ tests. $P$ values <0.05 were considered significant.

## Results

### *Establishment of transgenic lines*

To study the effects of elevated VEGF levels in the brain, we designed a transgenic mouse model system in which VEGF expression is specifically increased in neurons driven by the rat NSE promoter or regulatory sequences derived from the gene for the $\varepsilon_2$ subunit of the mouse NMDAR (Fig. 1A). Three rounds of pronuclei microinjections yielded eight transgenic NSE-VEGF and seven NMDAR-VEGF founders which were confirmed by PCR of tail DNA. Three of the NSE-VEGF

**Table 1** *Human VEGF$_{165}$ transgene and endogenous mouse VEGF expression*

| Promoter | Line | tg/ntg | hVEGF | *n* | mVEGF | *n* |
|---|---|---|---|---|---|---|
| NSE | V1 | tg | 21.74 ± 1.38 | 4 | 1.52 ± 0.27** | 3 |
| | | ntg | 0.40 ± 0.62 | 5 | 3.48 ± 0.74 | 5 |
| | V2 | tg | 4.90 ± 1.68 | 7 | NA | |
| | | ntg | ND | 1 | NA | |
| | V3 | tg | 16.57 ± 4.04 | 5 | 2.52 ± 1.35 | 5 |
| | | ntg | 0.29 ± 0.57 | 4 | 3.40 ± 0.89 | 3 |
| NMDAR | V4 | tg | 1.14 ± 0.78 | 8 | 3.05 ± 1.44 | 6 |
| | | ntg | 0.51 ± 0.62 | 6 | 3.39 ± 2.31 | 4 |
| | V5 | tg | 2.57 ± 2.33 | 3 | NA | |
| | | ntg | ND | 2 | NA | |
| | V6 | tg | 2.19 ± 0.93 | 8 | 3.12 ± 1.13 | 6 |
| | | ntg | 0.68 ± 0.88 | 3 | 3.03 ± 1.18 | 3 |
| | V7 | tg | 0.96 ± 0.21 | 3 | NA | |
| | | ntg | ND | 3 | NA | |
| | V8 | tg | 1.15 ± 1.0 | 3 | NA | |
| | | ntg | ND | 2 | NA | |

Human and mouse VEGF protein levels (ng/g total protein) were quantified in the brain of non-transgenic (ntg) and transgenic (tg) mice from all generated mouse lines (V1–V8) using commercially available ELISA kits. Means ± SD (**$P < 0.01$ versus non-transgenic control). NA = not analysed; ND = not detectable.

founders and five of the NMDAR-VEGF founders transmitted the transgene to their offsprings and were used to establish eight distinct heterozygous breeding lines: TgN(NSEVEGF) 1651–1653 (V1–V3) and TgN(NMDARVEGF) 1654–1658 (V4–V8) (Table 1). PCR analysis of living offspring revealed that 60% of heterozygous mice from the NSE-VEGF lines V1 and V3 were non-transgenic, while only 40% of the offsprings were transgenic, indicative of some embryonic lethality in these two lines. Transgenic offsprings derived from all other lines were born at equal frequency compared with their non-transgenic littermates (data not shown).

## *Characterization of transgenic mice*

Offspring from all eight lines were analysed for VEGF protein expression in the brain. ELISA of human VEGF in brain tissue showed a wide variation in level of expression (Table 1), depending on the promoter type, and presumably transgene copy number and integration site. Mean mouse VEGF protein level in non-transgenic animals (3.3 ± 1.3 ng of VEGF/g total protein) was comparable in all lines examined, except for line V1, in which mouse VEGF levels differed between transgenic and non-transgenic animals (Table 1), indicative of attenuation of endogenous VEGF production when human transgene levels are high. Quantitatively, the maximal level of total VEGF expression in the brain with each of the two promoters increased by 650% in line V1 and 70% in line V6, respectively, compared with the endogenous mouse VEGF protein level. Animals originating from the five highest expressing lines V1–V3, V5 and V6 were analysed further for transgene mRNA expression in adult brain. *In situ* hybridization using a human VEGF probe revealed a cross-reactivity with the endogenous mouse VEGF mRNA. Expression analysis showed that in all transgenic animals analysed, VEGF was targeted appropriately to neuronal cells and strongly expressed in dentate gyrus and hippocampus, but was also found in cortex (Fig. 1B and D). In addition, a scattered expression all over the brain was detected, which was also present in non-transgenic control mice (Fig. 1E), thus representing the endogenous mainly astrocytic expression of mouse VEGF mRNA as described before (Marti and Risau, 1998; Schoch *et al*., 2002). All subsequent work was performed in heterozygous transgenic animals and non-transgenic littermates derived from the highest VEGF-expressing NSE-VEGF line V1. Non-transgenic and transgenic animals exhibited normal growth and development without detectable anatomical, physiological or behavioural abnormalities (Vogel *et al*., 2004). Physiological monitoring with regard to body weight, blood gases and pH, haematocrit, heart rate and arterial blood pressure revealed no significant differences between the experimental groups (Vogel *et al*., 2004).

