Title

Delayed VEGF Treatment Enhances Angiogenesis and Recovery After Neonatal Focal Rodent Stroke

Journal

Translational Stroke Research, 4(2)

ISSN

1868-4483

Authors

Dzietko, M
Derugin, N
Wendland, MF
et al.

Publication Date

2013-04-01

DOI

10.1007/s12975-012-0221-6

Peer reviewed

Published in final edited form as:
Transl Stroke Res. 2013 April ; 4(2): 189–200. doi:10.1007/s12975-012-0221-6.

Delayed VEGF Treatment Enhances Angiogenesis and Recovery After Neonatal Focal Rodent Stroke

M. Dzietko,
Department of Pediatrics, University of California, San Francisco, San Francisco, CA, USA

Klinik für Kinderheilkunde I, Universitätsklinikum Essen, Essen, Germany

N. Derugin,
Department of Neurology, University of California, San Francisco, 521 Parnassus Avenue, San Francisco, CA 94143-0663, USA

M. F. Wendland,
Department of Radiology, University of California, San Francisco, CA, USA

Z. S. Vexler, and
Department of Neurology, University of California, San Francisco, 521 Parnassus Avenue, San Francisco, CA 94143-0663, USA

D. M. Ferriero
Department of Pediatrics, University of California, San Francisco, San Francisco, CA, USA

Department of Neurology, University of California, San Francisco, 521 Parnassus Avenue, San Francisco, CA 94143-0663, USA

D. M. Ferriero: donna.ferriero@ucsf.edu

Abstract

Neonatal stroke occurs in one in 4,000 live births and leads to significant morbidity and mortality. Approximately two thirds of the survivors have long-term sequelae including seizures and neurological deficits. However, the pathophysiological mechanisms of recovery after neonatal stroke are not clearly understood, and preventive measures and treatments are nonexistent in the clinical setting. In this study, we investigated the effect of vascular endothelial growth factor (VEGF) treatment on histological recovery and angiogenic response to the developing brain after an ischemic insult. Ten-day-old Sprague–Dawley rats underwent right middle cerebral arterial occlusion (MCAO) for 1.5 h. Diffusion-weighted MRI during occlusion confirmed focal ischemia that was then followed by reperfusion. On group of animals received 5-bromo-2-deoxyuridine and sacrificed at postnatal day (P)18 or P25. A second group of animals was treated with VEGF (1.5 μg/kg, icv) or phosphate-buffered saline (PBS) at P18 and perfusion fixed at P25. Based on Nissl and iron staining, a single VEGF injection reduced the injury score, compared to the animals that underwent MCAO and PBS injection. Furthermore, neurodegeneration represented by neuronal nuclei staining was markedly diminished. In addition, animals treated with VEGF revealed a positive trend in endothelial proliferation and a significant increase in total vessel volume in the peri-infarct region of the caudate. The number of Iba1-positive microglial cells was significantly reduced after a single VEGF injection, and myelin basic protein expression was enhanced in the caudate after ischemia without an effect of VEGF treatment. In conclusion, delayed treatment with

Correspondence to: D. M. Ferriero, donna.ferriero@ucsf.edu.
ZS Vexler and DM Ferriero are co-senior authors.
Conflict of Interest None.

VEGF ameliorates injury, promotes endothelial cell proliferation, and increases total vascular volume following neonatal stroke. These results suggest that VEGF has a neuroprotective effect, in part by enhancing endogenous angiogenesis. These data contribute to a better understanding of neonatal stroke.

Keywords

VEGF; Neuroprotection; Angiogenesis; Neonatal stroke

Introduction

Ischemic stroke in the full-term newborn is the second most common neurological disease after hypoxic ischemic encephalopathy, leading to significant lifelong morbidity and mortality [1]. Follow-up studies of human neonates with documented stroke revealed that as many as two thirds of the survivors develop long-term sequelae including seizures and neurological deficits [2]. It has been shown in imaging studies and animal models that damage to the brain does not only occur at the time of injury but continues to evolve over a period of days to weeks [3]. But the mechanisms behind this evolution of injury and activated endogenous repair mechanisms are not fully understood. In addition, there is no accepted treatment available for neonatal stroke. Efforts in animal studies to protect the immature brain against the consequences of stroke have largely focused on attenuation of acute cell death. Ischemic stroke manifests as an ischemic core of rapid cell death, surrounded by a vulnerable peri-infarct region. Within the peri-infarct region, reparative angiogenesis and neuronal repopulation occur in close proximity, facilitating mutually supportive neuron–endothelial cell interactions [4]. Additionally, angiogenic blood vessels serve as a physical scaffold for neuronal progenitors, enabling neurons to migrate toward the peri-ischemic regions [5]. Most recent trends in stroke research emphasize neuro-genesis and vasculogenesis as a complex, dynamic coupling with sophisticated cross-talk between neurons and endothelial cells via vascular endothelial growth factor (VEGF), neurotrophins, and their cognate receptors on both neurons and endothelial cells [4, 6]. Modulating this neurovascular niche might be a potential target for ischemic brain injury, especially in the developing brain, which has an exceptional potential for repair and recovery. A question yet to be answered is whether the induction of endogenous neurogenesis and angiogenesis in the developing brain might be helpful in repairing the ischemic brain, or if these processes are only short lived and ineffective in replacing dying cells. Furthermore, little is known about endogenous regeneration in the developing rodent brain after ischemic injury, and published data are conflicting. Hayashi et al. reported a significant increase of 5-bromo-2-deoxyuridine (BrdU) and doublecortin-positive cells in the striatum at 7, 14, and 21 days after ischemic/hypoxic injury in the neonatal rat brain, suggesting amplified neurogenesis [7]. In contrast, we described impaired neurogenesis following ischemic injury using a GFP-expressing lentivirus injected intraventricularly in the neonatal rat stroke model [8]. So far, the timing of neurogenesis and angiogenesis in the developing brain after perinatal stroke is not well defined yet to efficiently apply therapies aimed at these events. In adult animals, it has become apparent that the time course of administration of the growth factor VEGF is critical to induce neuroprotective effects. An early administration of VEGF induces brain edema, whereas a late application seems to have the desired effect of protection to the brain [9]. Thus, there is a clear need for a stroke therapy that is both neuroprotective and promotes brain repair without being harmful. We have previously described a non-hemorrhagic ischemic stroke model in the immature rat using transient middle cerebral artery occlusion (MCAO) [10, 11]. This model creates a reproducible injury verifiable by diffusion-weighted MRI in the ipsilateral cortex without inducing histological changes in the contralateral hemisphere. In the present study, therefore, we investigated the effects of cerebral ischemia

in postnatal day (P)10 rats on the neurovascular component using two different time points of BrdU injections and, additionally, after a single, delayed VEGF injection.

Materials and Methods

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco, and performed in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services, publication number 85–23, 1985).

