Promoting stroke recovery using delayed growth factor delivery: Contribution of ipsilesional versus contralesional pyramidal tract plasticity

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I. LIST OF ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
Apaf1	apoptotic protease activating factor 1
ATP	adenosine triphosphate
Na^+/K^+ ATPase	sodium-potassium adenosine triphosphatase
BBB	blood-brain barrier
BDA	biotinylated dextran amine
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
CB	cascade blue
CBF	cerebral blood flow
cDNA	cyclic deoxyribonucleic acid
CD 31+	cluster of differentiation 31
CD 45	cluster of differentiation 45
CNS	central nervous system
CSPGs	chondroitin sulphate proteoglycans
CST	corticospinal tract
CY3	cyanine dye 3
CY2	cyanine dye 2
CT	threshold cycle
DAB	3,3'-diaminobenzidine
DAPI	4'-6-diamidino-2-phenylindole
DCX	doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
dpi	days post ischemia
DNase	deoxyribonuclease
dATP	desoxyadenosintriphosphat
ECM	extracellular matrix

ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
Epo	erythropoietin
Eph B_1	ephrin B1
ENSCs	endogenous neuronal stem cells
F.I.	fold induction
fMRI	functional magnetic resonance imaging
GAGs	glucoseaminaoglycans
GAP-43	Growth Associated Protein 43
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
G-CSF	granulocyte-colony stimulating factor
HIF-1	hypoxia-induced factor 1
HRP	horseradish peroxidase
Iba-1	ionized calcium binding adaptor protein
i.c.v.	intracerebroventricular
IGF-1	insulin growth factor
iNOS	inducible nitric oxide synthase
IL-1β	interleukin-1β
IL-1ra	interleukin-1 receptor antagonist
IL-6	interleukin-6
iNOS	inducible NO synthase
IP3	inositoltrisphosphat
KDR	vascular endothelial growth factor receptor-2
LDF	laser Doppler flow
LIF	leukemia-inhibitory factor
LSD	least significant differences
LTD	long term depression
LTP	long term potentiation
MARCKS	myristoylated alanine-rich C-kinase substrate
MCAO	middle cerebral artery occlusion
MCx	motor cortex
MEAB	modified enzymatic activation buffer

MEG	magnetoencephalography
MMPs	matrix metalloproteases
MMP9	matrix metalloproteases 9
MPT	mitochondria permeability transition
mRNA	messenger RNA
NAD+	β-nicotinamide adenine dinucleotide
NA	nicotinamide
NaCl	natrium chloride
$NaNO_2$	natrium nitrite
Ncan	neurocan
Ncam	neural cell adhesion molecule
NeuN	neuron-specific nuclear protein
NMDA	N-methyl-D-aspartate
N_2O	nitrous oxide
NO	nitric oxide
NOS	nitric oxide synthase
NSC	neuronal stem cells
Ntn1	netrin-1
O_2	oxygen
ParCx	parietal cortex
PARP	poly-(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline tween 20
PCA	postconceptional age
PET	positron emission tomography
PFA	paraformaldehyd
PH	*pondus Hydrogenii (latein)*
PID	peri-infarct depolarization
PIGF	placenta induced growth factor
PPv	posterior periventricular area
PVDF	polyvinylidenfluorid
RMS	rostral migratory stream
RNA	ribonucleic acid

RNS	reactive nitric species
ROI	region of interest
ROS	reactive oxygen species
rt-PA	recombinant tissue-plasminogen activator
RT-PCR	real-time polymerase essay
SAINT-II	Stroke Acute Ischemic NXY Treatment
S.D.	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGZ	subgranular zone
SPRR1A	small proline-rich protein 1A
STDP	spike timing depending plasticity
Str	striatum
SVZ	subventricular zone
TBST	tris-buffered saline containing 0.5% Triton X-100
TBS-Tw	tris-buffered saline containing 0.1% Tween
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TLDA	TaqMan low density arrays
TMS	transcranial magnetic stimulation
TNF-α	tumor necrosis factor alpha
TUNEL	transferase dUTP nick end labeling
VEGF	vascular growth factor

II. LIST OF FIGURES

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IV. ABSTRACT

Stroke incidence is increasing due to the rapidly aging population in developed countries. Whereas untreated acute middle cerebral artery occlusion (MCAO) causes death in 20% of patients and long-term disability in more than 70% of patients, acute stroke therapy with rapid vessel recanalization significantly reduces mortality without influencing functional recovery beyond the acute stroke phase. This lack of functional recovery suggests a need for innovative therapies that can restore function after stroke.

The purpose of these studies was to examine the effects of delayed administration of the growth factors erythropoietin (Epo) and vascular endogenous growth factor (VEGF) on functional neurological recovery and pyramidal tract plasticity in mice.

The first study investigated how subacute delivery of Epo, starting at 3 days after stroke onset and continuing for 30 days (1 I.U. /day or 10 I.U. /day; via mini osmotic pump), influenced neuronal survival, axonal sprouting and neurological function recovery in C57Bl6/j mice submitted to 30-min MCAO.

Epo administered in a 10 I.U. /day dose, in contrast to the 1 I.U. /day administration, showed a significant increase in neuronal survival and CD31 + newly-formed capillaries. This vascular growth enabled further neuroregeneration processes. Functional behavioral tests showed a significant improvement of motor coordination (RotaRod test) and grip strength (Grips strength test) among mice with 10 I.U. /day Epo administration, with no improvement for the low dose group.

