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Cell Type-Specific Expression of Neuropilins in an MCA-Occlusion Model in Mice Suggests a Potential Role in Post-Ischemic Brain Remodeling

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Abstract. Neuropilin-1 and -2 (NP-1/NP-2) are transmembrane receptors that play a role in axonal guidance by binding of class III semaphorins, and in angiogenesis by binding of the vascular endothelial growth factor isoform $VEGF_{165}$ and placenta growth factor (PLGF). We investigated the expression pattern of NP-1/NP-2, their co-receptors, vascular endothelial growth factor receptor-1 and -2 (VEGFR-1, VEGFR-2), and their ligands, class III semaphorins, VEGF and PLGF, following experimental cerebral ischemia in mice. By means of in situ hybridization and immunohistochemistry we observed loss of expression of class III semaphorins in neurons in the infarct/peri-infarct area. In contrast, we observed high expression of NP-1 in vessels, neurons, and astrocytes surrounding the infarct. VEGF and PLGF were upregulated in different cell types following stroke. Our results suggest a shift in the balance between semaphorins and VEGF/PLGF, which compete for NP-binding. Possibly, the loss of semaphorins facilitates binding of the competing ligands (VEGF/PLGF), thus inducing angiogenesis. In addition, the observed expression patterns further suggest a neurotrophic/neuroprotective role of VEGF/PLGF.

Key Words: Angiogenesis; Class III semaphorins; Neuropilin; Placenta growth factor; Stroke; Vascular endothelial growth factor.

INTRODUCTION

Experimental models and studies in stroke patients have shown that ischemia is associated with growth of new blood vessels (1–4) and with neuronal remodeling (5, 6). Interestingly, on the molecular level, the molecules regulating angiogenesis and neuronal regeneration partly overlap. Neuropilin receptors (NPs) for example are expressed on endothelial cells and on neurons. Neuronal NPs are involved in axonal guidance where they become activated by class III semaphorins. Endothelial NPs aid angiogenesis by binding of the vascular endothelial growth factor isoform $VEGF_{164}$ (murine equivalent to the human $VEGF_{165}$) and the VEGF-homologue placenta growth factor (PLGF) (7). Interestingly, Semaphorin 3A and $VEGF_{165}$ compete for the binding to NPs (8–10), suggesting that on one hand the angiogenesis factor VEGF might act on neuronal cells, and on the other hand that Semaphorin 3A could have a role in vascular development. Indeed, a dual role of NPs in vascular and neuronal development is supported by gene deletion studies. NP-1-null mutant mice die at embryonic day 13.5 and show disorganization of nerve pathways (11) and impaired vascularization in the central (CNS) and peripheral nervous system (PNS) (12). A similar neuronal phenotype is observed in transgenic mice with inactivation of Sema 3A, the prototype and founding member of the class III semaphorins (13). These mice show no apparent anomalies in neural vascularization (12, 14). NP-2 shares a 47% homology with NP-1 and is expressed in overlapping, yet distinct populations of neurons in the embryonic nervous system and in many adult tissues (15, 16). In contrast to NP-1-null mutant mice, many NP-2-null mutant mice are viable into adulthood. However, they also show disorganization or even complete lack of major neuronal fiber tracts in the brain (17).

Both NPs have relatively short intracellular segments that lack cytoplasmic signal transduction domains, which indicates that they probably do not function as independent receptors. Putative co-receptors include plexins A1, A2, and A3, VEGFR-1, and VEGFR-2. Class III semaphorins (i.e. Sema3A, 3B, 3C, 3D, 3E, and 3F), which mediate axonal guidance, require plexins for their function (18, 19). The sustained expression of class III semaphorins, together with the constitutive expression of both NPs in specific subsets of adult neurons, suggests that these interactions may participate in the regulation of adult structural brain plasticity (20, 21). Pasterkamp et al postulated that the upregulation of semaphorins, especially Sema3A, after CNS injury contributes to the inhibitory effects exerted by scars on the outgrowth of injured CNS neurites (21).

VEGFR-1 and VEGFR-2 are tyrosine kinase receptors that bind the most potent endothelial cell mitogen VEGF with high affinity (22–25). VEGFR-2 appears to be the major signal transducer for VEGF in endothelial cells

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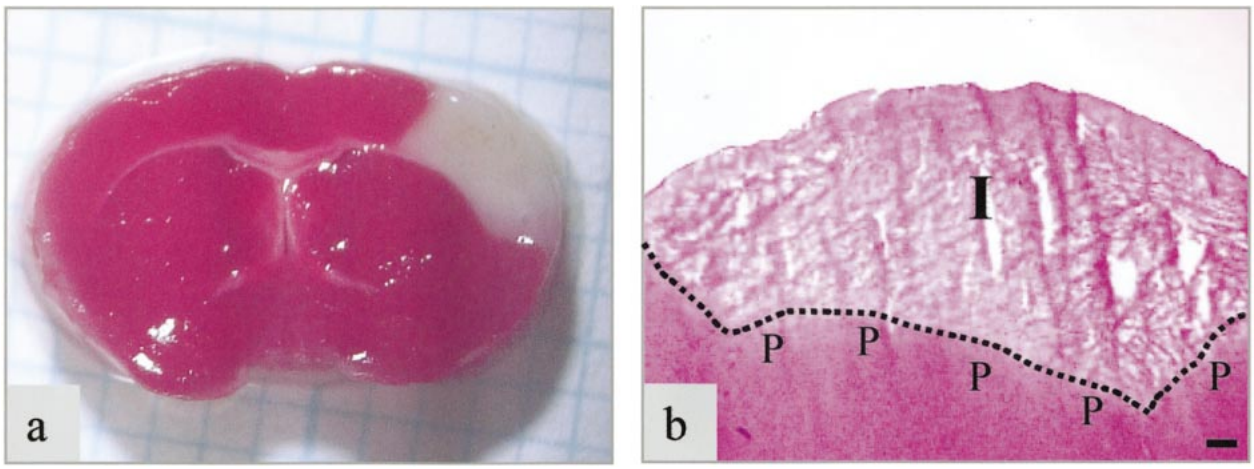

Fig. 1. a: TTC-stained mouse brain 24 h after MCAO. The infarct is identified as unstained (white) necrotic area surrounded by stained (red) viable tissue. Picture is taken on scale paper. b: H&E staining 24 h post-MCAO. I = infarct area; P = peri-infarct area. Scale bar, 50 μm.

(ECs). NP-1 acts as a co-receptor for VEGFR-2, thereby enhancing the binding capacity of VEGF to this receptor (26). In addition, an increase in the density of NP-1 on cells enhances VEGF binding to NP-1 (27).