## *LDF during and after ischaemia*

LDF measurements during and after 90 and 30 min of MCA occlusion exhibited no differences between transgenic and non-transgenic mice. In all animal groups, thread insertion resulted in a decline of blood flow to ∼25% of pre-ischaemic levels. After thread retraction, blood flow rapidly reached pre-ischaemic values in animals submitted to 90 min ischaemia. In animals subjected to 30 min ischaemia, a hyperperfusion reaction was found shortly after reperfusion (to ∼120–140% of pre-ischaemic levels).

## *VEGF overexpression attenuates post-ischaemic neurological deficits and ischaemic injury*

Reproducible neurological deficits and brain infarcts were noticed after 90 min MCA occlusion. VEGF overexpression significantly reduced infarct volume (Fig. 2A and B) and improved neurological abnormalities (Fig. 2C) at 24 h after stroke. To rule out that differences in infarct size in transgenic mice were due to macroscopic changes in the size of the MCA territory which exist in different mouse strains (Maeda *et al*., 1999), animals received latex infusions to assess the localization of anastomotic lines between the MCA and ACA territories (Fig. 3A). Latex infusions revealed that anastomotic lines did not differ between transgenic and non-transgenic mice, either at the striatal or at the thalamic level (Fig. 3B), demonstrating that the observed differences in the localization of the infarct border (Fig. 3C) were indeed caused by VEGF overexpression.

After 30 min of ischaemia, neurological deficits were always mild irrespective of the genotype of the mice (deficit score $\leqslant 1$ in all animals). However, widespread neuronal injury was detected in the striatum, but not the overlying

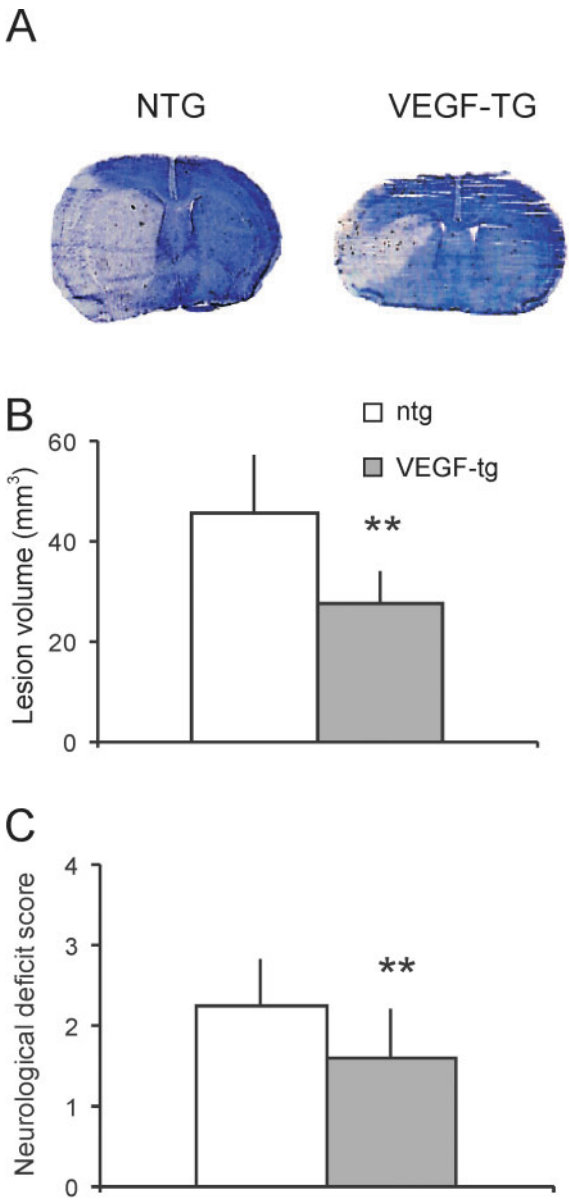


**Fig. 2** Brain-specific overexpression of VEGF reduces infarct size and improves neurological deficits. Mice underwent 90 min of MCA occlusion followed by 24 h reperfusion, and infarct size was measured by cresyl violet staining (**A**). Infarct quantification revealed significantly reduced infarct volumes in VEGF transgenic mice (VEGF-tg) compared with non-transgenic controls (ntg) (**B**). The smaller infarcts were associated with a significant reduction in neurological deficit scores (**C**). Values are means ± SD ($n$ = 11 animals/group). $^{**}P < 0.01$ compared with non-transgenic mice.

cortex at 3 days of reperfusion. VEGF overexpression significantly reduced the density of injured cells in the striatum, as judged from the reduced number of TUNEL-positive, i.e. DNA fragmented, cells. Quantification revealed a 41% reduction in the density of DNA fragmented cells in transgenic mice (Fig. 4A). This tissue protection coincided with a reduction of caspase-3 activity in the same area (Fig. 4B). Thus, VEGF overexpression resulted in neuroprotection after 24 h which was sustained after 3 days and accompanied by improvements in neurological behaviour.