Animal Model

Sprague-Dawley dams with a dated litter of pups composed of both male and female neonatal rats were purchased from Charles River Laboratories (Wilmington, MA, USA). A transient 1.5-h right middle cerebral artery occlusion was performed in 10-day-old pups. Since the P10 rat exhibits similar brain maturity to the human term newborn, this age was selected to study the pathophysiology of neonatal stroke [12]. This procedure was originally described for P7 rats [13, 14] and modified for P10 rats [15]. Briefly, each pup was weighed and anesthetized with 3 % isoflurane in a mixture of 70 % N_2O and 30 % O_2. Temperature was maintained at 36–37 °C with a combination of heating blanket and overhead light. A coated 6–0 Dermalon filament was inserted into the internal carotid artery and advanced 8.5 to 9.5 mm, depending on animal size, to occlude the middle cerebral artery. The wound was closed, and animals were returned to the dam. Reperfusion was achieved after removal of the coated filament under anesthesia, and application of gel foam to the arteriotomy in the internal carotid artery. Pups were examined by spin-echo echo-planar diffusion-weighted imaging (DWI) at 75–80 min during occlusion to document successful occlusion and assure that the typical arterial territory exhibited DW hyperintensity The entire brain was imaged with serial 2-mm thick coronal sections as previously described [10,14]. The incidence of the desired injury pattern, DWI abnormalities in the cortex and caudate, without injury in the brain stem, was seen on MRI in approximately 80 %. Animals that exhibited ischemic injury in atypical regions such as the brain stem, or without cortical involvement, were excluded from the study. Starting at P15 for animals sacrificed at P18 and at P22 for animal sacrificed at P25, 5-bromo-2-deoxyuridine (Sigma, St. Louis, MO, USA) injections, 50 mg/kg per dose intraperitoneally, were performed twice daily for three consecutive days.

VEGF Administration

A subgroup of animals received a single intracerebroventricular injection of human recombinant vascular endothelial growth factor (1.5 μg/kg, Sigma, St. Louis, MO, USA) at P18. The drug was dissolved in phosphate-buffered saline (PBS)/0.4 % bovine serum albumin (BSA) and stored frozen. A Hamilton syringe was placed in the right lateral cerebral ventricle according to predetermined coordinates (5-mm rostral and 3 mm lateral from lambda, depth 3 mm), and validity of injection was determined by using blue dye in one separate litter before drug administration. Injections were administered over 30 s. Control animals received PBS with 0.4 % BSA. VEGF-treated animals were then sacrificed at P25. Most pups that underwent MCAO showed poor suckling during the first 2 to 3 days after surgery and were gavage fed with milk formula, with weights measured daily for 14 days to ensure adequate weight gain.

Histology and Immunofluorescence

Eight or 15 days after MCAO, animals were perfused and postfixed with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH7.4); brains were removed and postfixed in the same solution overnight. The tissue was then transferred to 30 % sucrose in 0.1 M phosphate buffer and submerged for 3 days. Rat brains were flash frozen, and coronal

sections (thickness 12 μm,) were cut on a cryostat through the forebrain from the anterior extent of the lateral ventricles (Fig. 14, Paxinos [16]) through the posterior extent of the dorsal hippocampus (Fig. 40, Paxinos [16]). Every 40th slice was used for the determination of the injury score. Eight sections per brain were analyzed. The 18- and 25-day post-MCAO brains were analyzed using both cresyl violet and Pearl's iron-stained sections. For degree of injury and to confirm stroke size and location, four representative sections from the anterior and posterior caudate and four sections from the anterior and posterior cortex were scored. Sections were graded on a scale of 0 to 3 with 0=no injury; 1=few small areas of focal injury; 2=multiple areas of focal injury, and 3=widespread injury with loss of architecture. These four regional scores were summed to provide a scale of 0 to 12 as previously described [15].

Double immunofluorescence was performed on adjacent sections at the level of the anterior commissure because this brain region revealed the most consistent injury. Brain slices were blocked in 10 % normal goat serum (NGS)/PBS-Tween (PBST) and incubated overnight in 2 % NGS/PBST with antibodies directed to Iba1 (1:100; Wako, Richmond, VA, USA), neuronal nuclei (NeuN) (1:200; Millipore, Billerica, MA, USA), BrdU (1:200; Abcam, Cambridge, MA, USA), glial fibrillary acidic protein (GFAP) (1:50; ImmunO, Solon, OH, USA), and platelet endothelial cell adhesion molecule (PECAM) (1:500; courtesy of Dr. Peter Newman, Milwaukee, WI, USA), followed by appropriate Alexa Fluor 488 and 568 secondary antibodies (1:100; Invitrogen, Carlsbad, CA, USA). Sections were then counterstained with Hoechst 33342 and mounted with ProLong Gold agent (Invitrogen, Carlsbad, CA, USA). Tissue analyzed for BrdU incorporation was pretreated with 2 N HCl for 30 min at 37 °C followed by 0.1 M boric acid for 10 min at room temperature. Z-stacks of 17 images captured at 1-μm intervals (×25 objective, Plan-Apo lens, NA 0.75, camera resolution 1,024×1,024 pixels, field dimensions 541×412×17 μm), using a Zeiss Imager fluorescence microscope (Jena, Germany) equipped with Voocty software (Perkn Elmer, Waltham, USA), were deconvolved and analysis performed in eight regions of interest (ROI). These ROIs were in the ischemic and peri-infarct area of the cortex and caudate with matching contralateral tissue serving as controls (Fig. 1b). We used automated protocols for signal intensity threshold (>2 SD background in each channel), as previously described [17]. 5-BrdU-positive cells and PECAM-positive vessels with overlap >50 % surface was considered co-localized.

Statistical Analysis

Values are presented as mean ± standard error of the mean (SEM). Comparisons among groups were made using one-way analysis of variance (ANOVA) with the Tukey–Kramer post hoc test or unpaired Student's *t* test as appropriate. Statistical significance was determined at $p<0.05$.

Results

Delayed VEGF Treatment Ameliorates Brain Injury and Neurodegeneration After MCAO

We performed a single injection of VEGF to P18 old rat pups into the ipsilateral ventricle. These animals were allowed to survive for one additional week. The treatment significantly reduced histological injury score (Fig. 1a, cresyl violet and iron-stained brain sections). To further characterize neurodegenerative effects of MCAO, brains were analyzed by means of NeuN staining at P18 and P25. Histological evaluation of VEGF-untreated brains revealed profound neurodegeneration in the anterior cortex and caudate. In animals sacrificed at P18, the number of NeuN-stained cells was not diminished (Fig. 2c), but a significant decrease of NeuN-stained cells occurred predominantly in the ischemic region of the caudate at 2 weeks after the injury (Fig. 2a, b, and d). VEGF treatment ameliorated this effect (Fig. 2g). While

the number of NeuN-positive cells was reduced in the PBS-treated animals, the number of NeuN-positive cells was maintained in rats treated with VEGF, but comparison of PBS and VEGF treatment in the ischemic hemisphere did not reach statistical significance (Fig. 2e – h). No differences were identified in the peri-infarct region (data not shown).

VEGF Treatment Does Not Alter Endothelial Proliferation 2 Weeks After Injury

In order to investigate whether MCAO has an impact on endothelial proliferation in the caudate and cortex of the adolescent rat, co-labeling techniques with PECAM and BrdU were used. The number of BrdU/PECAM-stained vessels in the injured caudate was higher than the number in the uninjured caudate at both time points (Fig. 3a, b). In the same animals, the number of co-labeled BrdU/PECAM cells in the peri-infarct region of the caudate tended to be higher, compared to the contralateral site at P25, but did not reach statistical significance (Fig. 3b – d). No differences were identified in the peri-infarct region at P18 (Fig. 3a) or in the cortex at either time points (data not shown). VEGF did not significantly affect endothelial proliferation (Fig. 3e, f).

VEGF Therapy After MCAO Increases Total Vessel Volume in the Caudate

We then focused on the total vessel volume per cubic micrometer as a measurement of angiogenic response after MCAO. In the ipsi- and contralateral caudate, 8 and 15 days after surgery, MCAO alone had no impact on the total vessel volume (Fig. 4a, b). However, we found an increase of the total vessel volume in the peri-infarct area of the caudate, suggesting induction of angiogenesis by intervention with VEGF (Fig. 4f). The remaining regions were not affected by a single, delayed VEGF injection (Fig. 4e).