To investigate the neurological changes underlying these results, two anterograde tract tracers (dextrane amines) were injected in the motor cortex ipsilateral and contralateral to the ischemic lesion, in mice treated with the higher Epo dose. Histological evaluation of the tracers, both at the level of rubral and facial nucleus, showed that functional recovery in these animals was due to an increase of contralateral projections, accompanied by a compensatory decrease of ipsilateral projections.

In the second study, VEGF was investigated due to its dual actions on vessels and neurons, which have potential for promoting long distance axonal plasticity in the ischemic brain.

Mice were submitted to 30 minutes MCAO, followed by the intraventricular delivery of normal saline or VEGF (0.004 or 0.02 µg/day) starting 3 days post-ischemia. The outcome parameters were functional neurological recovery, long distance axonal plasticity by anterograde tract tracing and cellular and molecular responses examined by histochemistry, RT-PCR and Western blots.

VEGF promoted neurological recovery when administered at the higher dosage, by stimulating long distance axonal plasticity in the contralesional but not ipsilesional pyramidal tract system. This observation was accompanied by deactivation of matrix metalloproteinase-9 (MMP9) in the ipsilesional brain tissue and downregulation of axonal growth inhibitors and guidance molecules in the contralesional brain tissue.

The results support the concept that brain plasticity is consistent with coordinated axonal growth responses both ipsilateral and contralateral to the site of stroke. Considering that Epo is well tolerated in humans, clinical studies are now conceivable in which Epo is applied in patients in the post-acute stroke phase.

1. INTRODUCTION

1.1. Pathophysiology of acute ischemic injury

Stroke is a neurovascular pathology characterized by a sudden or gradually progressing obstruction of a large brain artery with induction of a core of necrosis. Currently, stroke affects more than 2 million people in the United States, and is the third leading cause of death and the leading cause of disability (Rosamond et al., 2007).

From a pathophysiological point of view, stroke can be divided into two categories: ischemic and haemorrhagic. Due to the prevalence of ischaemic stroke in clinical practice, the focus of preclinical and clinical studies was positioned on ischemic stroke. Thromboembolic events cause blood restriction in a specific brain region, leading to an ischemic lesion. The impact of the ischemic lesion on functional outcome is mainly shaped by the collateral flow of the region, the degree of vessel occlusion (complete versus incomplete) (Saver, 2008), duration of the occlusion (transient versus permanent), location (brain regions vary in susceptibility to ischemia; Hashimoto et al., 2008) and co-morbidities (arterial hypertension, diabetes, atherosclerosis; Hossmann, 2006). These factors play an important role in the transition from reversible to irreversible injury and represent the main target in acute stroke treatment strategies.

1.1.1. Anatomic considerations of blood flow regulation and collateralization

In comparison with other organs, the central nervous system (CNS) has a redundancy of arterial supply (Hossmann, 2006). The four extracranial arteries (the paired carotids and the vertebral arteries) are inter-connected intracranially by the main six collaterals (right and left anterior, middle and posterior cerebral arteries)

forming two rescuing systems at the level of the circle of Willis (see Figure 1) and the leptomeningeal anastomoses of Heubner (Hossmann, 2006).

The circle of Willis provides low-resistance connections between the origins of the six arteries, offering a compensation mechanism in the case of extracranial obstruction of the carotid and/ or vertebral arteries (Doyle et al., 2008). The circle of Willis provides a rescue mechanism against global ischemic processes in the central eloquent brain regions, also providing collateral flow under conditions of focal cerebral ischemia (Hossmann, 2006) (Figure 1).