## *VEGF causes a mild BBB dysfunction*

As VEGF is a potent inducer of vascular leakage leading to brain oedema formation, we next analysed histological brain swelling indicating major damage of the BBB as well as BBB permeability. To this end, sodium fluorescein was injected as a permeability marker at 24 h and 3 days after ischaemia, and animals were sacrificed 30 min thereafter. Brain swelling was not influenced by VEGF overexpression (Fig. 5A). However, sodium fluorescein extravasation revealed an increased BBB permeability in transgenic mice as compared with control animals both after 90 min ischaemia/24 h reperfusion (Fig. 5B) and after 30 min ischaemia/3 days reperfusion (Fig. 5C), although the absolute level of BBB damage was far less pronounced in the latter group.

## *VEGF overexpression and angiogenesis*

Vascular density was significantly increased in non-ischaemic transgenic mice compared with non-transgenic controls in both the cerebral cortex (143 ± 12 versus 97 ± 14 vessels/square; $P < 0.01$) and striatum (91 ± 10 versus 67 ± 8; $P < 0.01$), coinciding with the increased capillary density previously shown in other brain areas (Vogel *et al.*, 2004). After 90 min ischaemia/24 h reperfusion, the number of VEGF receptor-2-positive endothelial cells indicating new vessel growth was equal in transgenic and non-transgenic mice, in both the ischaemic and surrounding non-ischaemic tissue (data not shown). At 3 days reperfusion (after 30 min MCA occlusion), the density of newly formed blood vessels was higher in ischaemic compared with adjacent non-ischaemic areas; however, only in transgenic, but not non-transgenic animals (Fig. 6A). Quantification of vessel profiles revealed a significant increase in vessel density both in the cerebral cortex (Fig. 6B), and, even more pronounced, in the striatum of transgenic mice (Fig. 6C). Thus, the higher VEGF levels in the transgenic mice resulted in a faster and more pronounced angiogenic response, which was most distinct in areas exhibiting neuronal injury (i.e. the striatum).

## *Increased vessel density promotes haemodynamic steal flow*

An increased vessel density will facilitate neuronal survival only when it is associated with an increased CBF, resulting in a rise of oxygen and nutrient delivery. We therefore analysed regional CBF using the IAP autoradiography technique in transgenic and non-transgenic mice after 90 min ischaemia/24 h reperfusion. Surprisingly, we found a dramatic decrease in CBF in the ischaemic MCA territory of VEGF-overexpressing, compared with non-transgenic mice (Fig. 7). Quantitative