The purpose of the present study was to investigate the expression pattern of NP-1 and NP-2 as well as their co-receptors and ligands in an experimental stroke model. We performed in situ hybridization and immunohistochemical studies in order to investigate the temporal and spatial expression patterns. Furthermore, we characterized the expressing cell types by double labeling methods. We observed reduced expression of class III semaphorin in neurons and upregulation of NPs, VEGF, PLGF, and the NP-co-receptors VEGFR-1, VEGFR-2 in vascular cells. These observations are in agreement with the post-ischemic phenomenon of vascular proliferation and neuronal remodeling. Our observations also support the notion that VEGF exerts not only an angiogenic but also a neurotrophic/neuroprotective effect following cerebral ischemia.

MATERIALS AND METHODS

Murine Cerebral Infarction Model

All experiments were performed in mice of either sex (BalbC, SPF Facility of the Center for Transgene Technology and Gene Therapy, KU Leuven, Belgium) weighing 25–40 g. Animals were housed under standard conditions with free access to mice chow and tap water before and after surgery. Irreversible occlusion of the left MCA was performed as described previously (28). Briefly, animals were anesthetized by intraperitoneal injection of ketamine (75 mg/ml, Apharmo, Arnhem, The Netherlands) and xylazine (5 mg/ml, Bayer, Leverkusen, Germany). Atropine (1 mg/kg, Federa, Brussels, Belgium) was administered intramuscularly, and body temperature was maintained at 37°C by means of a rectal probe connected to a heating pad. Using an operating microscope, a U-shaped incision was made between the left ear and left eye. The top and back segments of the temporal muscle were transected and the skull was exposed by retraction of the temporal muscle. A small opening (1–2 mm diameter) was made in the region over the MCA with a handheld drill. The MCA was occluded by ligation with 10-0 nylon thread (Ethylon, Norderstedt, Germany) and transected distally to the ligation point. Finally, retracted soft tissues were replaced, wounds were sutured, and the mice were put back into their cages. Body temperature was maintained during surgery and until animals regained consciousness. Thereafter, rectal temperature was checked every 10–15 min during the following 2 hours (h) and, if necessary, it was corrected to 37°C by placing a heating pad below the cage. Following occlusion of the middle cerebral artery (MCAO) the animals were allowed to survive for 3 h, 6 h, 12 h, 24 h, 3 days (d), 7 d and 14 d (n = 4–6 per time). Animals

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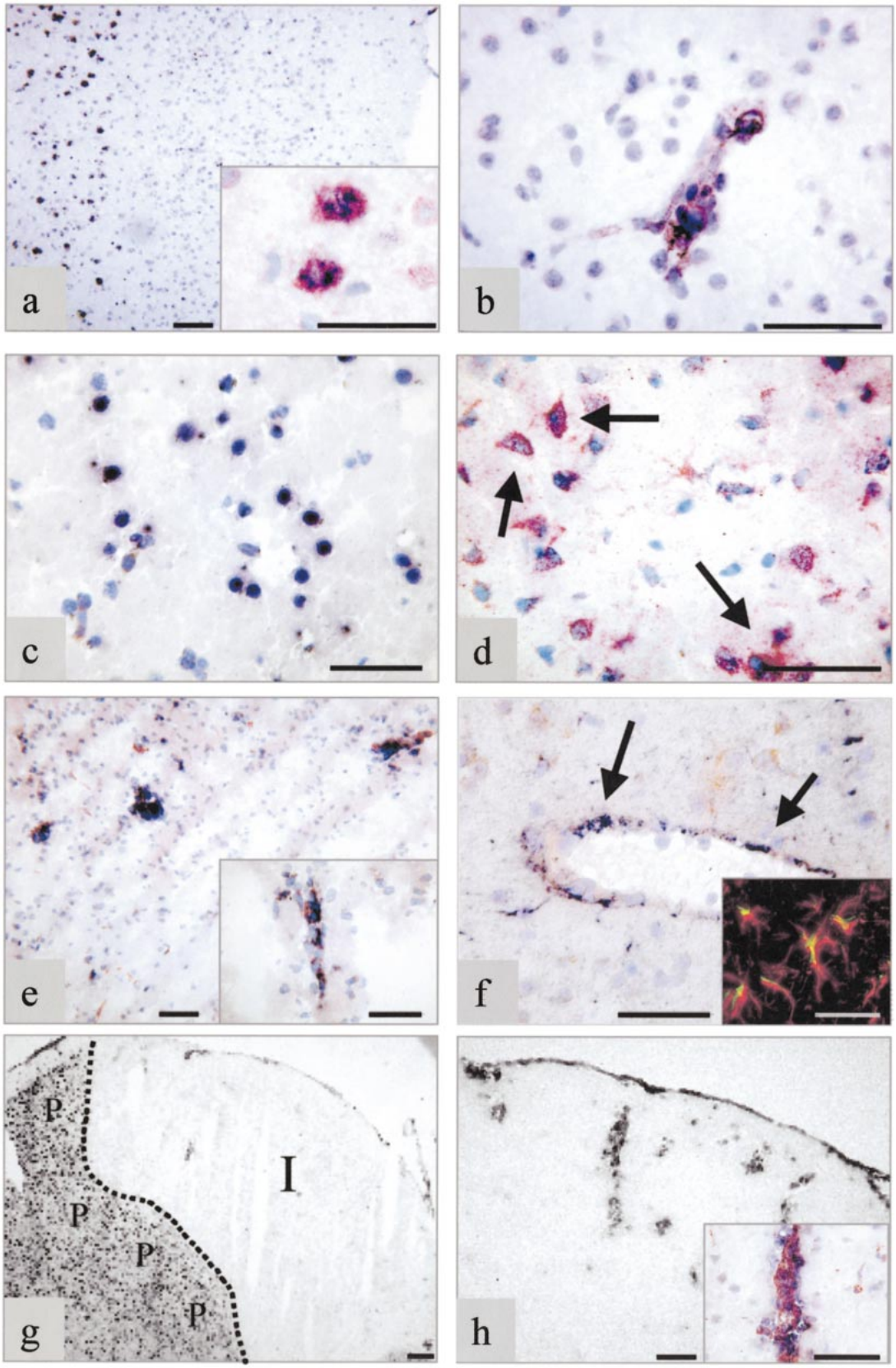
Fig. 2. In situ hybridization and immunohistochemistry for NP-1 in normal (a, b) and infarcted (c–f) mouse brain. NP-1 mRNA expression (shown in black) in layer V of the cerebral cortex (a). Insert in (a) shows a high magnification view of a NP-1 mRNA-positive neurons (double labeled with HuC/HuD (shown in red). b: NP-1 mRNA-positive vessel (black color) double labeled with lectin, red color. c: NP-1 mRNA (black) is upregulated in cells of the infarcted area (12 h MCAO). d: NP-1 immunohistochemistry (red) combined with immunohistochemistry for the neuronal marker NeuN (black). Picture is taken from the peri-infarct area 24 h following stroke. Double labeled neurons are indicated by arrows. e: NP-1 mRNA (black) is upregulated in vessels surrounding the infarcted area (3 d MCAO), insert shows double stained vessel (lectin shown in red). f: 3 d MCAO: expression of NP-1 protein (black) in a vessel of the peri-ischemic zone (indicated by arrows), insert in (d) shows NP-1 (green) /GFAP (red) double labeled astrocytes (yellow) of the peri-infarct area. g, h: 3 d following MCAO: NP-2 mRNA expression