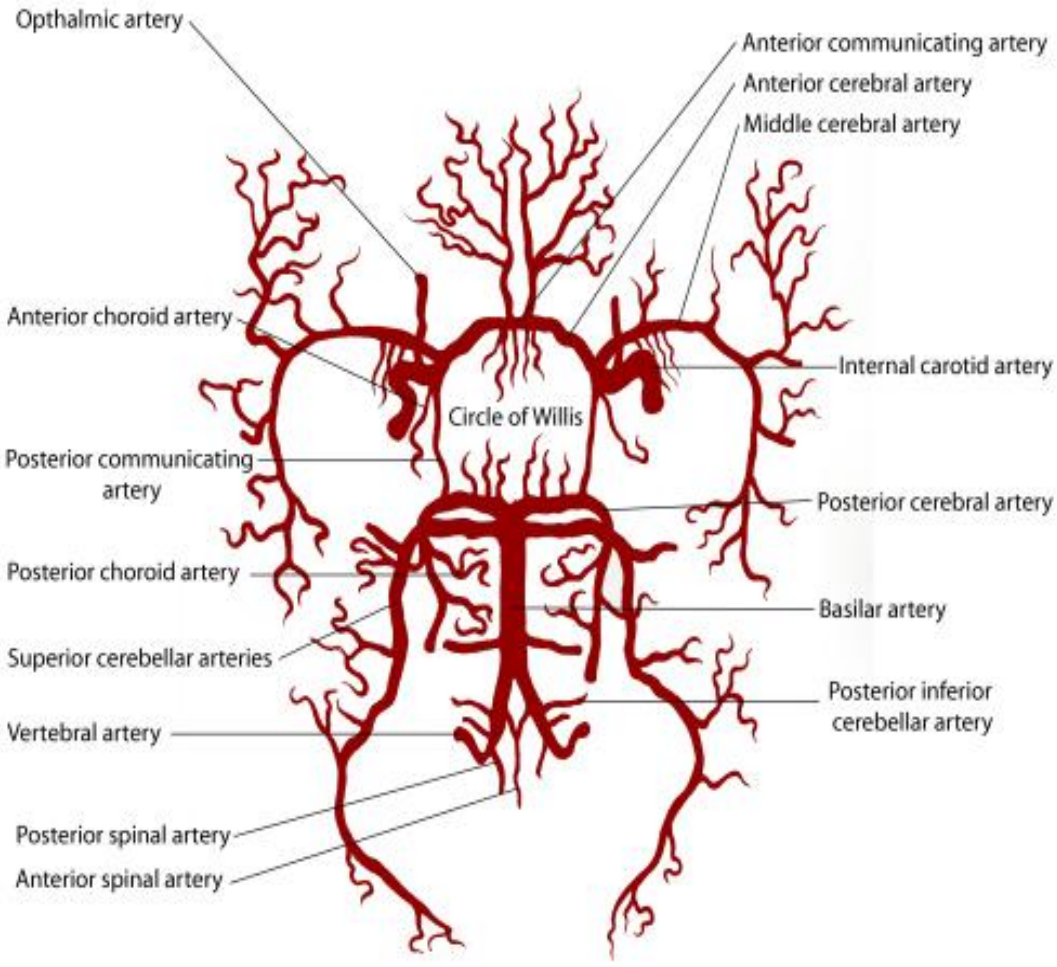

Figure 1. Anatomy of cerebral arteries: the main six cerebral arteries and their interconnections within the circle of Willis. (Figure copied from http://neuro4students.wordpress.com/pathophysiology)

The degree and the suddenness of vascular occlusion play an important role in the process of collateralisation. In case of an embolic complete vascular occlusion, the already anatomically present anastomotic devices may not resist the increased pressure from the main supplying artery and may fail to produce a functional collateralisation (Simons, 2005). On the opposite, a partial occlusion over time, for example due to atherosclerosis processes in the vessels, will allow the anastomotic vessels to undergo remodelling of the vascular wall by means of arteriogenesis, which will allow them to bypass the normal routes in case of critical occlusion of the main artery (Busch et al., 2003).

In addition to the anatomical back-up represented by collaterals, the human brain possesses another important mechanism that determines the size of ischemic injury: the autoregulatory response. In case of reduced blood pressure, the brain redistributes blood supply by autoregulatory dilatation of the resistance vessels and dilatation triggered by metabolic acidosis surrounding the necrotic core in case of ischaemia. Due to autoregulation, blood flows through the dilated vessels passively, representing the first rescue mechanism after a vessel occlusion (Dirnagl and Pusinelli, 1990).

1.1.2. The concept of ischemic core and penumbra

The human brain requires 20% of total oxygen consumption, used mainly for the generation of adenosine triphosphate (ATP), which is necessary for the Na^+/K^+ ATPase pump involved in restoration of ionic gradients (Edvison and Krause, 2002). In case of an energy failure, the neuronal membrane cannot repolarize and leads to release of the excitatory neurotransmitter glutamate along its concentration gradient from the pre- towards postsynaptic membranes (Schubert and Piasecki, 2001). This excess glutamate in the extracellular space leads to activation of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, stimulating further membrane depolarisation, with an increase in intracellular calcium and resulting activation of calcium dependent proteases, lipases and DNases, a process called excitotoxicity (Stout et al., 1998; Olney, 1969).

These metabolic changes are produced in the core of ischemia, where the cerebral blood flow (CBF) is most severely compromised, and then spreads in a radial manner to the surrounding tissue. The ischemic core is surrounded by a rim of tissue with impaired electrical activity but preserved cellular metabolism and viability, called the penumbra (Astrup et al. 1977; Astrup et al, 1981; Hossmann, 1993, 1994). Whereas the ischemic core evolves rapidly, and is characterised by necrotic debris, the penumbra region has a moderate reduction of CBF, providing a rescue potential which depends on the speed of reperfusion of this tissue after ischemia (Baron et al, 1995; Wise et al, 1983) (Figure 2).

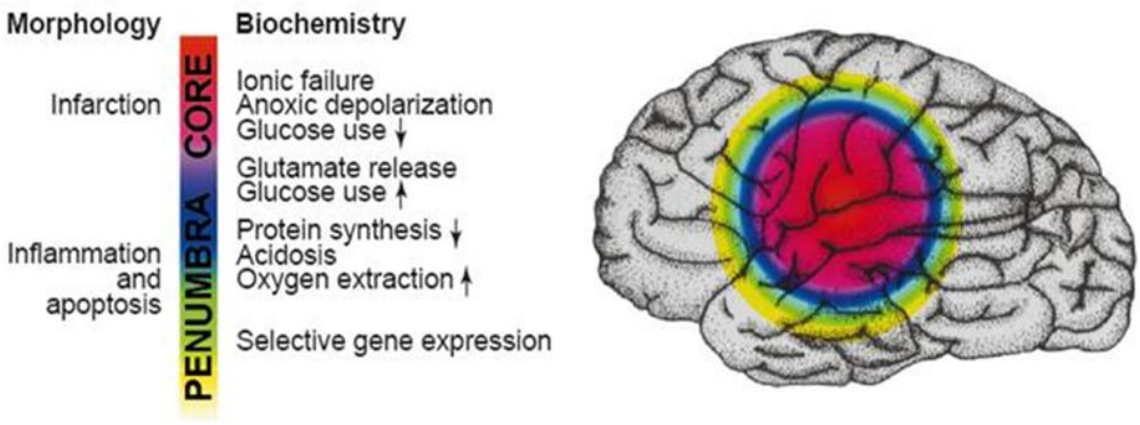

Figure 2. Ischemic penumbra and biochemical events in radial sprouting from the core (figure copied from Dirnagl, et al., 1999)