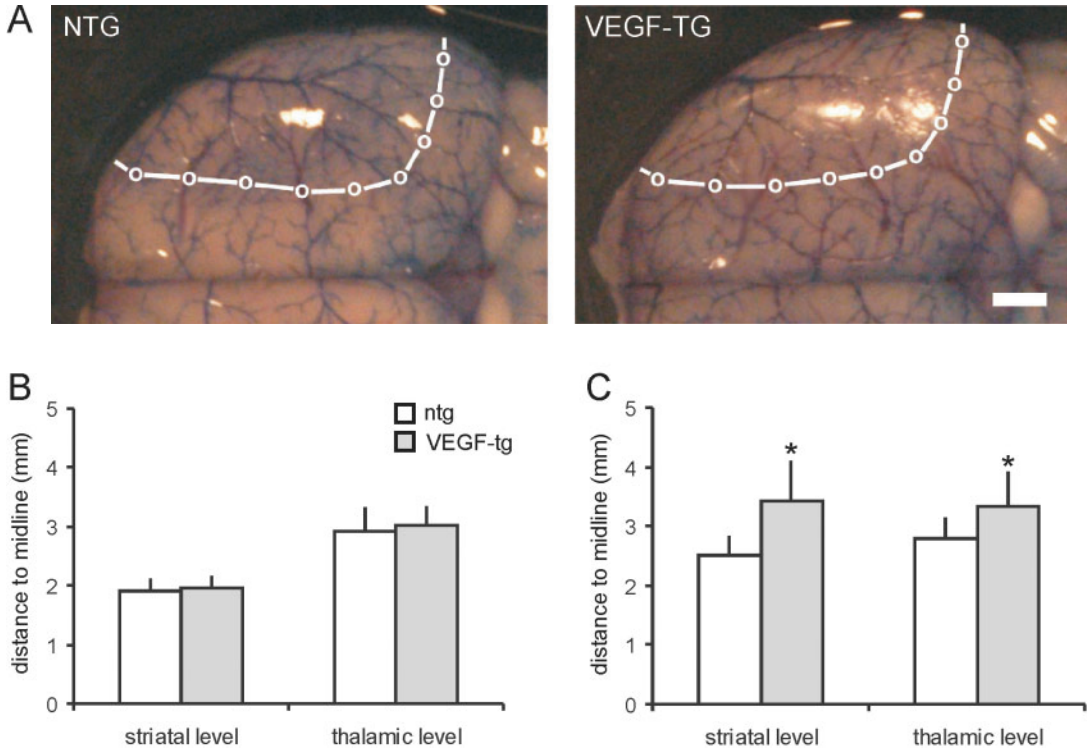


**Fig. 3** No changes in the gross anatomy of the middle cerebral artery (MCA) territory between VEGF-overexpressing and control mice. The distance from the midline of the anastomotic line between the MCA and anterior cerebral artery (ACA) vascular territory was determined at the level of the striatum and thalamus. To this end, latex mixed with carbon black was injected through the cannulated aorta. Anastomotic lines between the MCA and ACA territories were determined by tracing peripheral branches on dorsal brain surfaces of VEGF transgenic (VEGF-tg) and non-transgenic (ntg) control mice (**A**) to the points at which vessels were connected (indicated by dotted lines). The lack of difference indicates that both mouse groups have the same MCA territory size (**B**). The significant differences in the localization of the infarct border as determined by cresyl violet staining in transgenic mice compared with controls (**C**) thus reflects a neuroprotective effect of VEGF. Values are means ± SD [$n$ = 3 animals/group (latex studies) and $n$ = 11/group (cresyl violet staining)]. *$P < 0.05$ compared with non-transgenic mice. Bar = 1 mm.

analysis revealed a reduction of blood flow values in the parietal cortex by 58% (Table 2). Blood flow was also reduced in the sensory cortex by 46%. In the motor cortex, i.e. in the area close to the infarct border, CBF reduction was only 16% and not significantly different from non-transgenic animals (Table 2). Thus, the reduction in CBF appears to be most prominent in the ischaemic core, but less pronounced towards the ischaemic border. On the other hand, we noticed a significant increase in regional CBF by 59% in the non-ischaemic cingulate cortex, which is supplied by the ACA, in VEGF-overexpressing animals compared with controls (Fig. 7; Table 2). CBF in the remote basal forebrain did not differ between transgenic and non-transgenic animals (Table 2). These data suggest that the higher vessel density in transgenic animals promotes a pronounced steal of blood flow from ischaemic to non-ischaemic areas which becomes more apparent towards the core of the ischaemic area.

## Discussion

The main finding of the present study is that brain-selective VEGF overexpression provides neuroprotection against focal cerebral ischaemia, reflected by smaller infarct volume, reduced disseminated neuronal injury and better neurological outcome, although regional blood flow is significantly decreased due to a haemodynamic steal effect. Our data indicate that the beneficial effect of VEGF is due mainly to its direct neuroprotective action, i.e. by inhibition of cell death pathways, rather than angiogenic function.

### *VEGF overexpression and angiogenesis*

VEGF is the major angiogenic factor during development (Risau, 1997), but, in adaptation to tissue hypoxia, can also induce new vessel growth in the adult organism, allowing increased transportation of oxygen and nutrients to the tissue at risk (LaManna and Harik, 1997). In a model of permanent focal cerebral ischaemia, we previously have shown that VEGF gene expression increased in the penumbra as early as 6 h after the onset of ischaemia, resulting in new vessel growth in the ischaemic area within 48 h after MCA occlusion (Marti *et al*., 2000). In line with these findings, we show here that apart from the constitutively increased vessel density in transgenic mice, VEGF overexpression stimulates new vessel growth in ischaemic areas. Our data indicate a synergistic