VEGF Intervention Counteracts a Profound Glial Response After MCAO

A MCAO of 90 min in P10 rats leads to a persistent glial response 1 and 2 weeks later based on marked Iba1 and GFAP immunolabeling predominantly in the ischemic region of the caudate (Fig. 5a, b). A single injection of VEGF reduced the number of Iba1-positive microglial cells in the ischemic caudate (Fig. 5c, d, and f). Nevertheless, VEGF treatment had no significant effect on tissue volume occupied by GFAP-positive cells in the caudate (Fig. 5e).

Myelin Basic Protein Volume Is Augmented in the Ipsilateral Caudate 2 Weeks After MCAO

To follow the development of the white matter after ischemic injury, we measured myelin basic protein (MBP) expression at the two different time points. The presence of MBP-positive fibers in the cortex and caudate was not reduced 1 week after MCAO. However, we discovered a significantly increased MBP volume in the ischemic region of the caudate in 25-day-old animals (Fig. 6a, c, and d). In the peri-infarct region of the caudate, the pattern of MBP staining showed the same trend but did not reach statistical significance (Fig. 6b). We then analyzed the effect of VEGF on myelination. Area occupied by MBP-positive fibers was significantly increased in the ipsilateral caudate of PBS-treated rats. In animals injected with VEGF, no significant difference in area occupied by MBP in ipsi- and contralateral caudate was apparent. Comparison of the ischemic caudate after intervention with PBS and VEGF revealed no significant difference (Fig. 6e). No further effect was detected in the peri-infarct region (Fig. 6f).

Discussion

Our study demonstrates that delayed VEGF therapy after transient MCAO in P10 rats ameliorated ischemic injury in the neonatal brain, in part by decreasing neuronal cell death and supporting the vasculature. In addition, VEGF treatment counteracted the profound glial

response associated with injury. This is the first study of VEGF intervention in a neonatal transient MCAO model. These data are in keeping with our previous report of enhancement of injury after VEGF receptor inhibition in this model [17].

In a wide range of adult animal model systems, the concept of modulating angiogenesis has been evaluated as a treatment for ischemic stroke [18]. In particular, exogenous VEGF has shown a promising efficacy in different models of cerebral ischemia. It is strongly angiogenic and neuroprotective by inhibiting apoptosis and decreasing oxidative stress [19, 20]. VEGF therapy promotes memory and learning [21] and synaptic plasticity [22]. Consequently, VEGF is still explored as a potential agent for ischemic stroke. But, the literature for use of VEGF as a treatment option in the newborn population is extremely sparse.

We explored the effect of exogenous VEGF on the degree of injury, primarily in the caudate, because that is the core of the injury in this stroke model. We used a delayed intervention strategy 7 days after MCAO because studies using acute applications exhibit unfavorable effects of VEGF like exacerbated brain infarcts due to enhanced brain edema [9].

Our data do concur with adult data showing that VEGF treatment improves ischemic injury [19, 20]. Because experimental studies in the past few years have pointed out that tissue remodeling after stroke is a highly dynamic process [23], which involves close and finely tuned interactions between neuronal, vascular, and glial cells [24], we studied the effects on all three cell types in our model.

In adult animal models, 2 weeks after ischemic injury, many neurons still co-label with TUNEL and activated caspase-3 both in the penumbra and ischemic core, suggesting a continuum of neuronal cell death which is still suitable for intervention [25]. By using a delayed protocol in our model, VEGF protected the brain against neuronal death at 15 days after MCAO represented by preservation of NeuN staining predominantly in the ischemic cortex compared to the contralateral hemisphere. A possible explanation for this region-specific response could be that there is more severe injury in the caudate compared to the cortex. In addition, the comparison of PBS against VEGF treatment revealed no significant difference, suggesting only a minimal neuroprotective effect on NeuN-positive cells after late treatment. These data suggest that the cortex may be a region which might be salvageable even with late treatment, whereas the ischemic caudate may only be rescued through more immediate interventions.

VEGF is well established as a promoter of angiogenesis in different experimental models [18, 26, 27]. In addition, patients suffering from stroke with a higher density of blood vessels in the penumbra appear to have reduced morbidity and survive longer [28], suggesting a beneficial effect of enhanced formation of new cerebral vessels. This process seems to be fundamental for providing nutritional and trophic support to adjacent tissues, maintaining blood–brain barrier (BBB) integrity and tissue repair after injury. In our experimental model, ischemic injury triggered only a slight increase in BrdU-positive newly born vessels in the peri-infarct and ischemic region of the caudate at both time points. VEGF intervention had surprisingly no further impact on newly born vessels, but it increased the total vessel volume in an area surrounding the ischemic core in the caudate. This difference from well-established results in the adult literature may be attributed to the maturity of the brain, different timings, route of administration, and concentrations of exogenous VEGF used. The best time point for VEGF application to enhance angiogenesis seems to be very critical and still needs to be determined. The region-specific angiogenic response after VEGF treatment in the caudate did not match with the effect on NeuN-positive cells which

was predominantly present in the cortex. This suggests additional nonvascular mechanisms being responsible for the neuroprotective effect of late VEGF therapy. Although we believe that angiogenesis is an active process 7 days after the insult, it may continue for several weeks [9, 25]. After VEGF intervention in an adult rodent model, newly formed vessels were first visible at 4 days and peaked at 12 days after VEGF administration [29]. For this reason, the timing of injections may be another possible explanation for failure of supporting angiogenesis. Considering that in our experiments, VEGF administration was performed 5 days before BrdU injection, not all newly born endothelial cells might be captured. Morphological analysis of cerebral vessels in the rodent brain described in the literature revealed a formation of enlarged and thin-walled blood vessels in the peri-infarct region. These vessels develop into smaller vessels and extend into the ischemic core by sprouting or intussusception during 2 to 28 days after the onset of cerebral ischemia [30]. During our evaluation, we might have predominantly captured enlarged thin-walled vessels that would explain our finding of increased total vessel volume at the border of the infarct area only.

There is increasing evidence that post-ischemic inflammation plays an important role in brain ischemia. Chemokines and cytokines released from multiple cell types, including microglia, reactive astrocytes, and activated endothelial cells [17, 31], may play an important role in vessel susceptibility to injury and modulate angiogenesis as well. However, whether inflammatory processes are deleterious or beneficial to recovery is presently a matter of debate and controversy. A role for GFAP-reactive astrocytes in angiogenesis has been well documented by inducing key vascular regulators, including VEGF and angiopoietin 1 and its receptors [32]. In addition, injury-induced astrocytosis has been found to be associated with neo-angiogenesis [33], but the role of exogenous VEGF on inflammation in the brain is controversial. VEGF itself may modulate immune responses in the central nervous system and modulate opening the BBB [34]. VEGF has also been reported to reduce inflammation after stroke when excreted from transplanted stem cells [35] and has been shown to be beneficial when injected in a model of viral pneumonia [36].

We also found a strong glial reaction 1 and 2 weeks after the insult in the ischemic caudate as previously reported [14]. VEGF intervention had no significant impact on astrogliosis in the immature brain, but the number of Iba1-positive cells was significantly lower in the ischemic caudate, suggesting cell type-specific effects of VEGF. Diverse VEGF responses described in the literature can be also explained by different ways of VEGF administration in the literature, but the neonatal BBB, in particular, might also respond differently to a VEGF application [37].