Based on the dynamics of the penumbra, a therapeutic time window was defined for treating acute ischemic stroke. Although the acute delivery of recombinant tissue-plasminogen activator (rt-PA) in the first 4.5 hours after stroke correlated in clinical studies with a reduced evolution of stroke disease (Hacke W. et al., 2008), other neuroprotective treatments failed repetitively in clinical studies. Whereas chemical thrombolysis with rt-PA may induce intracerebral haemorrhage after stroke, mechanical thrombolysis is proven to increase the therapeutic window and to reduce functional disabilities, and is currently the gold standard for acute stroke therapy (Gralla et al., 2012; Gralla et al., 2006). The reperfusion of the ischemic tissue in this therapeutic window aims to save the penumbra and minimize ischemic damage, and to prevent vasogenic edema and haemorrhagic complications after revascularization.

1.1.3. Progression of ischemic injury

Neuroprotection therapy after stroke failed in the translation phase, so a better understanding of the mechanisms leading to the progression of injury is urgently needed. A series of molecular events characterize the cascade of ischemic injury: excitotoxicity, oxidative and nitrative stress, dysfunction of endoplasmatic reticulum and mitochondrial disturbances (Doyle et al., 2008).

The immediate release of excitatory neurotransmitters after stroke and simultaneous activation of their receptors results in an increased calcium concentration inside the cell, leading to mitochondrial calcium overload and activation of calcium-dependent catabolic enzymes (Choi, 1994). The main neurotransmitter involved in this process is glutamate (Schubert and Piasecki, 2001; Stout et al., 1998). Glutamate activates metabolic processes by stimulation of Inositoltriphosphate (IP3) dependent transduction pathways, modifying their responses in the brain (Kiessling and Gass, 1994) and thereby inducing excitotoxic injury (see Figure 3).

Because neurons have low levels of endogenous antioxidants, they are extremely vulnerable to reactive oxygen species. The activity of nitric oxide synthase (NOS) is stimulated under hypoxic conditions, leading to an increase in nitric oxide (NO) and peroxynitrate concentration (Kunz et al, 2007). Oxidative stress correlates with over-activation of poly (ADP-ribose) polymerase-1 (PARP-1), a DNA repair enzyme, which catalyses the transformation of β-nicotinamide adenine dinucleotide (NAD+) into nicotinamide (NA) and long polymers of poly (ADP-ribose) (PARP) (Sairanen et al., 2009). Over-activation of intracellular PARP-1 leads to a depletion of NAD+, causing an impairment of NAD+ dependent cellular processes such as anaerobic glycolysis and mitochondrial respiration, resulting in ATP starvation and neuronal death (Gingsberg, 1997).

The second phase of oxidative stress is observed during the reperfusion-induced injury phase, and is characterised by activation of matrix metalloproteases (MMPs), which affect the integrity and functionality of the blood brain barrier (BBB) (Valable et al., 2005). These second phase activities may lead to parenchymal haemorrhage and vasogenic brain edema, and may also explain why the therapeutic

window of thrombolytic therapy has a time-dependent functional limitation (Crack and Taylor, 2005).

The increase of cytosolic calcium activity leads also to increased permeability of inner mitochondrial membranes, which leads to formation of MPT (mitochondrial permeability transition) pores (Siesjö et al., 1999). This causes swelling of mitochondrial matrix, allowing release of pro-apoptotic mitochondrial proteins which activate the cysteine protease caspase 3, an important initiator of apoptotic cell death (Krajewski et al., 1999) (Figure 3).