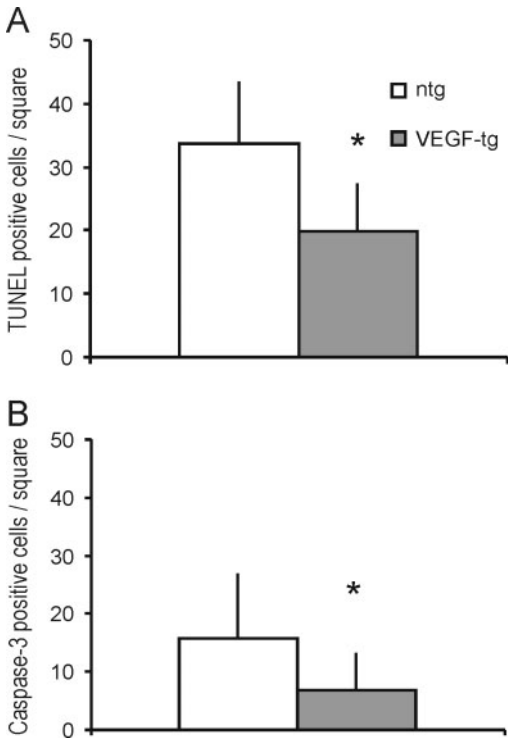


**Fig. 4** VEGF overexpression reduces disseminated neuronal cell death. Mice underwent 30 min of MCA occlusion followed by 3 days reperfusion, and disseminated cell death was analysed in the striatum by visualization of DNA strand breaks labelled by TUNEL staining and caspase-3 activation detected by staining with an antibody against activated caspase-3. Quantification revealed significantly reduced DNA fragmented (**A**) as well as activated caspase-3-positive (**B**) cells in VEGF transgenic mice compared with non-transgenic controls. Values are means ± SD ($n$ = 7–8 animals/group). *$P < 0.05$ compared with non-transgenic mice.

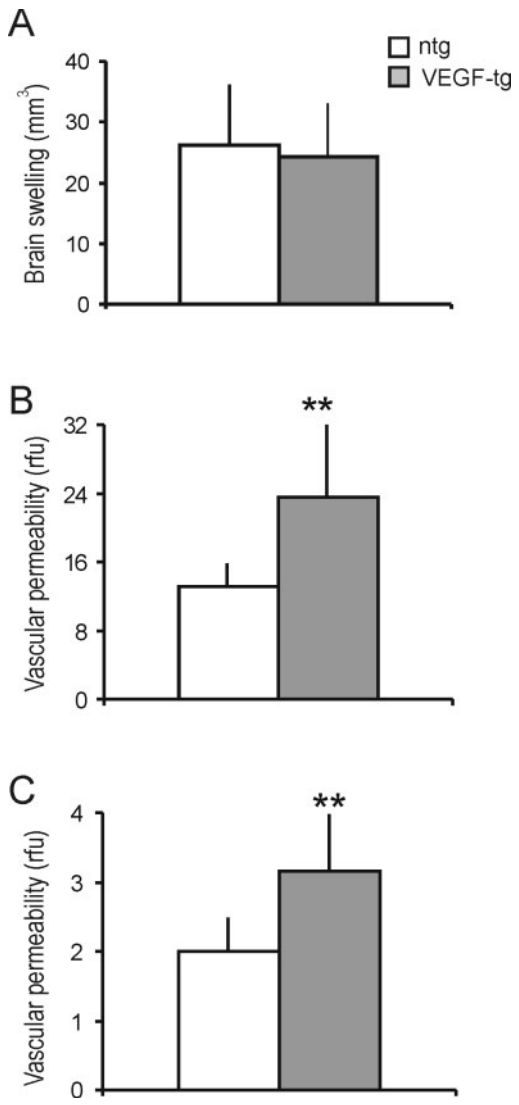


**Fig. 5** VEGF overexpression has no effect on brain swelling, but induces mild BBB dysfunction. Analysis of cresyl violet-stained brain sections after 90 min ischaemia/3 days reperfusion revealed no changes of brain swelling in VEGF transgenic mice (**A**). However, determination of subtle changes in BBB function by quantification of sodium fluorescein extravasation revealed a significant increase in vascular permeability in transgenic mice, both after 90 min MCA occlusion/24 h reperfusion (**B**) and after 30 min ischaemia/3 days reperfusion (**C**). Note that vascular leakage was less pronounced after mild ischaemia (**C**). Values are means ± SD [$n$ = 6–8 animals (sodium fluorescein) and $n$ = 11 animals (brain swelling)/group]. **$P < 0.01$ compared with non-transgenic mice.

action of transgenic human $VEGF_{165}$ and hypoxically induced mouse VEGF, thus further increasing vessel density in ischaemic areas.