(black) is strongly reduced in the infarcted tissue, but not in the peri-infarct zone (e). Vessels invading the infarcted area express NP-2 mRNA (f) (insert: double stained NP-2 mRNA-positive vessel, lectin shown in red. I = infarct area; P = peri-infarct area. Scale bars, 50 μm.

were decapitated under deep anesthesia (500 mg/kg Nembutal, Knoll, Ludwigshafen, Germany). Brains were removed within 5 min after decapitation and either frozen in OCT embedding medium (Sakura Finetec, Torrance, CA) on dry ice and stored at −80°C until further processing, or placed in a matrix for direct sectioning into 1-mm segments. Sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, Deisenhofen, Germany) in saline, incubated for 30 min at 37°C, and placed in 4% formalin in PBS. With this procedure the necrotic infarct area remains unstained (white) and is clearly distinguishable from stained (brick red) viable tissue. The sections were photographed and subjected to planimetry. The infarct volume was defined as the sum of the unstained areas of the sections multiplied by their thickness.

In Situ Hybridization

Ten-μm-thick frozen sections were melted on silanized (3-Aminopropyltriethoxysilane, Fluka, Deisenhofen, Germany) glass slides, dried at 50°C, and fixed for 15 min in 4% paraformaldehyde/PBS followed by dehydration through ethanol (30%, 60%, 80%, 95% and 100% ethanol, 5 min each). Slides were incubated in 0.2 M HCl for 10 min at room temperature followed by digestion with Proteinase K (10 μg/ml) (Sigma) for 10 min at room temperature and acetylation with 0.1 M triethanolamine (Sigma) mixed with 0.25% acetic anhydride (Fluka) for 10 min at room temperature. Sections were then prehybridized in 4 × SSC, 0.02% SDS, 5 × Denhardt's solution, 50% ultra-pure formamide (Life Technologies, Inc., Karlsruhe, Germany), 5% dextran sulfate (Sigma) and 0.5 mg/ml yeast tRNA (Sigma) for 5 h at room temperature. Hybridization was carried out with a digoxigenin-labeled (Boehringer, Mannheim, Germany) cRNA generated by in vitro transcription using the following cDNA templates: a murine 600-bp KpnI-BamHI NP-1 cDNA fragment, a murine 3,000-bp EcoRI-xhoI NP-2 cDNA fragment, a murine 2,300-bp SacII-SacI semaphorin3A cDNA fragment, a murine 2,500-bp EcoRI-xhoI semaphorin3C cDNA fragment, a murine 1,600-bp EcoR1-NotI Sema 3F fragment, a 583-bp BamH1-EcoR1 VEGF cDNA fragment (encoding murine VEGF164), a murine 1.2-kb HindIII-xHo PLGF cDNA fragment, a 2.6-kb EcoR1-BamH1 cDNA encoding part of the murine flk-1 extracellular domain, and a 2.1-kb EcoR1-Xba1 cDNA encoding part of the murine flt-1 extracellular domain. Probes with cRNAs larger than 1,000 bp were subjected to alkaline hydrolysis to yield probes with an average size of 500 bp. Labeled cRNA probes were used at a concentration of 0.5 ng RNA/μl. Hybridization with sense probe served as control. Tissue sections were incubated in a humidified chamber under glass coverslips at 70°C (hybridization oven, Biometra, Göttingen, Germany) for 12–16 h. Post-hybridization stringency washes included 0.2 × SSC for 30 min at 70°C, 2 × SSC for 2 min at room temperature, 0.2 × SSC for 15 min at 70°C, and 2 × SSC for 5 min at room temperature. Each wash was carried out twice. Hybridized probes were detected by an anti-digoxigenin antibody conjugated to alkaline phosphatase (diluted 1:500, 1 h at room temperature) (Boehringer) using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution as substrate (Boehringer). Color reaction time ranged from 2 h to 2 d, after which slides were rinsed in PBS, counterstained with hematoxylin, finally rinsed in aqua dest and mounted in Elvanol.

Immunohistochemistry

Immunohistochemistry was carried out as described previously (3). The following probes were used: rabbit polyclonal anti-mouse NP-1 (Dr. H. Fujisawa, Nagoya, Japan), goat polyclonal anti-human neuropilin (Santa Cruz, Heidelberg, Germany), rabbit polyclonal anti-human PLGF-2 (a kind gift of Dr. H. Weich, Braunschweig, Germany), rabbit polyclonal anti-human VEGF-A20 (Santa Cruz), goat polyclonal anti-human Sema3A (Santa Cruz), biotin-labeled lectin from lycopersicon esculentum (10 μg/ml; Sigma Chemical Co.), mouse monoclonal anti-mouse VEGFR-2 (Santa Cruz), rat anti-mouse VEGFR-2 (a kind gift of Dr. H. Kataoka, Kyoto, Japan), rabbit polyclonal anti-human VEGFR-1 (Santa Cruz), rat anti-VEGFR-1 antibody (Imclone, New York, NY), mouse monoclonal anti-rat ED1 (Serotec), monoclonal anti-GFAP (Boehringer), rabbit polyclonal anti-human von Willebrand Factor (DAKO, Glostrup, Denmark), mouse monoclonal anti-rat CD31 (Serotec), rat anti-mouse F4/80 (Serotec), mouse monoclonal anti-mouse neuronal nuclei (NeuN) antibody (Chemicon International Inc., Temecula, CA), and mouse monoclonal anti-human neuronal protein HuC/HuD (anti-Hu), (Molecular Probes, Eugene, OR). In all immunohistochemical experiments, omission of the primary antibody served as control. According to the primary antibody the following secondary antibodies were used: biotinylated rat anti-mouse, rabbit anti-mouse, donkey anti-goat, mouse anti-rat, goat anti-rat, or goat anti-rabbit antibodies (Dianova, Hamburg, Germany, diluted 1:300 in 5% BSA/ PBS/ 0.1% Triton), respectively. For mouse monoclonal antibodies we used the Vector M.O.M. immunodetection kit (Vector, Burlingame, CA) in order to reduce unspecific background staining. Sections stained for neuropilin (Santa Cruz), Sema3A, and PLGF-2 were additionally treated with TSA Biotin System (NEN, Life Science Products, Boston, MA) for signal amplification. Thereafter, slides were washed and incubated with peroxidase conjugated streptavidin (Vector, Vectastain Kit ABC) for 1 h at room temperature and then rinsed in PBS/0.1%Triton. The immunoperoxidase reaction was visualized with 3,3′-diaminobenzidine-HCl (DAB) buffer tablets (Sigma) or 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) and 0.006% H_2O_2. The slides were briefly counterstained with hematoxylin, finally rinsed in aqua dest, and mounted in Elvanol.