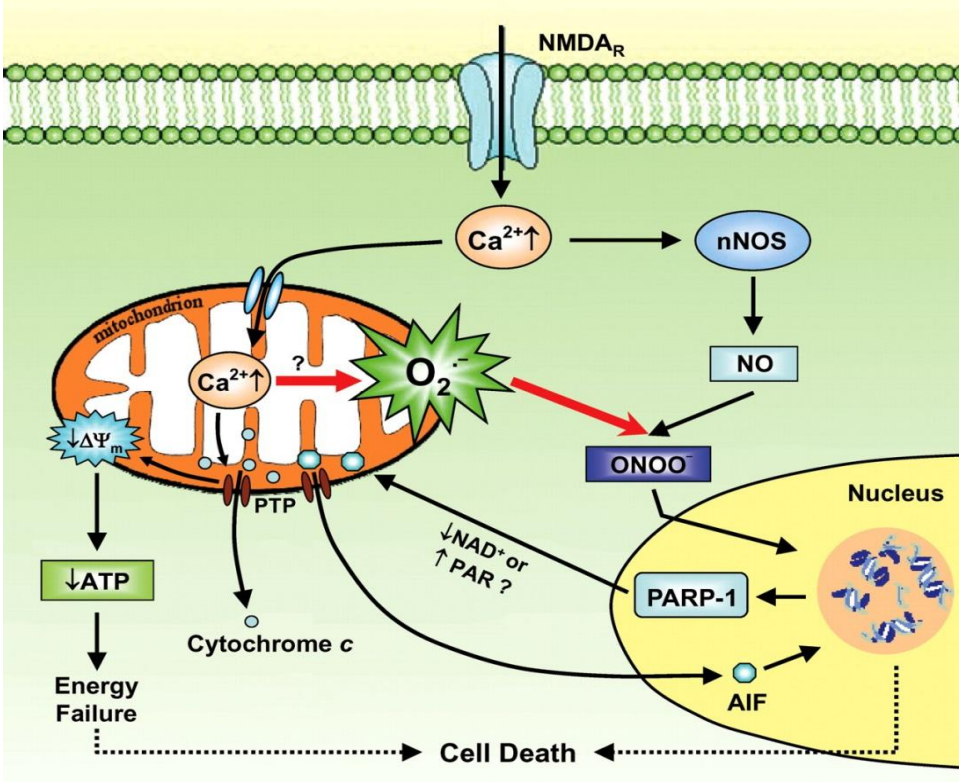

Figure 3. Excitotoxicity after stroke (figure copied from Duan et al., 2007)

Depending upon the pathophysiological mechanism, stroke leads to two types of edema: cytotoxic (caused by cellular swelling) and vasogenic (caused by leakage of the BBB) (Hossmann, 1989, 1993).

Alteration of ionic balance in the necrotic core lesion with an increase of the intracellular sodium gradient causes intracellular water accumulation by means of osmotic gradient dependent forces (Todd, 1986). This leads to a cytotoxic

oedematous transformation of the ischemic core, which is later augmented by the disruption of the brain-blood barrier (BBB) approximately six hours after ischemia. Whereas cytotoxic oedema results from acute changes in ionic balance after stroke, the vasogenic edema reaches a peak approximately two days after stroke (Walz et al., 2002). The clinical relevance of brain oedema is exemplified by the so-called malignant infarct, which causes swelling of the infarcted region over the anatomical borders of the skull, and may result in life-endangering transtentorial herniation and midbrain compression if a decompressive craniectomy is not immediately performed (Hacke et al, 1996; Bardutzky and Schwab, 2007).

The complex pro-inflammatory reaction after stroke was shown to cause an increase in the structural ischemic damage and it is a target for neuroprotective treatments in stroke (Dirnagl, et al., 1999). The inflammatory processes in the brain differ from other tissues due to the brain's evolutionary privilege in having an anatomical and physiological barrier (the BBB) and an immunological barrier (reduced expression of major histocompatibility complex class-I and class-II antigens on specific cells in the CNS) (Schwartz, et al., 1999). These particular characteristics are responsible for the dual role of inflammation during pathophysiologic processes in the brain such as in ischemic stroke.

Different inflammatory mechanisms were shown to negatively influence the ischemic damage in the brain, producing microvascular obstruction, increase of acidosis, elimination of toxins, production of reactive oxygen species (ROS) (del Zoppo et al., 1991; Dirnagl et al., 1999). These processes induce BBB disruption, leading to release of histamine and proteases with activation of inflammatory cells in the perivascular space, which create a proinflammatory milieu in the brain parenchyma due to cytokine production (tumour necrosis factor [TNF], Il-1β) (Iadecola and Anrather, 2011).

After stroke, programmed cell death occurs in the ischemic penumbra and is triggered by oxygen reactive free nitric radicals, death receptor activation, DNA damage and protease activation. There are two important pathways for induction of apoptotic death: the intrinsic and the extrinsic pathways (Hossmann, 1989, 1993). The apoptotic cascade starts with the intracellular release of cytochrome c from the

outer mitochondrial membrane in response to ionic imbalance and mitochondrial swelling. After release from the outer membrane, cytochrome c binds to ATP, procaspase 9 and apoptotic protease activating factor 1 (Apaf1), creating an apoptosome (Krajewski et al., 1999). The apoptosome is responsible for DNA degradation and p53 activation, leading to an increase of Bax expression which further promotes mitochondrial pore formation and mitochondrial swelling (Brad et al., 2009; Mattson et al., 2000). The extrinsic cascade involves death receptors independent of the release of cytochrome c. Death-receptors activate caspase-8 and caspase-10, which finally involve caspase 3 and cause DNA degradation (Namura et al., 1998).

1.2. Clinical aspects of stroke research

The human brain possesses endogenous rescue mechanisms such as: 1) long time arteriogenesis of collateral vessels that enable rapid re-routing of blood flow in case of a sudden obstruction (Busch et al, 2003); 2) glial scar formation in the close vicinity of the ischemic core with neuroprotection potential (Trandelenburg and Dirnagl, 2005); 3) tissue-remodeling by reactivation of ontogenetic repair mechanisms (Cramer and Chopp, 2000). Trying to sustain these endogenous strategies leads to three therapeutic stroke research strategies: acute chemical or mechanical thrombolysis, neuroprotection therapies and neurovascular restorative strategies. Because our study focuses on the third aspect, Chapter 1.3 is dedicated to the restorative mechanisms and therapeutic strategies after stroke.