VEGF-induced angiogenesis has been considered as treatment in ischaemic disease (Rivard and Isner, 1998). Preliminary clinical studies for the treatment of peripheral arterial occlusive diseases (Baumgartner *et al*., 1998) and myocardial ischaemia (Hendel *et al*., 2000) were promising; however, results from the larger VIVA trial were somewhat disappointing (Henry *et al*., 2003). A reason for the relatively poor beneficial effect might be the absence of increased blood flow in the ischaemic tissue, as suspected in this trial (Henry *et al*., 2003). We now were able to show in our mice overexpressing VEGF that a higher capillary density does not automatically result in an improvement of blood flow. It has been suggested that vascular remodelling might be suboptimal after isolated VEGF application and that additional angiogenic factors, such as angiopoietins and platelet-derived growth factor (PDGF), are needed for a normal remodelling process (Bruick and McKnight, 2001). Indeed, we previously showed that CBF in transgenic animals was scarcely increased despite a nearly 3-fold increase in capillary density (Vogel *et al*., 2004). Nevertheless after maximal vasodilatation, CBF in transgenic animals was increased to a significantly greater extent compared with controls, demonstrating that an additional vessel capacity that is provided by isolated VEGF overexpression can be recruited and results in increased organ perfusion (Vogel *et al*., 2004). However, based on our current data, we conclude that the surplus in vessel density and thus the capacity to increase CBF is ineffective in the MCA territory. On the

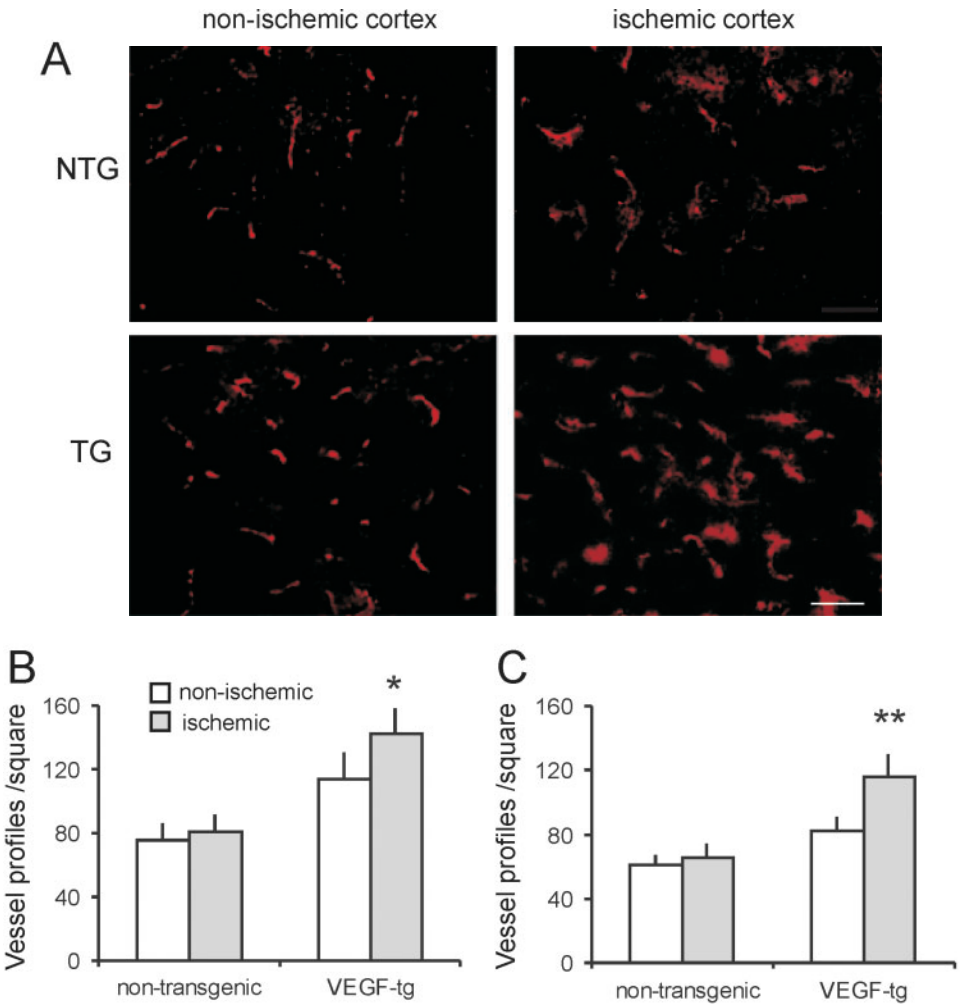


**Fig. 6** Increased angiogenesis in ischaemic brain tissue of VEGF-overexpressing mice. Formation of new blood vessels was detected after 30 min ischaemia/3 days reperfusion by immunohistochemistry for VEGF receptor-2, which mainly stains newly formed blood vessels. VEGF receptor-2-positive endothelial cells were detected in both transgenic and non-transgenic mice, in the ischaemic as well as non-ischaemic cortex (**A**). Quantification of VEGF receptor-2-positive vessels in cerebral cortex (**B**) and striatum (**C**) revealed a significantly higher number of stained vessels only in the ischaemic tissue of transgenic animals, but not in non-ischaemic areas nor in non-transgenic mice, suggestive of increased angiogenesis in ischaemic areas of VEGF-overexpressing mice. Values are means ± SD ($n$ = 6–8 animals/group). *$P$ < 0.05 and **$P$ < 0.01 compared with non-transgenic mice. Scale bar = 200 μm.