Double Labeling Experiments

Combined In Situ Hybridization/Immunohistochemistry: In situ hybridization was carried out as described above, using the different murine probes. Color reaction time ranged from 6–16 h, after which slides were rinsed in PBS and overlaid for 30 min with PBS containing 5% Bovine Serum (Fraction 5, Sigma) and then for 1 h with 20% NGS/PBS to block nonspecific binding. Immunohistochemistry was carried out essentially as described above, using the following probes: monoclonal anti-GFAP antibody GFAP (5 μg/ml; Boehringer), polyclonal anti-von Willebrand Factor (29 μg/ml; DAKO) and biotin-labeled lectin from lycopersicon esculentum (10 μg/ml; Sigma), mouse monoclonal anti-human neuronal protein HuC/HuD (anti-Hu), (Molecular Probes), rat anti-mouse F4/80 (Serotec).

TABLE 1
Semiquantitative Assessment of Neuropilin/Neuropilin-Ligand mRNA Expression Patterns in Normal Mouse Brain

NP-1	NP-2	VEGF	PLGF	Sema 3A	Sema 3C	Sema 3F	
+	++	−	++	+	++	++	neurons
+	−	−	−	−	−	−	vessels

Summary of expression patterns of NPs and NP-ligands in normal mouse brain. −, no staining; +++, increasing staining levels.

After six washes in PBS/0.1% Triton, slides were incubated for 1 h at room temperature with biotinylated rat anti-mouse, goat anti-rabbit or mouse anti-rat secondary antibodies (Dianova; dilution 1:200 in 10% NGS/PBS, respectively). Slides were rinsed three times in PBS/ 0.1% Triton and then incubated for 1h at room temperature with peroxidase conjugated streptavidin (Vector, Vectastain KIT ABC). Thereafter, slides were rinsed 4 times with PBS/0.1%Triton and then incubated with 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) and 0.006% H_2O_2. Color developed within 20–30 min. Sections were rinsed in aqua dest, counterstained with hematoxylin and mounted in Elvanol.

Double Immunofluorescence Labeling: In order to characterize the cell types, double immunofluorescence labeling was performed. Briefly, cryosections were washed thoroughly in PBS before and after each incubation. Nonspecific binding sites were blocked by incubation in 20% normal goat serum and 5% BSA/PBS. Goat anti-mouse Alexa Fluor™568, goat anti-mouse Alexa Fluor™488, goat anti-rabbit Alexa Fluor™568, goat anti-rabbit Alexa Fluor™488, donkey anti-goat Alexa Fluor™568 (Molecular Probes; dilution 1:200 in 5% BSA/PBS, respectively) and mouse anti-rat Rhodamine Red™-X conjugated antibody (Dianova; dilution 1:200 in 10% NGS/PBS, respectively) were used as secondary antibodies. Evaluation of double staining was performed using a fluorescence microscope.

RESULTS

Cerebral Infarction Size

In order to verify successful occlusion of the MCA, a set of 8 infarcted brains was analyzed 24 h following ischemia with TTC staining (Fig. 1). Ligation of the left MCA induced a cerebral infarct with a volume of 7.91 $mm^3 \pm 1.1$ SEM (n = 8) in wild-type (50% SV129/50% Swiss) mice.

Expression of NP-1 mRNA and Protein

In normal mouse brain, NP-1 mRNA was constitutively expressed in many cells throughout the brain. Double labeling showed NP-1 mRNA-positive neurons, especially in cortical layer V (Fig. 2) and the hippocampal formation. In addition, vessels (Fig. 2) as well as the choroid plexus were NP-1 mRNA-positive. Neuropilin-1 protein was detectable in low amounts in many neurons throughout the brain and astrocytes surrounding the ventricles.

Following MCAO, NP-1 mRNA was first upregulated in injured neurons in the infarct core (12–24 h following MCAO) (Fig. 2), but then disappeared with complete lack of expression at 3 d MCAO. However, NP-1 mRNA was gradually upregulated in vessels surrounding and invading the infarct (starting 24 h and peaking 3 d following MCAO), as well as in neurons and astrocytes of the infarct border zone, with a peak of expression 3 d following MCAO (Fig. 2). In addition, immunohistochemistry showed NP-1 upregulation in vessels, neurons, and astrocytes surrounding the infarcted tissue (peak of expression 3–7 d after MCAO) (Fig. 2). Results are summarized in Tables 1–3.

Expression of NP-2 mRNA

NP-2 mRNA was expressed in all neurons of normal mouse brain. Following MCAO, NP-2 mRNA was gradually upregulated in vessels surrounding and invading the infarcted area (Fig. 2). Expression started 24 h after MCAO and peaked at 3 d. Twenty-four h following MCAO, neurons of the infarcted tissue still expressed NP-2 mRNA. At 3 d following MCAO, NP-2 mRNA was no longer detectable in these ischemic neurons. However, neurons of the border zone still expressed high levels of

TABLE 2
Semiquantitative Assessment of Neuropilin/Neuropilin-Ligand mRNA and VEGF Protein Expression Patterns 24 Hours Following MCAO

NP-1	NP-2	VEGF mRNA	VEGF protein	PLGF	Sema 3A	Sema 3C	Sema 3F	Cell type
								Neurons
++	+	−	−	+++	−	−	−	infarct area
+	++	+	+	++	+/−	+/−	+/−	peri-infarct area
								Vessels
+	+	−	−	+	−	−	−	infarct area
+	+	−	+	−	−	−	−	peri-infarct area

Summary of expression patterns of NPs and NP-ligands 24 h following MCAO. VEGF mRNA and protein expressions are listed separately, because due to secretion of the protein, cellular mRNA and protein levels do not necessarily overlap. −, no staining; +++, increasing staining levels.