Emerging data indicate that axonal remodeling is a critical aspect of brain repair and contributes to improvements of neurological deficits after stroke [38, 39]. A substantial increase in MBP-positive oligodendrocytes in the peri-infarct area during stroke recovery suggested that stroke induces axonal regrowth and myelination [40]. We also found a substantial increase of MBP-positive fibers 2 weeks after the stroke, but predominantly, in the ischemic caudate. Interestingly, intervention with VEGF abolished the proliferating effect. From experimental stroke models in adult animals, it is known that VEGF regulates migration of oligodendrocyte precursor cells, but proliferation is not supported [41, 42]. VEGF has also been shown to have a detrimental effect on the periventricular white matter in the neonatal brain, emphasizing a possible dual role in the immature brain [43].

Taken together, our data suggest that delayed VEGF treatment enhances recovery after neonatal focal rodent stroke. Intervention with VEGF may support angiogenesis and interfere with microglia cells and the inflammatory response, at the expense of mature MBP-positive oligodendrocytes.

Acknowledgments

This work was supported by a grant NS35902 (DMF and ZSV) and was a recipient of PAS Travel Award 2012 (MD).

References

1. Nelson KB. Perinatal ischemic stroke. Stroke. 2007; 38:742–745. [PubMed: 17261729]
2. Sreenan C, Bhargava R, Robertson CMT. Cerebral infarction in the term newborn: clinical presentation and long-term outcome. J Pediatr. 2000; 137:351–355. [PubMed: 10969259]
3. Ferriero DM. Neonatal brain injury. NEJM. 2004; 351:1985–1995. [PubMed: 15525724]
4. Arai K, Jin G, Navaratna D, Lo EH. Brain angiogenesis in developmental and pathological processes: neurovascular injury and angiogenic recovery after stroke. FEBS J. 2009; 276:4644–4652. [PubMed: 19664070]
5. Ohab JJ, Fleming S, Blesch A, Carmichael ST. A neurovascular niche for neurogenesis after stroke. J Neurosci. 2006; 26:13007–13016. [PubMed: 17167090]
6. Madri J. Modeling the neurovascular niche: implications for recovery from CNS injury. J Physiol Pharmacol. 2009; 60:94–104.
7. Hayashi T, Iwai M, Ikeda T, Jin G, Deguchi K, Nagotani S, et al. Neural precursor cells division and migration in neonatal rat brain after ischemic/hypoxic injury. Brain Res. 2005; 1038:41–49. [PubMed: 15748871]
8. Spadafora R, Gonzalez FF, Derugin N, Wendland M, Ferriero D, McQuillen P. Altered fate of subventricular zone progenitor cells and reduced neurogenesis following neonatal stroke. Dev Neurosci. 2010; 32:101–113. [PubMed: 20453463]
9. Zhang ZG. VEGF enhances angiogenesis and promotes blood- brain barrier leakage in the ischemic brain. J Clin Invest. 2000; 106:829–838. [PubMed: 11018070]
10. Derugin N, Wendland M, Muramatsu K, Roberts TPL, Gregory G, Ferriero DM, et al. Evolution of brain injury after transient middle cerebral artery occlusion in neonatal rats editorial comment. Stroke. 2000; 31:1752–1761. [PubMed: 10884483]
11. Mu D. Regulation of hypoxia-inducible factor 1alpha and induction of vascular endothelial growth factor in a rat neonatal stroke model. Neurobiol Dis. 2003; 14:524–534. [PubMed: 14678768]
12. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. Early Hum Dev. 1979; 3:79–83. [PubMed: 118862]
13. Derugin N, Ferriero DM, Vexler ZS. Neonatal reversible focal cerebral ischemia: a new model. Neurosci Res. 1998; 32:349–353. [PubMed: 9950062]
14. Derugin N, Dingman A, Wendland MF, Fox C, Bollen A, Vexler ZS. Magnetic resonance imaging as a surrogate measure for histo-logical sub-chronic endpoint in a neonatal rat stroke model. Brain Res. 2005; 1066:49–56. [PubMed: 16336947]
15. Shimotake J, Derugin N, Wendland M, Vexler ZS, Ferriero DM. Vascular endothelial growth factor receptor-2 inhibition promotes cell death and limits endothelial cell proliferation in a neonatal rodent model of stroke. Stroke. 2010; 41:343–349. [PubMed: 20101028]
16. Paxinos, G. The rat brain in stereotaxic coordinates. 4th ed.. San Diego: Academic; 1998.
17. Faustino JV, Wang X, Johnson CE, Klibanov A, Derugin N, Wendland MF, et al. Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. J Neurosci. 2011; 31:12992–3001. [PubMed: 21900578]
18. Greenberg DA, Jin K. From angiogenesis to neuropathology. Nature. 2005; 438:954–959. [PubMed: 16355213]
19. Kaya D, Gürsoy-Özdemir Y, Yemisci M, Tuncer N, Aktan S, Dalkara T. VEGF protects brain against focal ischemia without increasing blood-brain permeability when administered intracerebroventricularly. J Cereb Blood Flow Metab. 2005; 25:1111–1118. [PubMed: 15829918]
20. Sun Y. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest. 2003; 111:1843–1851. [PubMed: 12813020]

21. Wang Y, Galvan V, Gorostiza O, Ataie M, Jin K, Greenberg DA. Vascular endothelial growth factor improves recovery of sensorimotor and cognitive deficits after focal cerebral ischemia in the rat. Brain Res. 2006; 1115:186–193. [PubMed: 16928361]
22. Wang Y-Q, Cui H-R, Yang S-Z, Sun H-P, Qiu M-H, Feng X-Y, et al. VEGF enhance cortical newborn neurons and their neurite development in adult rat brain after cerebral ischemia. Neurochem Int. 2009; 55:629–636. [PubMed: 19540294]
23. Chen J, Chopp M. Neurorestorative treatment of stroke: cell and pharmacological approaches. J Am Soc Exp NeuroTher. 2011; 3:466–473.
24. Chopp M, Li Y, Zhang J. Plasticity and remodeling of brain. J Neurol Sci. 2008; 265:97–101. [PubMed: 17610903]
25. Krupinski J, Stroemer P, Slevin M, Marti E, Kumar P, Rubio F. Three-dimensional structure and survival of newly formed blood vessels after focal cerebral ischemia. NeuroReport. 2003; 14:1171–1176. [PubMed: 12821803]
26. Ma Y, Zechariah A, Qu Y, Hermann DM. Effects of vascular endothelial growth factor in ischemic stroke. J Neurosci Res. 2012
27. Hermann DM, Zechariah A. Implications of vascular endothelial growth factor for postischemic neurovascular remodeling. J Cereb Blood Flow Metab. 2009; 29:1620–1643. [PubMed: 19654590]
28. Sc C, Chopp M. Recovery recapitulates ontogeny. Trends Neurosci. 2000; 23:265–271. [PubMed: 10838596]
29. Dellian M, Witwer BP, Salehi HA, Yuan F, Jain RK. Quantitation and physiological characterization of angiogenic vessels in mice. Am J Pathol. 1996; 149:59–71. [PubMed: 8686763]
30. Zhang ZG, Chopp M. Vascular endothelial growth factor and angiopoietins in focal cerebral ischemia. Trends Cardiovasc Med. 2002; 12:62–66. [PubMed: 11852252]
31. Denker SP, Ji S, Dingman A, Lee SY, Derugin N, Wendland MF, et al. Macrophages are comprised of resident brain microglia not infiltrating peripheral monocytes acutely after neonatal stroke. J Neurochem. 2007; 100:893–904. [PubMed: 17212701]
32. Acker T, Beck H, Plate KH. Cell type specific expression of vascular endothelial growth factor and angiopoietin-1 and -2 suggests an important role of astrocytes in cerebellar vascularization. Mech Dev. 2001; 108:45–47. [PubMed: 11578860]
33. Salhia B, Angelova L, Roncari L, Wu X, Shannon P, Guha A. Expression of vascular endothelial growth factor by reactive astrocytes and associated neoangiogenesis. Brain Res. 2000; 883:1–11. [PubMed: 11063982]
34. Proescholdt M, Heiss J, Walbridge S, Mühlhauser J, Capogrossi M, Oldfield E, et al. Vascular endothelial growth factor (VEGF) modulates vascular permeability and inflammation in rat brain. J Neuropathol Exp Neurol. 1999; 58:613–627. [PubMed: 10374752]
35. Horie N, Pereira MP, Niizuma K, Sun G, Keren-Gill H, Encarnacion A, et al. Transplanted stem cell-secreted vascular endothelial growth factor effects poststroke recovery, inflammation, and vascular repair. Stem Cells. 2011; 29:274–285. [PubMed: 21732485]
36. Olivier AK, Gallup JM, Geelen AV, Ackermann MR. Exogenous administration of vascular endothelial growth factor prior to human respiratory syncytial virus a2 infection reduces pulmonary pathology in neonatal lambs and alters epithelial innate immune responses. Exp Lung Res. 2011; 37:131–143. [PubMed: 21309731]
37. Fernandez-Lopez D, Faustino J, Daneman R, Zhou L, Lee SY, Derugin N, et al. Blood-brain barrier permeability is increased after acute adult stroke but not neonatal stroke in the rat. J Neurosci. 2012; 32:9588–9600. [PubMed: 22787045]
38. Li S, Overman JJ, Katsman D, Kozlov SV, Donnelly CJ, Twiss JL, et al. An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. Nat Neurosci. 2010; 13:1496–1504. [PubMed: 21057507]
39. Liu Z, Li Y, Zhang ZG, Cui X, Cui Y, Lu M, et al. Bone marrow stromal cells enhance inter- and intracortical axonal connections after ischemic stroke in adult rats. J Cereb Blood Flow Metab. 2010; 30:1288–1295. [PubMed: 20125183]
40. Ueno Y, Chopp M, Zhang L, Buller B, Liu Z, Lehman NL, et al. Axonal outgrowth and dendritic plasticity in the cortical peri-infarct area after experimental stroke. Stroke. 2012