contrary, CBF in the MCA area was even significantly reduced in transgenic animals compared with non-transgenic mice, while the higher vascular density resulted in an increased CBF outside the MCA territory in the ischaemia-remote cortex, such as the cingulate cortex, which is supplied by the ACA. These results indicate that blood flow in ischaemic areas is compromised by a steal of circulating blood to non-ischaemic areas. In ischaemic stroke, such steal phenomena are well known from vasodilator therapies, e.g. with calcium antagonists, which may improve blood flow outside an ischaemic region, but can cause a deterioration in perfusion of tissue at risk (Strandgaard and Paulson, 1990). In addition, it has been shown that adenovirus-mediated application of VEGF resulted in similar steal effects (Vogel *et al*., 2003). These data indicate that therapeutic angiogenesis for stroke patients by application of VEGF is far more complex than initially thought (Greenberg, 1998; Croll and Wiegand, 2001). Taken together, our findings argue against major haemodynamic benefits of angiogenesis, at least during one single stroke event. On the other hand, stimulation of angiogenesis may be beneficial when subsequent episodes of ischaemia in the same vascular territory are expected, e.g. in the clinical situation because of localized ulcerated plaques or arterial stenosis. However, proper vascular remodelling as well as the contribution of newly formed vessels for local CBF must be carefully investigated.

### *VEGF overexpression induces post-ischaemic neuroprotection*

Our data strongly indicate a powerful direct neuroprotective function of VEGF, as brain damage after focal ischaemia is significantly attenuated in VEGF-overexpressing mice, both

**Table 2** *Quantification of regional CBF in VEGF-overexpressing mice (tg) and controls (ntg)*

| | Cingulate cortex | Motor cortex | Sensory cortex | Parietal cortex | Dorsolateral striatum | Ventromedial striatum | Basal forebrain |
|---|---|---|---|---|---|---|---|
| ntg | 69.0 ± 29.9 | 75.3 ± 22.6 | 113.2 ± 34.9 | 75.5 ± 47.1 | 43.8 ± 44.4 | 57.1 ± 59.9 | 80.7 ± 19.5 |
| tg | 109.6 ± 36.1* | 63.2 ± 32.1 | 60.6 ± 31.6* | 32.0 ± 10.5* | 28.4 ± 38.6 | 27.3 ± 37.9 | 79.5 ± 36.3 |

Quantification of regional CBF was calculated from IAP autoradiographies. Values are mean ± SD ($n$ = 5 animals/group); *$P < 0.05$ compared with non-transgenic mice.

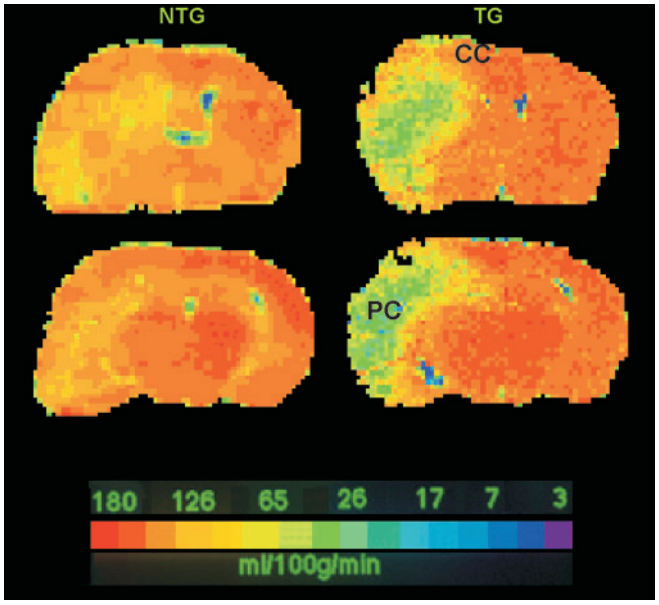


**Fig. 7** VEGF overexpression induces haemodynamic steal phenomena in the ischaemic brain. Regional CBF in various brain areas of non-transgenic and VEGF-overexpressing mice submitted to 90 min MCA occlusion/24 h reperfusion was determined by 4-iodo-*N*-methyl-[$^{14}$C]antipyrine autoradiography. Blood flow was markedly reduced in the ischaemic parietal cortex (PC), and increased in the non-ischaemic cingulate cortex (CC) in VEGF transgenic mice.