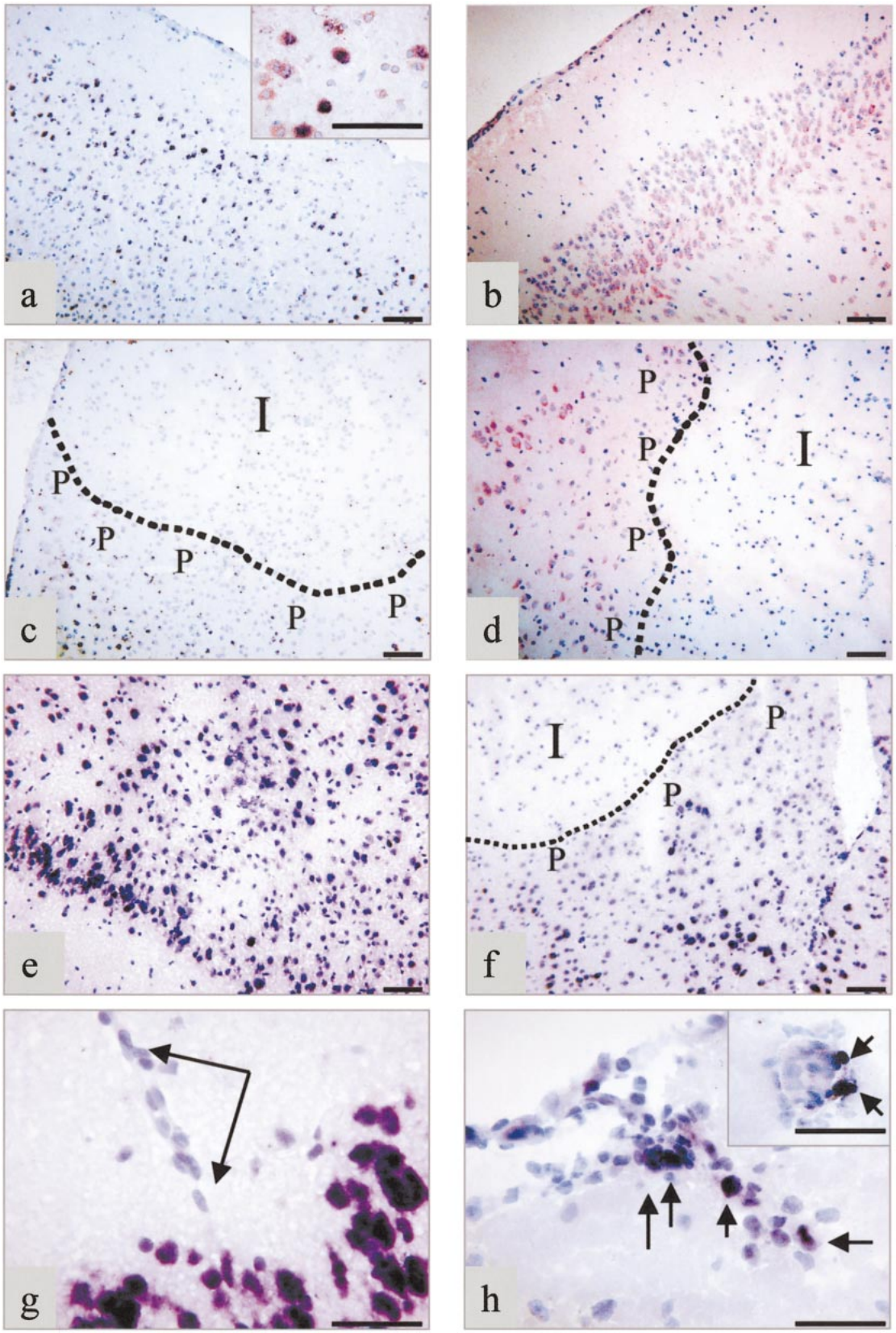


Fig. 3. a–d: In situ hybridization (a, c) and immunohistochemistry (b, d) for Sema3A in normal (a, b) and infarcted mouse brain (c, d). Sema3A mRNA (shown in black) is mainly detectable in layer II and III of the cerebral cortex (a). Insert in (a) shows Sema3A mRNA-positive neurons double labeled with HuC/HuD (red). b: Sema3A-immunopositive cortical neurons (red).

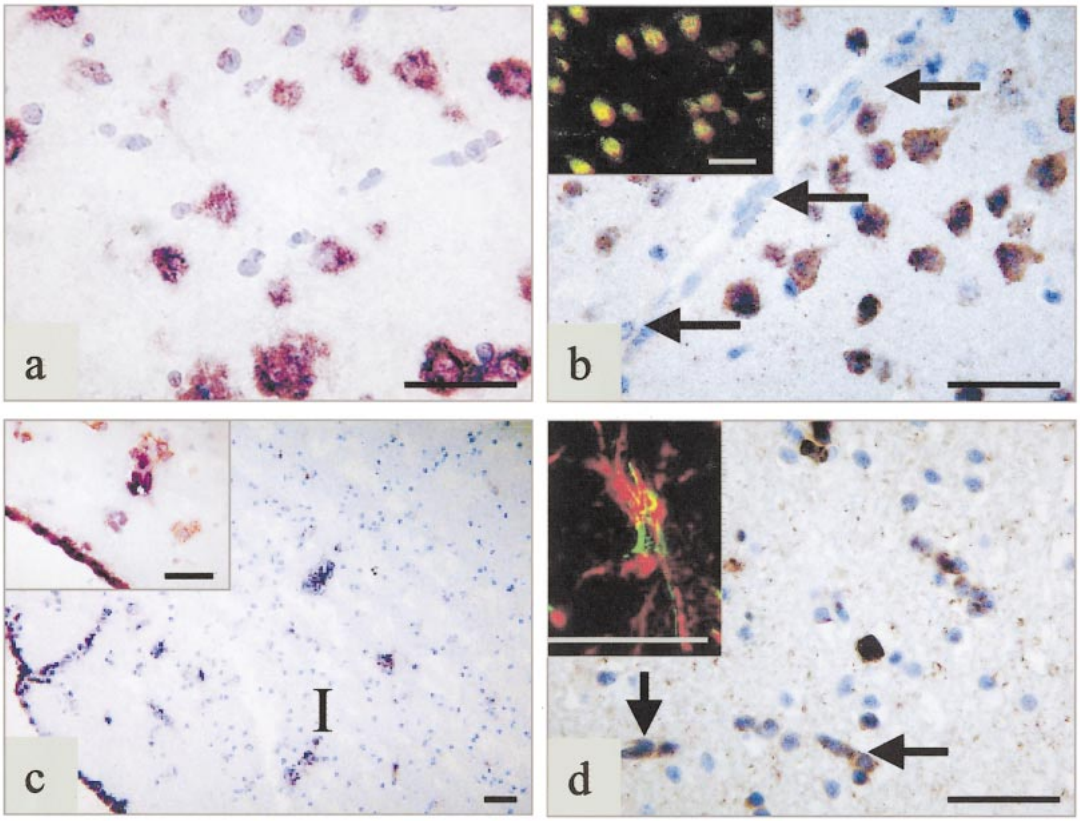


Fig. 4. PLGF expression in normal mouse brain (a, b) and 3 d following MCAO (c, d). a: PLGF mRNA (black) is almost exclusively expressed in neurons (double labeling with HuC/HuD shown in red). b: PLGF-immunostaining (brown), arrows indicate PLGF-negative vessel. Insert in (b) shows PLGF (red)/NeuN (green) double labeled neurons (yellow). Three d after MCAO (c, d). PLGF mRNA (black) is expressed in vessels invading the infarcted tissue (c). Insert shows double labeling of PLGF mRNA with lectin (red). d: Arrows indicate PLGF-immunopositive vessels (brown) in the infarcted tissue. Insert shows Np-1 (red)/PLGF (green) double labeled astrocyte (yellow). Scale bars, 50 μm.

NP-2 mRNA. Additionally, we detected NP-2 mRNA expression in macrophages of the infarcted tissue (7–14 d MCAO). Results are summarized in Tables 1–3.