41. Hayakawa K, Seo JH, Pham L-DD, Miyamoto N, Som AT, Guo S, et al. Cerebral endothelial derived vascular endothelial growth factor promotes the migration but not the proliferation of oligodendrocyte precursor cells in vitro. Neurosci Lett. 2012; 513:42–46. [PubMed: 22342913]

42. Hayakawa K, Pham LDD, Som AT, Lee BJ, Guo S, Lo EH, et al. Vascular endothelial growth factor regulates the migration of oligodendrocyte precursor cells. J Neurosci. 2011; 31:10666–10670. [PubMed: 21775609]

43. Kaur C, Sivakumar V, Ang LS, Sundaresan A. Hypoxic damage to the periventricular white matter in neonatal brain: role of vascular endothelial growth factor, nitric oxide and excitotoxicity. J Neurochem. 2006; 98:1200–1216. [PubMed: 16787408]

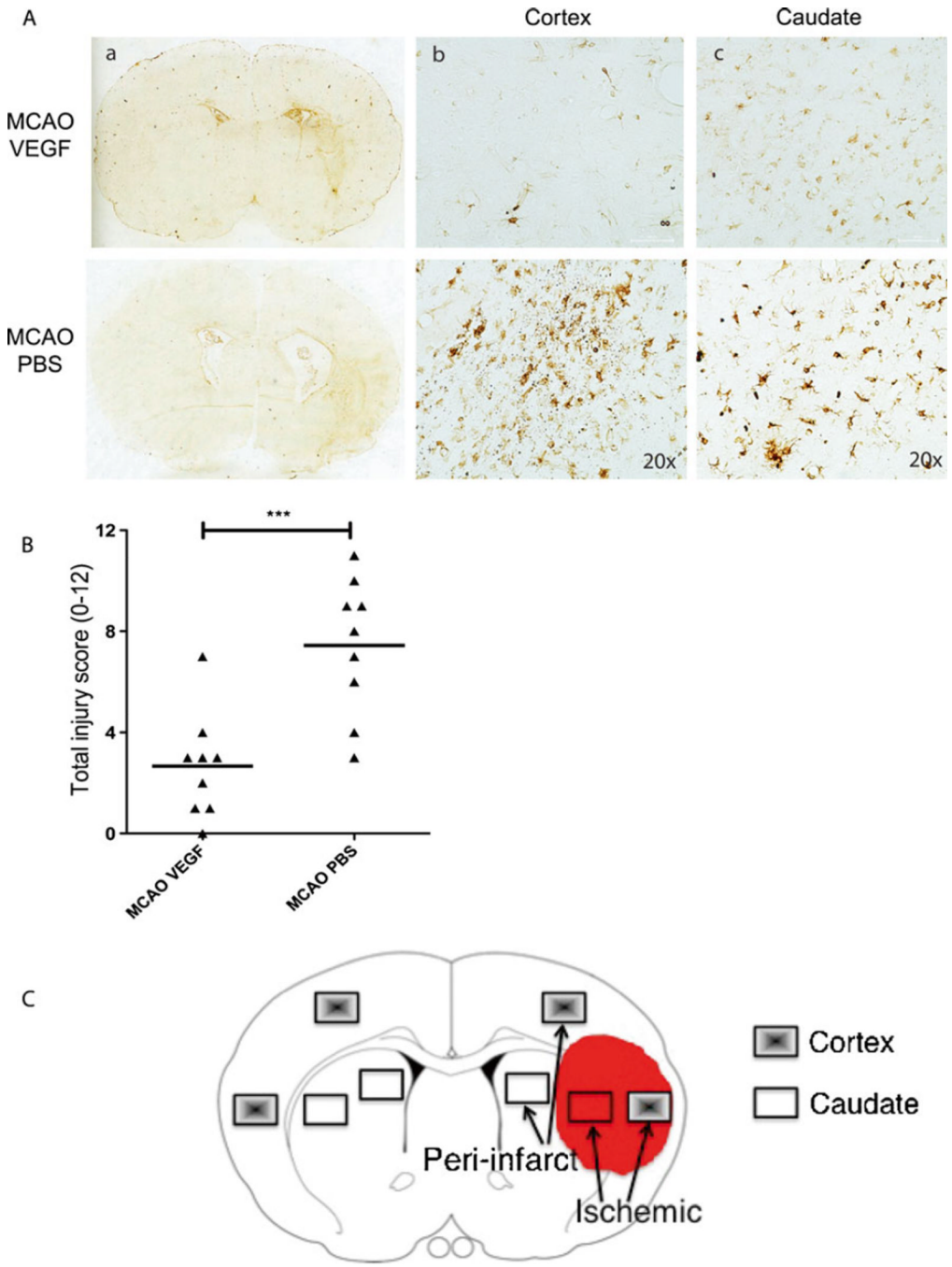

Fig. 1.

Delayed VEGF treatment protects the neonatal brain after stroke. P18 rats subjected to 1.5-h MCA occlusion–reperfusion at P10 were administered with either VEGF or PBS; 7 days later, brain tissues were sectioned, and mean injury score was measured with cresyl violet and Pearl's iron staining. **a** Representative images of the whole brain (*a*), anterior cortex (*b*), and caudate (*c*) show profound iron staining in the control (PBS) animals after MCAO, but not in the VEGF-treated rats. **b** The injury score of the ipsilateral hemisphere was lower in VEGF- than in PBS-treated brains (VEGF 2.7±0.7, PBS 7.4±0.9, ***p<0.001, mean ± SEM, Student's t test, n=9/group). **c** A scheme of regions of interest was used for creating z-stacks.