1.2.1. Thrombolysis and mechanical recanalization

Intravenous tissue plasminogen activator was proven to be the first successful stroke therapy in acute ischaemic stroke, but it successfully recanalizes MCAOs in only one-third of cases. Only a very rapid recanalization, achieved within minutes to hours before irreversible brain damage develops, has proven to be effective (Cohen, 2013). Mechanical thrombectomy detaches the intra-arterial thrombus and allows rapid revascularisation of the ischemic region, and can be used in a looser time-

window of up to eight hours after stroke (Fiehler, 2012). However this therapy still needs to be proven in prospective clinical trials and the side-effects are not yet fully characterised (Lo et al., 2003).

Chemical thrombolysis, either systemic or regional, by endovascular methods also proved to be effective in the treatment of acute ischemic stroke. It was reported that with increasing delay of the application of thrombolysis therapy, there was a decline in the therapeutic effect; the critical period was restricted to 3 to 4.5 hours after onset of symptoms (Hacke et al, 2008). Although it was reported that thrombolysis decreases mortality (Wardlaw et al, 2003), this treatment is effective in a narrow time-window after the onset of stroke (Brown et al, 2003). A new time-independent therapy is required in order to facilitate treatment for all stroke patients irregardless of the health and economic systems in the various countries.

Recent studies report that increasing delay of intravenous thrombolysis administration after stroke causes a decline in the therapeutic effect (Brinker et al. 2003; Vergouwen et al, 2012). In case of mechanical thrombolysis, a recent study confirmed that time to treatment is a predictor of outcome, but only when collaterals were excluded from the stroke model of the anterior circulation (Galimanis et al., 2012). Even in studies where it was proven that thrombolysis decreases mortality (Wardlaw et al, 2003), this treatment was restricted to patients treated within a specific time period after the onset of stroke (Brown et al, 2003).

The concept of “stroke care unit” was developed in order to increase the efficiency of stroke treatment, by reducing the time-to-diagnosis and time-to-treatment window, and led to a significant reduction in mortality and institutionalised care in the first 10 years (Indreadavik et al., 1999). Strategies for improving functional recovery after stroke have unfortunately not yet been established in clinics, leading to permanent functional impairment in the majority of patients.

1.2.2. Neuroprotection

Neuroprotection aims at reducing ischemic injury by preventing apoptosis, inflammation, metabolic distruptions and neurotransmitter disturbaces in the ischemic area. Despite intensive research and promising preclinical results, all the neuroprotective drugs failed in the clinical translation phase (Gingsberg, 1997). The most important candidates of this group are described breifly below.

Glutamate antagonists were proved to reduce the size of ischemic lesion (Iijima et al., 1992; Gill et al., 1992). Reduction of neuronal necrosis after stroke was linked to glutamate antagonists, antioxidant substances meant to stop the formation of reactive oxygen species (ROS), and substances meant to stop delayed neuronal death (e.g., apoptosis).

Aiming to interfere with the development of cytotoxic oedema, aquaporin channel inhibitors meant to block intracellular water accumulation were investigated. It was shown that aquaporine channel inhibitors reduced ischemic injury (Griesdale and Honey, 2004). However, it was later proven that aquaporine channel inhibitors merely delayed edema formation (Hossmann, 2006).

Interleukin (IL)1-beta was for a long time considered to be a promising neuroprotective target, since administration of interleukin 1-beta receptor antagonist (IL-1ra) reduced infarct size (Stroemer and Rothwell, 1998). However the transition of these therapies into the clinic failed repeatedly. The SAINT-II (Stroke Acute Ischaemic NXY Treatment) study tested the antioxidative agent NXY-059 as neuroprotectant in stroke patients. This study did not reveal efficacy in phase III trials (Savitz and Fisher, 2007; Savitz, 2007) and had to be interrupted.

1.3. Restorative mechanisms in post-acute stroke phase

Stroke remains the leading cause of serious motor disabilities in adults (Bonita and Beaglehole, 1994). Hemiparesis initially affects 80-90% of patients, and 45-60% of patients still exhibit motor deficits in the post-acute stroke phase (Dobkin, 1996). Thus, patient recovery is limited.

Post-ischemic endogenous responses of the CNS correspond to an enhanced sensitivity to rehabilitative (Biernaskie et al., 2004) and plasticity-promoting (Papadopoulus et al., 2002; Seymour et al., 2005) treatments, opening a time window in which ontogenetic brain repair mechanisms may successfully be reactivated (Cramer and Chopp, 2000; Buchli and Schwab, 2005; Hermann and Chopp, 2012).

The observation of endogenous regeneration potential after stroke, by means of neurogenesis (Jin et al., 2001), angiogenesis (Krupinski et al., 1994), functionally active stem cells (Gould et al., 1999), axonal sprouting (Reitmeir et al., 2011,2012) and synaptogenesis paved the way for post-acute treatments.