Expression of Class 3 Semaphorins

Sema3A mRNA and Protein Expression: Sema3A mRNA was expressed in neurons throughout the normal mouse brain, but showed strongest expression in neurons of cortical layers II and III (Fig. 3). Sema3A protein could be detected in all cortical neurons in low amounts (Fig. 3).

Following cerebral ischemia, expression of Sema3A mRNA and protein was strongly reduced in the infarcted tissue (Fig. 3) commencing 6 h after MCAO. In addition, neurons of the infarct border zone (peri-infarct area) expressed lower amounts of Sema3A compared with those of the corresponding area of the contralateral hemisphere (Fig. 3).

Sema3C mRNA, Sema3F mRNA Expression: Sema3C mRNA was expressed in neurons throughout the normal mouse brain with stronger expression in cortical neurons of layer II and V. Sema3C mRNA was almost completely absent in the infarcted tissue (Fig. 3) as early as 6 h following MCAO, with no recovery up to 14 d. The border zone of the infarcted area also showed reduction of Sema3C mRNA expression compared with the corresponding area of the contralateral hemisphere (Fig. 3).

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3 d following MCAO (c, d): Sema3A mRNA (c) and protein expression (d) is completely absent in the infarcted area (I = infarct area), and reduced in the peri-infarct area (P = peri-infarct area). e–f: Sema3C mRNA in situ hybridization (black) in normal mouse brain (e) and 3 d following MCAO (f). e: Sema3C mRNA is expressed in many cortical neurons. f: Sema3c mRNA expression is significantly reduced, mainly in the infarcted area (I), but also in the peri-infarct area (P). g, h: Sema3F mRNA in situ hybridization (black). g: Layer I/II of normal mouse cortex, neurons are Sema3F mRNA-positive, whereas vessels (arrows) show no mRNA expression. h: 3 d MCAO: cells in close association to vessels invading the infarct display Sema3F mRNA expression (Sema3F mRNA-positive cells are indicated by arrows). Scale bars, 50 μm, dotted lines indicate the infarct areas.

TABLE 3
Semiquantitative Assessment of Neuropilin/Neuropilin-Ligand mRNA and VEGF Protein Expression Patterns 3 Days after MCAO

NP-1	NP-2	VEGF mRNA	VEGF protein	PLGF	Sema 3A	Sema 3C	Sema 3F	Cell type
								Neurons
−	−	−	−	−	−	−	−	infarct area
++	++	+	+	++	+/−	+/−	+/−	peri-infarct area
								Vessels
+++	+++	−	−	+++	−	−	−	infarct area
++	++	−	+	++	−	−	−	peri-infarct area

Summary of expression patterns of NPs and NP-ligands 3 d following MCAO. VEGF mRNA and protein expressions are listed separately, because due to secretion of the protein, cellular mRNA and protein levels do not necessarily overlap. −, no staining; +++, increasing staining levels.

Sema3F mRNA could be detected in almost all neurons of normal mouse brain. Following cerebral ischemia we observed a similar expression pattern as described for Sema3C mRNA. However, 3 d following MCAO, Sema3F mRNA expression was additionally observed in cells in close association with vessels invading the infarcted area (Fig. 3). These cells most likely resemble macrophages as morphologically similar cells on serial sections expressed F4/80 (Tables 1–3).

Expression of Vascular Endothelial Growth Factor (VEGF) mRNA and Protein

In normal mouse brain, VEGF mRNA was detectable in the choroid plexus, in cerebellar Purkinje cells, and in scattered glial cells throughout the brain. Significant amounts of VEGF protein were detectable in the choroid plexus and in cerebellar Purkinje cells. A marked peri-ischemic upregulation of VEGF mRNA was observed 3 h following MCAO, with a peak of expression at 24 h. VEGF mRNA-positive cells surrounding the infarcted area were mainly identified as astrocytes. Single peri-infarct neurons showed weak VEGF mRNA expression. In addition, the pia above the infarcted area also expressed VEGF mRNA. VEGF protein was detectable in peri-ischemic cells 6 h after MCAO, with a peak of expression at 24 h to 3 d following cerebral ischemia. VEGF-immunopositive cells could be identified as microglial cells, astrocytes, neurons, and vessels.

Expression of Placenta Growth Factor (PLGF) mRNA and Protein

PLGF mRNA as well as PLGF protein were detectable in cells throughout the normal mouse brain. In double labeling experiments almost all PLGF expressing cells could be identified as neurons, whereas vessels and astrocytes did not express PLGF (Fig. 4). Following stroke, PLGF was first upregulated in ischemic neurons with a peak of expression 24 h following MCAO. Afterwards, it gradually decreased with complete lack of expression at 3 d following MCAO. However, PLGF mRNA and protein were both upregulated in vessels in and surrounding the infarcted area (peak of expression at 3 d following MCAO), as well as in neurons and astrocytes (only PLGF protein) of the ischemic border zone. In addition, macrophages of the infarcted tissue also showed PLGF expression 3–7 d following MCAO. Double labeling experiments revealed that the PLGF-expressing astrocytes of the peri-infarct area also expressed NP-1 protein.

Expression of VEGFR-1 mRNA and Protein

VEGFR-1 mRNA could be detected in vessels and in neurons throughout the normal mouse brain. Following cerebral infarction, mRNA was less in the affected neurons but strongly upregulated in vessels surrounding and invading the infarcted tissue, with a peak of expression at 3 d following MCAO. VEGFR-1 protein expression pattern was dependent on antibodies used and on tissue preparation. One polyclonal VEGFR-1 antibody stained all brain vessels on frozen sections, but marked neurons on paraffin sections. Another polyclonal VEGFR-1 antibody marked both vessels and neurons in normal mouse brain. However, following stroke, both antibodies detected a strong upregulation of VEGFR-1 expression in vessels surrounding the infarcted tissue.

Expression of VEGFR-2 mRNA and Protein

VEGFR-2 mRNA was detectable in scattered neurons throughout the brain, but specifically marked neurons of cortical layers II, III, and V. In addition, vessels were stained. Following 6 h MCAO, VEGFR-2 was downregulated in neurons of the stroke area, but was upregulated in vessels and neurons surrounding the infarcted tissue (peak at 3 d after MCAO). Antibodies against VEGFR-2 stained all vessels and neurons in normal mouse brain and showed strong upregulation of VEGFR-2 protein in vessels surrounding the infarcted tissue 3 d following occlusion of the MCA.

DISCUSSION

Both vascular proliferation (1–4) and neuronal remodeling (5, 6) follow cerebral ischemia. One interest-

ing molecular link between both processes may be the NP receptors (NP-1, NP-2), because they are involved in neural growth and angiogenesis (29). NPs are receptors for class III semaphorins, mediators of neuronal guidance, and for the angiogenesis factors VEGF/PLGF. The role of NPs in normal development is partly understood (11, 12, 17), but little information is available on their potential role under pathological conditions. Therefore we investigated the expression of the NPs, their ligands, and their co-receptors in normal and ischemic mouse brain and characterized the expressing cell types in situ.