Regions were identified by cresyl violet and iron staining. The *filled boxes* represent ischemic or peri-infarct regions in the cortex. The *clear boxes* represent ischemic or peri-infarct regions in the caudate. The corresponding contralateral regions served as internal controls

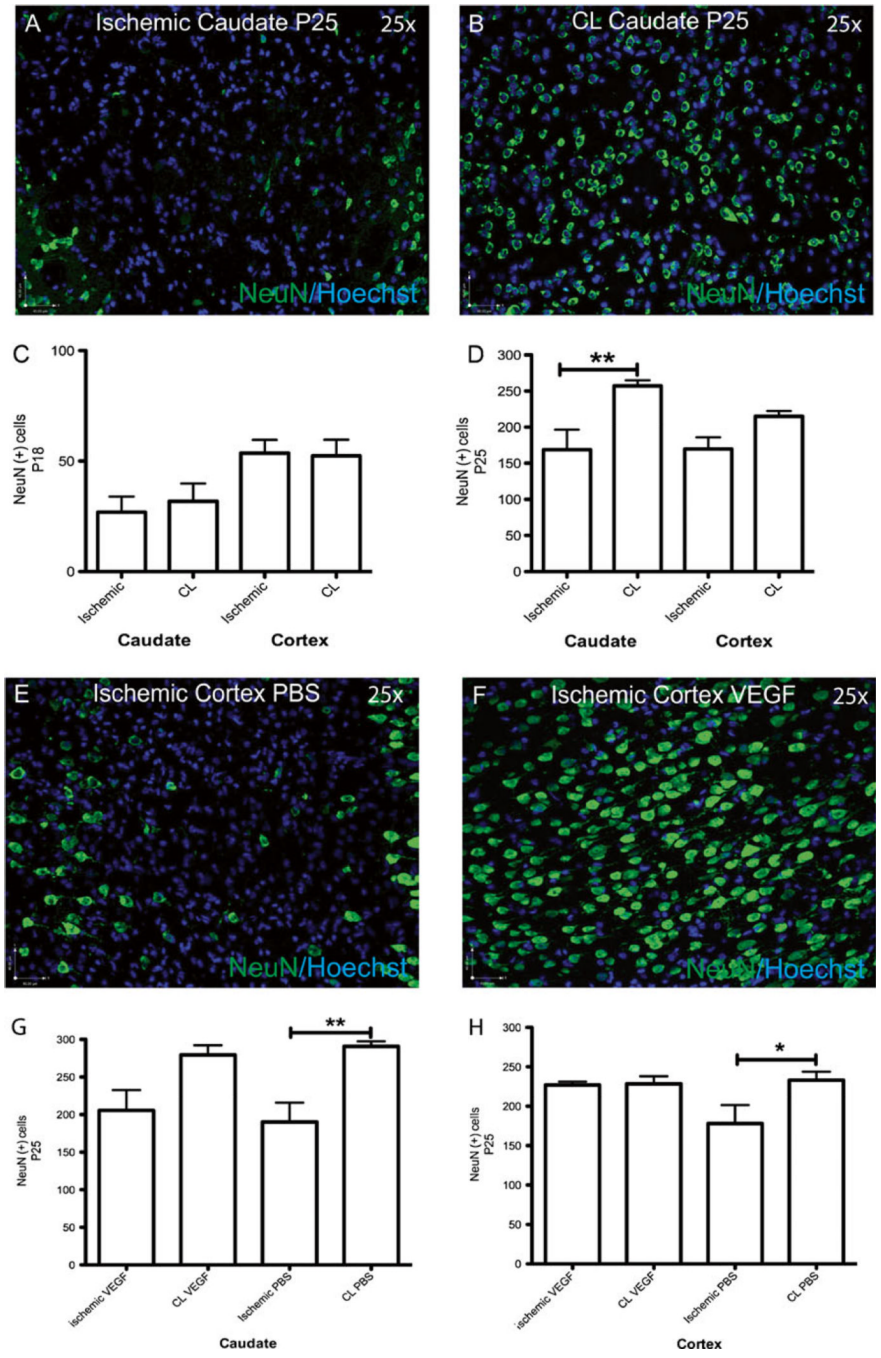

Fig. 2.

VEGF intervention ameliorates neurodegenerative effect of MCAO. A profound decrease of NeuN-positive cells was observed predominantly in the ischemic caudate at P25. The ischemic cortex tended to be affected (NS) (**a, d**). The corresponding contralateral (*CL*) regions remained unaffected (**b, d**). Animals surviving until P18 showed no significant decrease of NeuN cells in the caudate or in the cortex (c). VEGF therapy counteracted the neurodegenerative effect of MCAO predominantly not only in the cortex but also in the caudate?(**e–h**), (*p<0.05; **p<0.01; mean ± SEM, one-way ANOVA with Tukey's multiple comparison tests, n=8–10/group)