1.3.1. Endogeneous neurogenesis

Formation of neuronal stem cells (NSCs) starts in the gastrulation phase and continues throughout the embryonal phase by a continuous proliferation in the ventricular zone (Clark and Chiu, 2003). Radial glia cells, situated in the subventricular zone (SVZ) (Clark and Chiu, 2003), migrate to the primordial dentate hilus of the hippocampus, forming the subgranular zone (SGZ) and the dentate gyrus (DG) (Pleasure et al, 2000). In the adult brain these NSCs were found to proliferate in the SVZ, SGZ and the posterior periventricular (PPv) area (Gage, 2000; Alvarez-Buylla et al., 2002). This represents an endogenous pool which was shown to be activated by focal ischemia (Yan et al., 2006) both in the ipsilesional and in the contralesional hemisphere (Zhang et al., 2001 a,b), reaching a peak 1-2 weeks after stroke and returning to sham levels by 3-4 weeks (Dempsey et al., 2003, Zhu et al., 2003).

The process of neurogenesis includes three major anatomical steps: proliferation, migration and differentiation (Iwai et al., 2002) which are followed by functionality steps such as development of electric potential of the newly formed neuron and its integration into the cellular matrix environment.

Promotion of neuronal proliferation after stroke was intensively studied using different growth factors, and the most promising are Epo and VEGF. Ischemia was proven to stimulate the hypoxia-induced factor-1 (HIF-1) as a main player in the signal cascade after stroke. The smallest reduction in oxygen partial pressure in the brain leads to a strong activation of HIF-1. Both VEGF and Epo are responsible for the down-stream effects of HIF-1 (Pugh and Ratcliffe, 2003). Epo knock-out mice show deficiencies in post-ischemic neurogenesis (Tsai et al., 2006) and VEGF promotes neurogenesis both *in vitro* and *in vivo* (Wang et al., 2007).

Endogenous NSCs in the normal brain follow the route of rostral migratory stream (RMS) towards the olfactory bulb, whereas in the ischemic preconditioned brain, the NSCs migrate towards the injured areas in the brain (Thored et al., 2006, Arvidsson et al, 2009). Important mediators in this process of migration and maturation are the MMPs, especially MMP9, which is upregulated in the infarcted cortex of rats 7-14 days after infarct. MMP9 colocalizes with Doublecortin (DCX) and Bromo-deoxy-uridine (BrdU) positive cells migrating from the SVZ (Zhao et al., 2006). Wang et al. reported that conditioned medium from Epo-treated epithelial cell cultures significantly increased the neural progenitor migration which was impaired by MMP inhibitor (Wang et al., 2006).

Even when there is evidence for migration and maturation of NSCs to the ischemic lesion, the functionality of these neurons, their long-time survival, and their integration in the peri-neural and angiogenic milieu is still controversial. Such survival and integration is necessary in order to support beneficial recovery after stroke. VEGF and Epo are thought to be promising candidates to facilitate this functional integration. Up to now they have been proven to be stimulating factors for neurogenesis, angiogenesis, migration and maturation, so the only missing puzzle piece is their potential role in axonal sprouting with integration in peri-neural matrix.

1.3.2. Angiogenesis and neurovascular remodelling after stroke

A complex intercellular orchestration is needed to create permissive conditions for functionally relevant axonal and dendritic sprouting after ischemic injury in the brain. The vascular and nervous systems share multiple similarities in their development, both of them using long-distance projections to reach their target, being guided by gradients of growth factors (Hermann and Zechariah, 2009). The parallel tracking of blood vessels and nerves is especially obvious in peripheral nervous tissues. In the CNS, neurogenesis takes place in the embryologic vascular niches where endothelial cells (ECs) proliferate. Thus, both systems have to be taken into consideration as a homeostatic unit, especially in neuropathological events of the brain such as stroke (Hermann and Zechariah, 2009).

The formation of new vessels alone has less impact upon neural regeneration after stroke, unless functionality of the neurons is maintained. There are two important aspects that should be considered when discussing functional integrity of newly formed vessels: first they should lead to an increase in the cerebral blood flow proportional with the increase in vessel density, and second they should integrate in an incipient neurovascular niche by forming new physiologically active units in the process of regeneration after stroke.

Both neurogenesis and angiogenesis seem to take place in the same time-window, starting about 48 hours after the stroke occurs (Seylaz et al, 1999; Marti et al., 2002). This vessel formation is probably too late to influence injury development in stroke (Hermann and Zechariah, 2009), supporting the idea that vascular remodeling is more involved in restorative and plasticity processes.

Pharmacological interference with angiogenesis and neurogenesis may disrupt these systems, thereby hindering stroke recovery. Early post-ischemic administration of VEGF to ischemic rats increased BBB leakage and infarction volume in the ischemic brain (Zhang et al., 2000), whereas late-administration (48 hours) enhanced angiogenesis, decreased BBB leakage and improved stroke recovery (Chae et al., 2000).

1.3.3. Axonal plasticity

Efforts from the last decade showed that not only that the brain itself has an intrinsic capacity to regenerate after stroke, but also that extrinsic factors can be used to successfully stimulate regeneration. Recent data shows that the adult brain has intrinsic reorganisation and repair potential after stroke (Cramer and Chopp, 2000). This plasticity potential is represented at the anatomical level by: recruitment of axons that sustain the same function as the destroyed ones but have a different anatomical form, synaptogenesis, dendritic arborisation, fortification of functional silent synaptic connections, and long distance fiber sprouting (Carmichael et al, 2001, 2003). These events take place in the first days and weeks after the ischemic lesion, and their susceptibility to external therapeutic factors is negatively correlated with time.

An understanding of the molecular, cellular, anatomical and temporal self-repair capacity of the brain after stroke is necessary in order to develop cell- and pharmacologic-based therapies. In order to understand how we can stimulate plasticity after stroke, plasticity during ontogeny is an important consideration.