Vascular Expression of NPs, their Angiogenic Ligands (VEGF/PLGF) and Co-Receptors (VEGFR-1, VEGFR-2) in Normal Mouse Brain and Following Experimental Cerebral Ischemia

The neuropilin family consists of the 2 membrane-bound proteins, NP-1 and NP-2. Both receptors have relatively short intracellular segments that lack cytoplasmic signal transduction domains, which indicates that they probably do not function as independent receptor (30). For their angiogenic properties, the NPs form complexes with VEGFR-1 (27, 30). Additionally, it was shown that NP-1 enhances the binding of $VEGF_{165}$ to VEGFR-2 and $VEGF_{165}$-induced EC chemotaxis (26). In our MCAO model we observed a constitutive but low expression of NP-1 mRNA in vessels throughout the brain. Following stroke, expression of both NPs was upregulated in vessels surrounding and possibly invading the ischemic tissue. Upregulation started 24 h after MCAO and peaked at 3 d. The co-receptor VEGFR-2 as well as PLGF showed a similar spatial and temporal vascular expression pattern. VEGF mRNA was expressed in astrocytes in close vicinity to the NP-positive vessels surrounding the infarct area as well as in the pia mater above the ischemic zone. We previously reported VEGF immunoreactivity in rats subjected to MCAO, preferentially in microglia. The current studies, using a different animal model and different reagents, extended these observations. In situ hybridization for VEGF mRNA combined with immunohistochemistry for different cell markers revealed that astrocytes are the main source of VEGF mRNA following stroke in mice. In addition, a few neurons surrounding the infarcted tissue upregulated VEGF mRNA. Taken together, these observations suggest that VEGF secreted by astrocytes and neurons binds to the NP receptors in the nearby vascular endothelium. These expression patterns correlate with data obtained from infarcted rat brains, where post-ischemic EC proliferation started 24 h following MCAO in peri-infarct vessels and peaked at 3 d (31). Our results thus further support a proposed role for NPs in pathological angiogenesis (Fig. 6) (32, 33).

Neuronal Expression of NPs Class III Semaphorins Sema3A, 3C, 3F

Class III semaphorins are axonal guidance molecules that function via NPs and require plexins as co-receptors. It has been postulated that the sustained expression and interaction of NP-1/NP-2 and class III semaphorins in specific subsets of adult neurons may participate in the regulation of adult brain structural plasticity (20, 34). Here we report constitutive expression of both NPs and the investigated class III semaphorins in specific subsets of neurons throughout the brain. Our investigations focused on the parietal cortex, because this region is severely affected by MCAO. Following stroke, Sema3A, 3C, and 3F appeared to be strongly downregulated commencing 3 h following vessel occlusion in the infarcted and peri-infarct area. Although we cannot definitely exclude a loss of cell viability, several findings suggest that the mRNA and protein levels are indeed downregulated. Firstly, based on morphology up to 12–24 h following stroke, cells in the infarct area are still viable. Secondly, different cell types such as neurons and vascular cells are able to upregulate specific mRNAs (e.g. NP-1 mRNA) during the first 24 h following stroke. Sema levels stayed low until 14 d after MCAO, the latest time point that we investigated. In contrast to our findings, Pasterkamp et al described upregulation of Sema3A following penetrating brain injury (21). The authors suggested a possible role of Sema3A in the failure of the CNS to regenerate after such incidents. In line with this, Sema3A is absent or downregulated following injuries to the peripheral nervous system or neonatal brain when vigorous regeneration occurs (35–37). Stroke, however, is a cerebral insult that is accompanied by neuroanatomical remodeling (5, 6). After focal cortical ischemia, massive primary and secondary neuronal death provides a stimulus for undamaged neurons to sprout and establish new synaptic connections (5). In our MCAO model, Semaphorins 3A, 3C and 3F expression was significantly reduced in neurons of the infarcted area at early time points. Semaphorin expression also decreased in the border zone of the infarcted tissue, whereas NP-2 expression was unaltered in this area. Twenty-four h following MCAO, NP-1 mRNA was upregulated even in ischemic neurons. NP-1 protein was expressed in astrocytes of the ischemic border zone 3–7 d following MCAO. We speculate that the early and strong downregulation of the class III semaphorins, which are mainly functioning as chemorepellants, would favor conditions that promote neuronal remodeling in the peri-infarct area following experimental cerebral ischemia (Fig. 6).

Neurotrophic/Neuroprotective Role of VEGF/PLGF via NP Receptors?

Though vascular endothelial growth factor (VEGF) is a key player in the formation of new blood vessels and