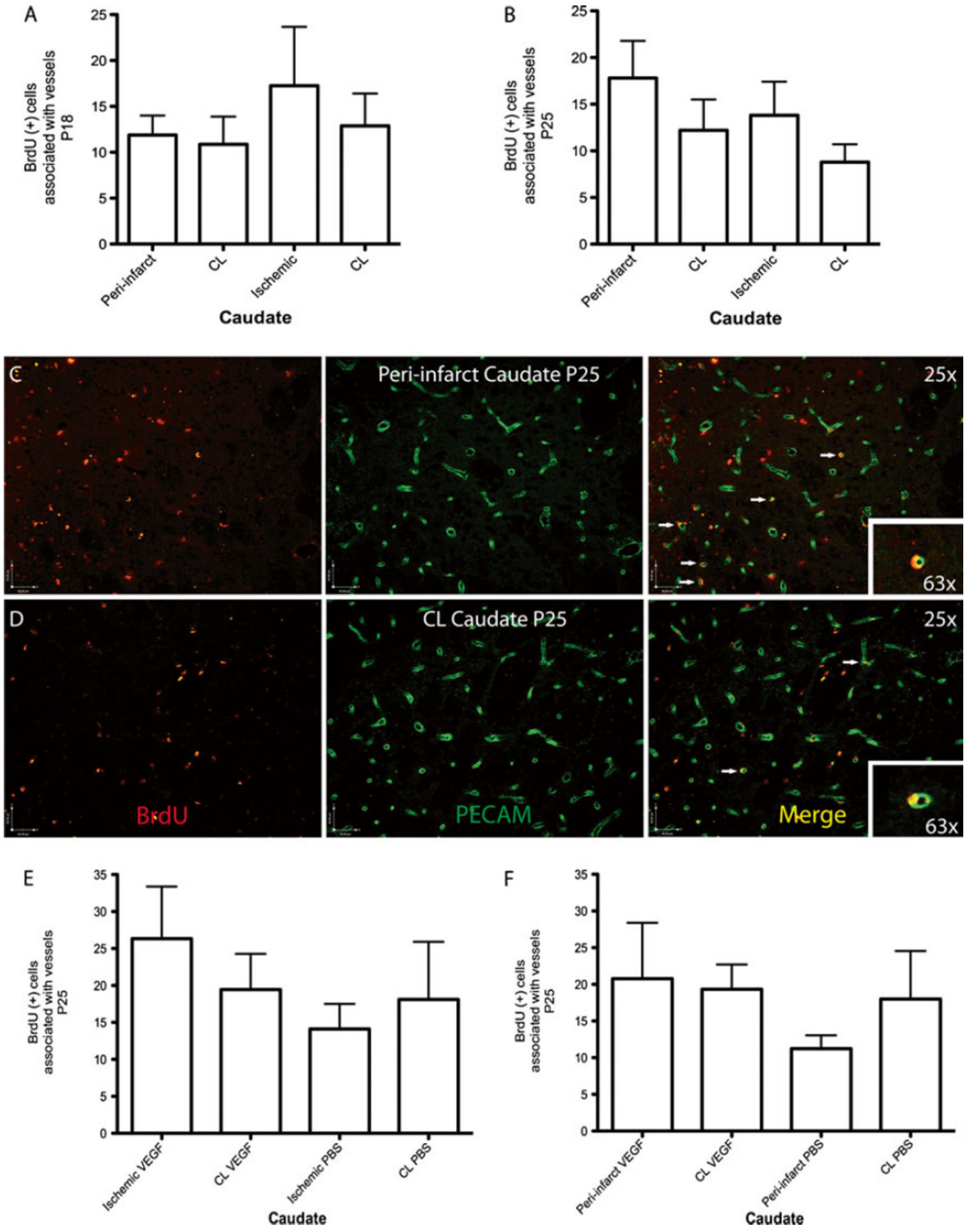

Fig. 3.

Angiogenic response after MCAO is variable in the caudate. The presence of proliferating BrdU-positive endothelial cells is enhanced in the ischemic and peri-infarct regions of the caudate, especially at P25 compared to the corresponding contralateral regions (**a, b**, and **c** vs **d**; *filled arrows* represent merged, yellow colored double-labeled BrdU+/PECAM+ endothelial cells). VEGF therapy 8 days after MCAO generated no additional angiogenic effects (**e, f**), (mean ± SEM, one-way ANOVA with Tukey's multiple comparison tests, n=5–9/group)

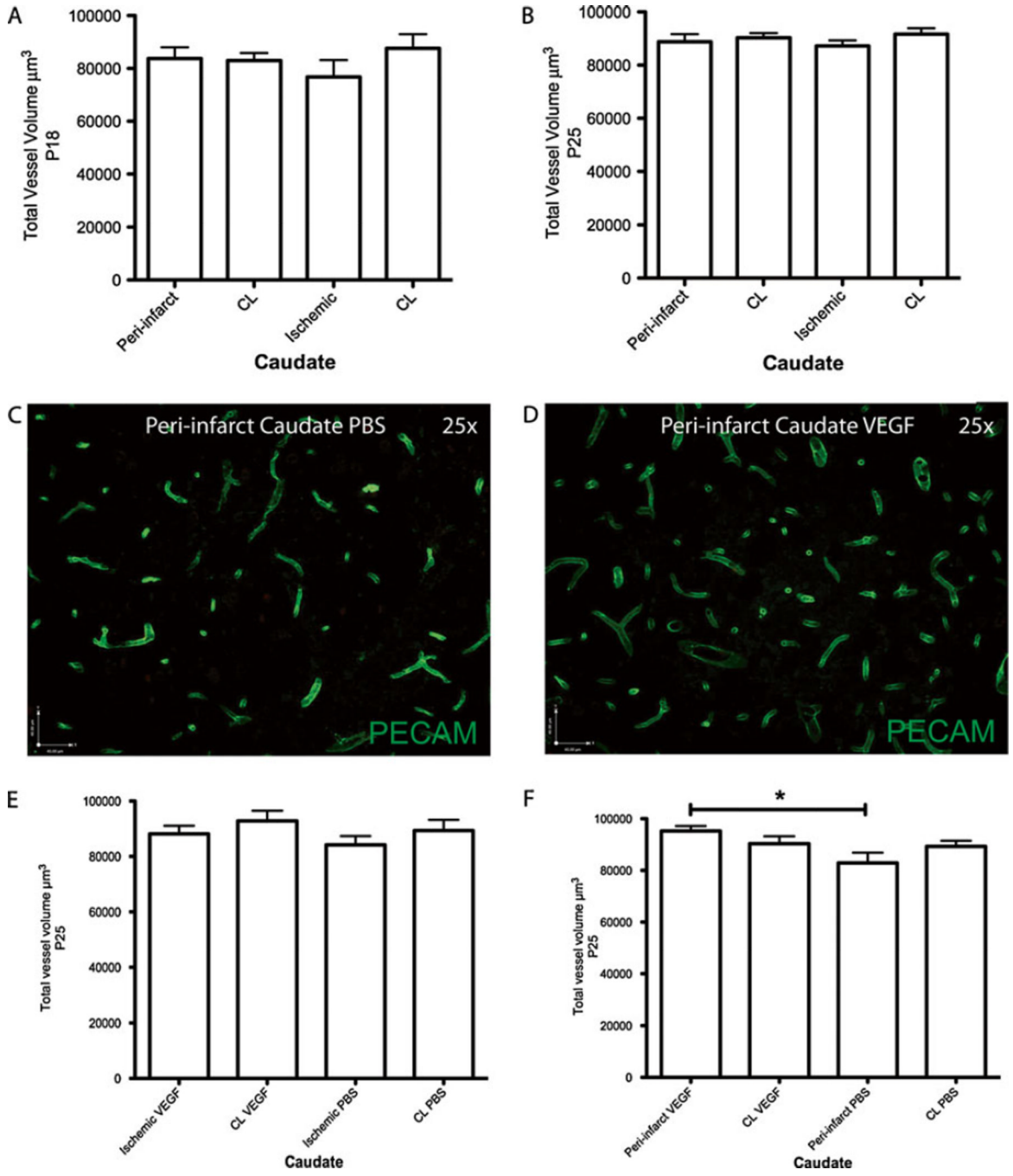

Fig. 4.

VEGF increases total vessel volume in peri-infarct region of the caudate. MCAO performed at P10 had no impact on total vessel volume measurements at P18 or P25 in the peri-infarct and ischemic caudate (**a, b**). Administration of VEGF 8 days after MCAO at P10 induced total vessel volume in the peri-infarct region in the caudate compared to PBS-treated animals. The vessel volume in the ischemic caudate remained unaffected (**c–f**), (*p<0.05, mean ± SEM, one-way ANOVA with Tukey's multiple comparison tests, n=9–10/group; *CL* contralateral region)