after longer lasting ischaemia (90 min) leading to brain infarction and after mild ischaemia (30 min) associated with disseminated neuronal injury. The protective effect of VEGF is associated with an inhibition of caspase-3 activity, suggesting that VEGF inhibited executive cell death pathways, in line with previous studies showing neuroprotection after VEGF treatment, both *in vitro* (Matsuzaki *et al*., 2001) and *in vivo* (Sun *et al*., 2003). It appears that VEGF receptor-2 as well as neuropilin-1 are transducing the protective signal (Matsuzaki *et al*., 2001), possibly leading to subsequent activation of phosphatidylinositol 3-kinase/Akt and extracellular-regulated kinase (ERK-1/-2) pathways (recently reviewed by Marti, 2002; Rosenstein and Krum, 2004). Recent data suggest that VEGF can also act on the neuronal microtubular content, which is involved with growth, stability and maturation of neural cells (Rosenstein *et al*., 2003). Taken together, VEGF could play a significant role in brain repair and emerges more and more as a central player in neurodegenerative disorders (Storkebaum and Carmeliet, 2004) with potent direct neuroprotective (Marti, 2002; Sun *et al*., 2003) and neurotrophic (Sondell *et al*., 1999; Rosenstein *et al*., 2003) functions.

## *VEGF overexpression causes mild BBB dysfunction*

Finally, when VEGF therapy for stroke patients is considered, the potent permeability-inducing effect of this factor (Senger *et al*., 1983) must be borne in mind. Oedema formation depends on VEGF dosage, application time and localization (topical or systemic), as well as on concomitant activation of VEGF receptors. In our VEGF transgenic animals, cerebral vessels appear tight and non-leaky (Vogel *et al*., 2004), probably due to the absence of VEGF receptor activation which is commonly associated with vasogenic oedema formation (Croll and Wiegand, 2001). Similarly, chronic intraventricular infusion of VEGF into normal non-ischaemic rat brain resulted in a dose-dependent increase in vessel density and vascular permeability, though without a corresponding increase of brain water content (Harrigan *et al*., 2002). Accordingly, we were unable to detect detrimental effects of VEGF overexpression on brain oedema formation after MCA occlusion although enhanced angiogenesis mildly increased BBB leakage as assessed by sodium fluorescein. The degree of vascular leakage was comparable with the effect observed when mice were subjected to severe systemic hypoxia (Schoch *et al*., 2002). It is difficult to predict whether clinical CNS symptoms associated with hypoxia, e.g. during exposure to high altitude (Hackett and Roach, 2001), could be the consequence of such a mild BBB dysfunction. BBB opening can disturb local ion concentrations and thus might interfere with proper neural function (Bradbury, 1993). Behavioural tests with VEGF transgenic mice, however, revealed no differences when compared with non-transgenic littermates (unpublished own observation), arguing against a significant effect of the mild BBB opening. Gross breakdown of the BBB will certainly cause vasogenic cerebral oedema and even result in the transmigration of activated leukocytes (Petty and Lo, 2002). In fact, when high doses of VEGF were delivered to the brain of normal rats, detrimental oedema formation causing animal death has been observed (Vogel *et al*., 2003).

Several reports deal with the effect of VEGF application to the CNS. It appears that application time and localization play crucial roles in the outcome after cerebral ischaemia.

Topical application of VEGF on the surface of the reperfused rat brain after 90 min MCA occlusion resulted in a significant reduction of infarct volume and oedema formation, associated with reduced neuronal damage (Hayashi *et al*., 1998). Local VEGF levels may not suffice to increase vascular permeability in sufficient microvessels to cause oedema formation. In analogy, a recent study has shown that local tissue concentrations of VEGF critically determine whether proper angiogenesis takes place or abnormal vasculature is formed (Ozawa *et al*., 2004), pointing to the importance of having the right amount of VEGF at the right place. Beneficial effects of systemic VEGF application, on the other hand depend on the time point of treatment.

When VEGF was delivered in the acute stage after stroke, the BBB was damaged and this damage may have compromised the beneficial neuroprotective actions of VEGF (van Bruggen *et al*., 1999). Late administration of VEGF, i.e. on days 1–3 of reperfusion, however, improved neurological outcome (Zhang *et al*., 2000; Sun *et al*., 2003). Finally, 6–12 days of VEGF pre-treatment using an intraventricularly applied adeno-associated virus gene transfer system was associated with an improvement of global brain ischaemia injury in gerbils (Bellomo *et al*., 2003).

All these data indicate the crucial importance of proper application time and dosage of VEGF (Zhang *et al*., 2000). It appears that the acute early increase in VEGF levels provokes oedema formation resulting in a detrimental outcome, while delayed activation or moderately augmented but steady VEGF levels favour its neuroprotective effect. The detailed study of acute changes elicited by exogenous VEGF application, however, will require transgenic mice with inducible VEGF expression in the brain (Dor *et al*., 2002).

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