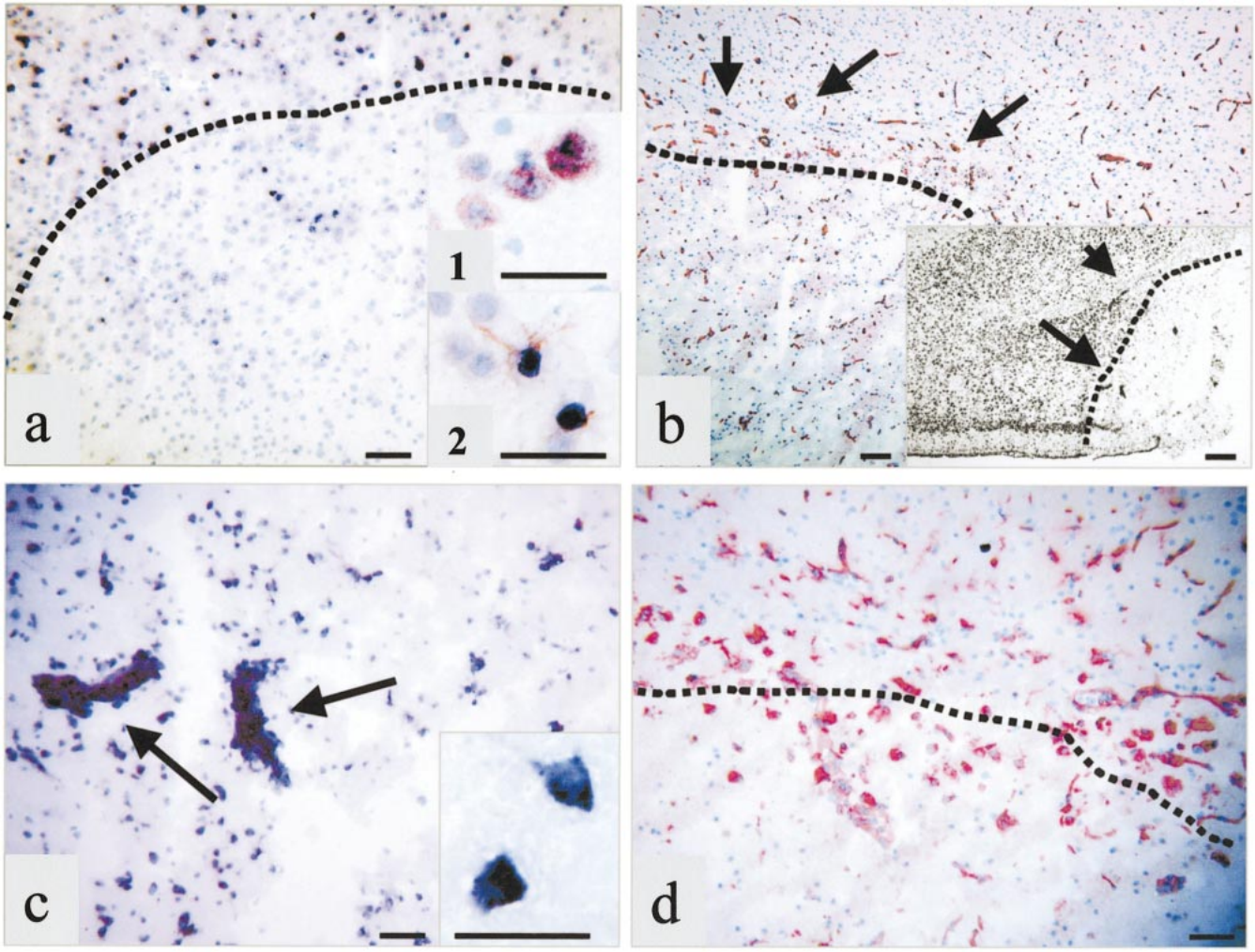

Fig. 5. a: VEGFmRNA expression in cells surrounding the infarcted tissue (black) 24 h following MCAO. Inserts show double labeling with the neuronal marker HuC/HuD (red) (5a1) or with the astrocytic marker GFAP (red) (5a2). b: VEGFR-1 immunohistochemistry (red) 3 d following stroke. High expression of VEGFR-1 in vessels surrounding the infarct is indicated by arrows. Insert shows upregulation of VEGFR-1mRNA in vessels surrounding the infarcted area (indicated by arrows). c: VEGFR-2mRNA expression in vessels invading the infarcted area (3 d MCAO), insert shows VEGFR-2mRNA expressing neurons of the peri-infarct area. d: VEGFR-2 immunohistochemistry (red) 3 d following infarction. Dotted lines demarcate the infarct areas. Scale bars, 50 μm.

primarily acts on endothelial cells (22–25) it also acts on other cell types, including neural cells (38–42). Several studies hint towards a neurotrophic/neuroprotective role of VEGF in the nervous system. Matsuzaki et al (40) demonstrated that VEGF rescued hippocampal neurons from glutamate-induced toxicity. Zhang et al (43) showed that application of recombinant human VEGF following experimental cerebral ischemia in rats improved functional neurological outcome of the infarcted animals. They postulated that in addition to VEGF-induced angiogenesis, VEGF may have direct effects on neuronal plasticity by its neurotrophic activity and by stimulating axonal outgrowth. Sondell and Schratzberger already described such an effect for the peripheral nervous system (38, 39). Our results give additional support to the hypothesis that VEGF and PLGF may exert a neurotrophic/neuroprotective role following experimental stroke. VEGF competes with Sema 3A for the binding to the NP receptors (8–10). In the peri-infarct area this balance seems to be shifted towards VEGF (Fig. 6). Here, the investigated class III semaphorins are significantly reduced at early time points, whereas the neuronal NP expression is not affected (NP-2) or even upregulated (NP-1) in the ischemic border zone. In addition, PLGF and VEGF are both upregulated in neurons of the peri-infarct area. We are currently conducting experiments in mutant mice with conditional inactivation of the VEGF gene in specific subsets of brain cells in order to further elucidate a possible neurotrophic/neuroprotective role of VEGF in cerebro-ischemic diseases.

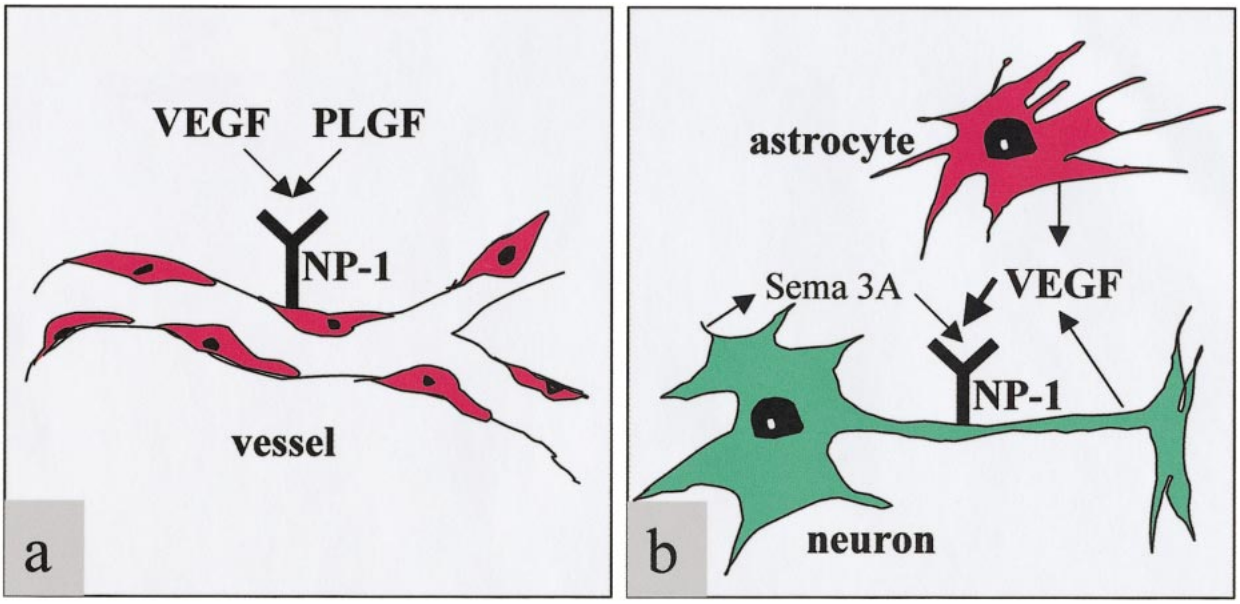

Fig. 6. Model for NP-1 function in post-ischemic remodeling processes. a: Upregulation of NP-1mRNA and protein in vessels surrounding the infarcted tissue as well as upregulation of its angiogenic ligands VEGF and PLGF suggests a role for NP-1 in post-ischemic angiogenesis. b: Shift in the balance between Sema3A (loss in peri-infarct neurons) and VEGF (upregulated in peri-infarct neurons and astrocytes) towards VEGF following cerebral ischemia. This might generate a microenvironment that favors neuronal remodeling processes and might exert neuroprotective functions.

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