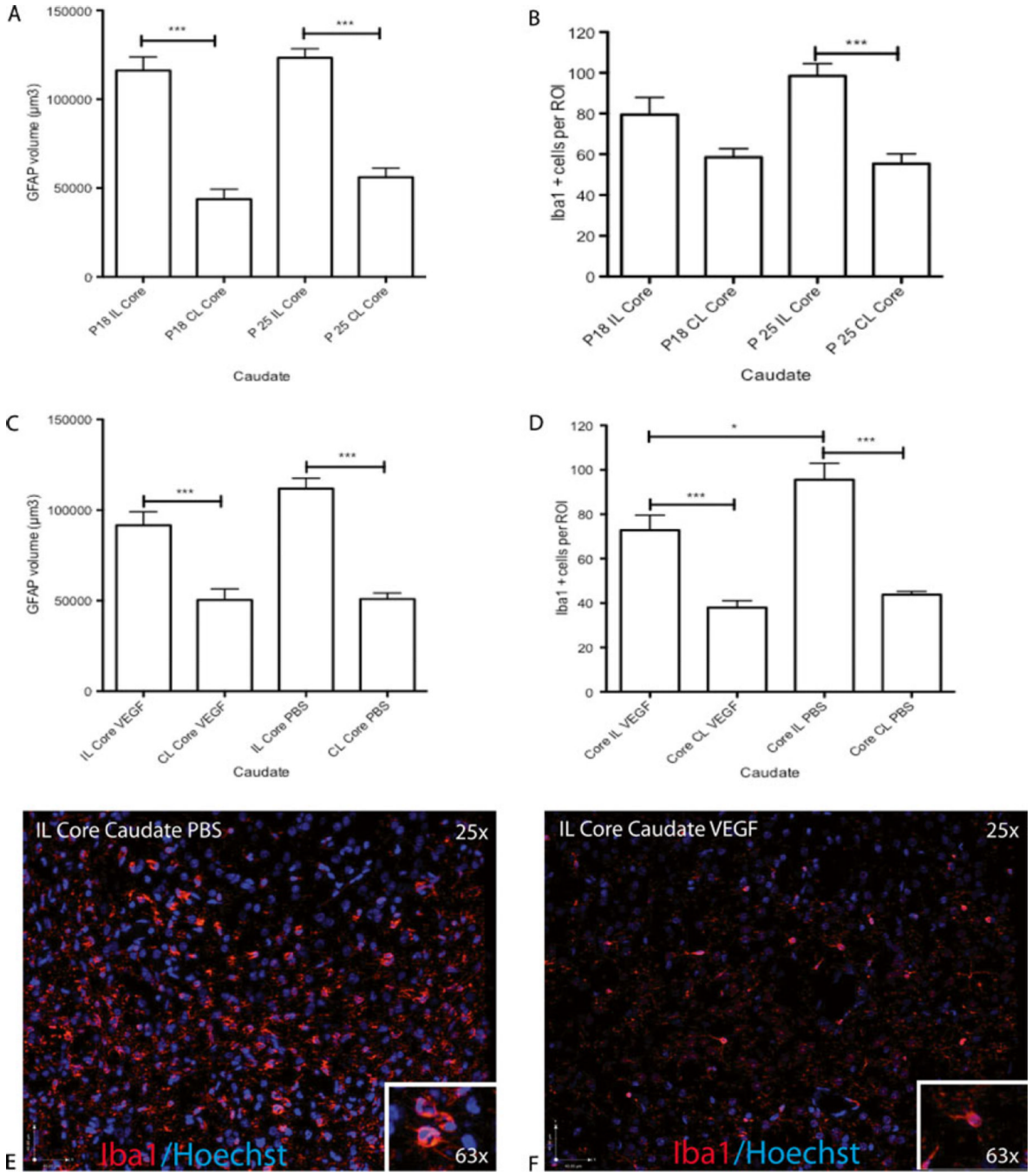

Fig. 5.

VEGF therapy reduces microgliosis in ischemic core of the caudate. MCAO triggered a profound accumulation of Iba1-positive cells and GFAP-positive cells at P18 and P25 (**a, b**). Microgliosis in VEGF-treated animals was diminished in the ischemic caudate. Astrogliosis represented by GFAP staining was unaffected (**c–f**); high power images (×63) show morphological changes of microglial cells (**e, f**), (*p<0.05; ***p<0.001; mean ± SEM, one-way ANOVA with Tukey's multiple comparison tests, n=9–10/group; *CL* contralateral region)

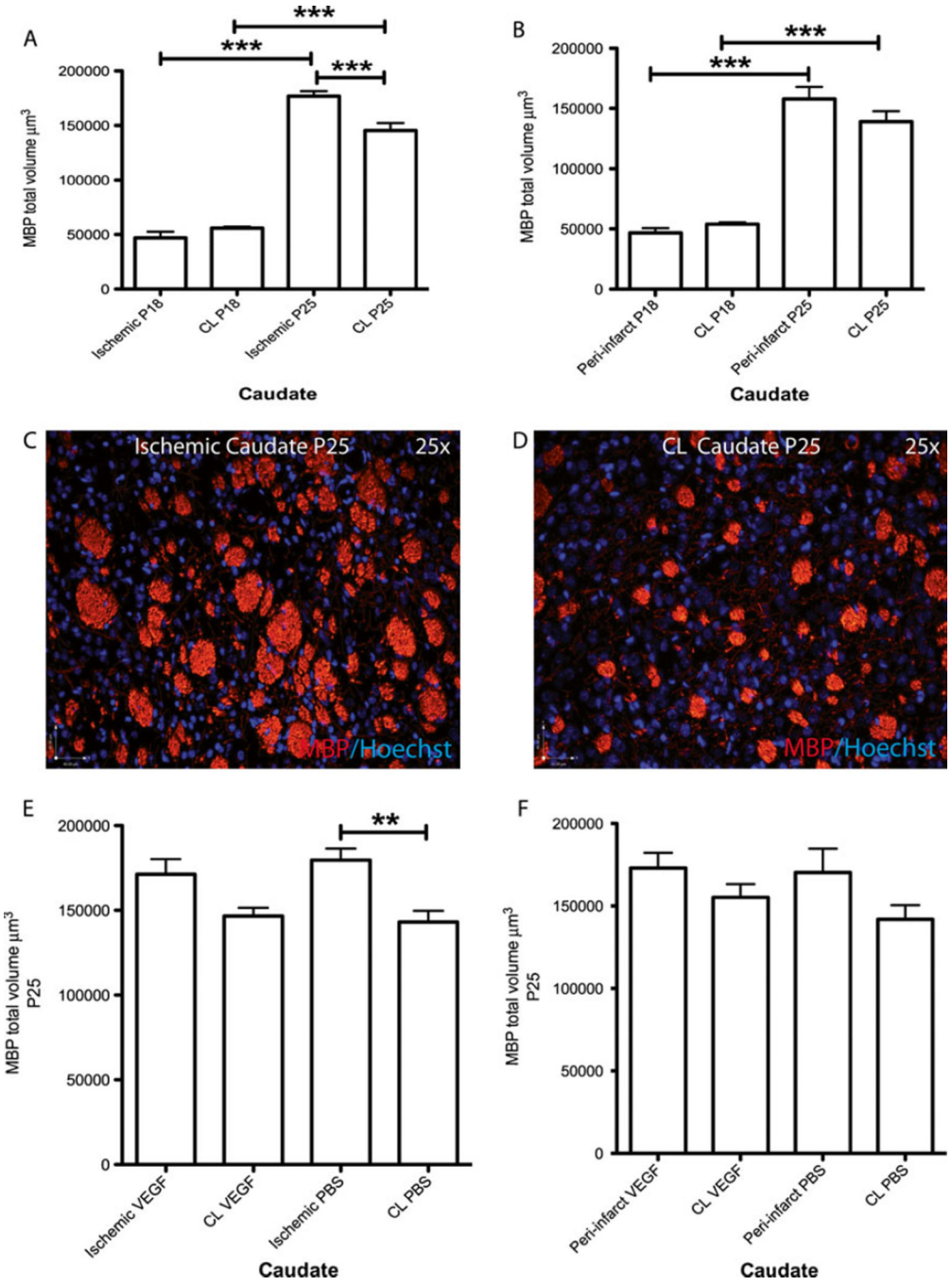

Fig. 6.

Enhancement of MBP-positive white matter fibers in the ischemic caudate after MCAO. Enhanced MPB staining is evident in the ischemic caudate compared to the corresponding contralateral region in P25 animals that underwent MCAO at P10. Note that such an effect was not present in injured P18 animals. At the same time, volume of MBP-positive fibers within the peri-infarct region in the caudate was not affected (**a–d**). VEGF therapy attenuated the increased MBP presence induced by MCAO in the ischemic caudate (**e–f**), (**p<0.01; ***p<0.001; mean ± SEM, one-way ANOVA with Tukey's multiple comparison tests, n=8–10/group)