1.3.3.1. Corticospinal system: evolution and ontogenetic development

Whereas non-mammals possess precocious motor skills immediately after birth, many mammals are born with only the simple motor behaviours necessary for survival, such as breathing and feeding-related behaviours (Muir, 2000). Human motor development is completed within the first few years after birth. Whereas the brainstem motor systems are well developed at birth, the corticospinal system is not (Kudo et al., 1993). The development and maturation of corticospinal connections in humans take place over years, forming the principal motor system for voluntary limb control (Eyre, 2003).

Human corticospinal axons reach the lowest cervical spinal cord by 24 weeks postconceptional age (PCA) (Stanfield, 1992). At about 40 weeks PCA the expression of neurofilaments begins and myelination of corticospinal axons is still ongoing (Stanfield, 1992). Nathan et al. demonstrated that 8 to 15% of the

corticospinal tract is represented by uncrossed axons (Nathan et al., 1996). These ipsilateral corticospinal tract fibers originate from the same cortical areas as the contralateral fibers and present a similar pattern of spinal innervation as the contralateral projection (Nathan et al, 1996).

1.3.3.2. Plasticity of corticospinal tract after stroke

Stroke is the leading cause of chronic disability, as many patients survive the acute event and undergo a first phase of innate spontaneous recovery which is usually insufficient for a complete recovery. Attempts to augment innate functional recovery after stroke are now in a focus of pre-clinical and clinical studies.

An exciting pathophysiologic aspect in stroke recovery is the opening of a time-limited neuroplasticity window due to reactivation of ontogenic mechanisms similar to ones described in the developing nervous system (Cramer and Chopp, 2000; Carmichael and Li, 2006). This attempt towards self-reorganisation of the CNS after stroke corresponds with an enhanced receptivity of the ischemic brain towards neuroregenerative pharmacologic therapy (Biernaskie et al., 2004; Brown et al., 2009; Carmichael et al., 2006). Extensive studies in non-human species provided deep insights into the understanding of the complex interactions taking place during brain remodelling after ischemic injury.

A series of temporo-spatial events have been suggested as important players in this reorganisation process. The peri-infarct region undergoes molecular changes with fibre sprouting of surviving neurons (Li and Chopp, 1999), synaptogenesis (Wall and Egger, 1971), dendritic arborisation (Jones and Schallert, 1992) and sprouting over long distances by unmasking of latent horizontal connections (Sanes and Donoghue, 2000). This long distance fiber sprouting implicates the non-ischemic hemisphere and other distal structures like the red nucleus and basilar pontine nuclei. These events are initiated after the “hyperacute and acute” post-stroke phase (up to 72 h after onset), unfold in the subacute recovery phase, and reach optimal efficiency in the first 2 weeks after parenchymal injury (Kreisel et al., 2006). This expansive temporospatial pro-plasticity conditioning of the brain towards more

responsiveness to brain remodelling opens new frontiers for delayed and chronic therapeutic neurorehabilitation possibilities after stroke.

Latent interneuronal network nodes connect the lesional or perilesional area with other distant secondary, associative or contralateral cortical areas in the brain, and are reactivated after stroke. Their degree of excitation is conditioned by the primary localisation of the stroke and by its size (Kreisel et al., 2006) (Figure 4). A subcortical stroke was shown to disrupt cortical inhibition in the unaffected hemisphere (Shimizu et al., 2002). Recruitment of these increased activation areas from the contralesional hemisphere correlated with a better recovery after stroke (Feeney and Baron, 1986). This remapping potential was shown to be greater in models of relatively small strokes that resemble the size of a survivable human stoke (Cramer and Crafton, 2006). The present study is based on 30 minutes MCAO as a stroke model, which produces a localised, reproducible and focal subcortical infarct.

The role of the contralateral hemisphere in functional recovery after stroke remains controversial. A series of events observed in the intact hemisphere after stroke, such as increased blood flow (Silvestrini et al., 1998), metabolism (Marshall et al., 2000), excitability (Iglesias et al., 1996), inflammatory markers (Haring et al., 1996), and sprouting of dendrites and synapses, also have a delayed onset in the subacute stroke phase, leading to the speculation that these events may provide the anatomical background for the plasticity-promoting phase initiated in the contralesional hemisphere (Jones and Schallert , 1992). These events correlated with improved functional outcome after stroke in non-human animal models, in clinical practice the degree of contralesional activation was observed to be more intense in patients with worse outcome after stroke (Calautti and Baron, 2003; Ward, 2011). The goal of the present study is to clarify the role and nature of interhemispheric interactions after subcortical stroke injury, and to examine their impact on post-stroke recovery.

Preclinical and clinical studies on unilateral ischemic brain damage have demonstrated that reorganization of the corticospinal tract in the intact hemisphere may increase functional outcome after stroke. Ischemia of the corticospinal tract leads to degeneration (Eyre, 2003). This leads to postsynaptic inactivation of spinal

cord motoneurons and subsequent postsynaptic degeneration, unless fibers of other subcortical nuclei take over the distal corticospinal function. In a non-human animal model, the rubrospinal tract was shown to take over the function of the corticospinal tract projections, thus promoting functional recovery after stroke (Papadopoulos et al., 2001). In humans the role of the rubrospinal tract is not well defined.

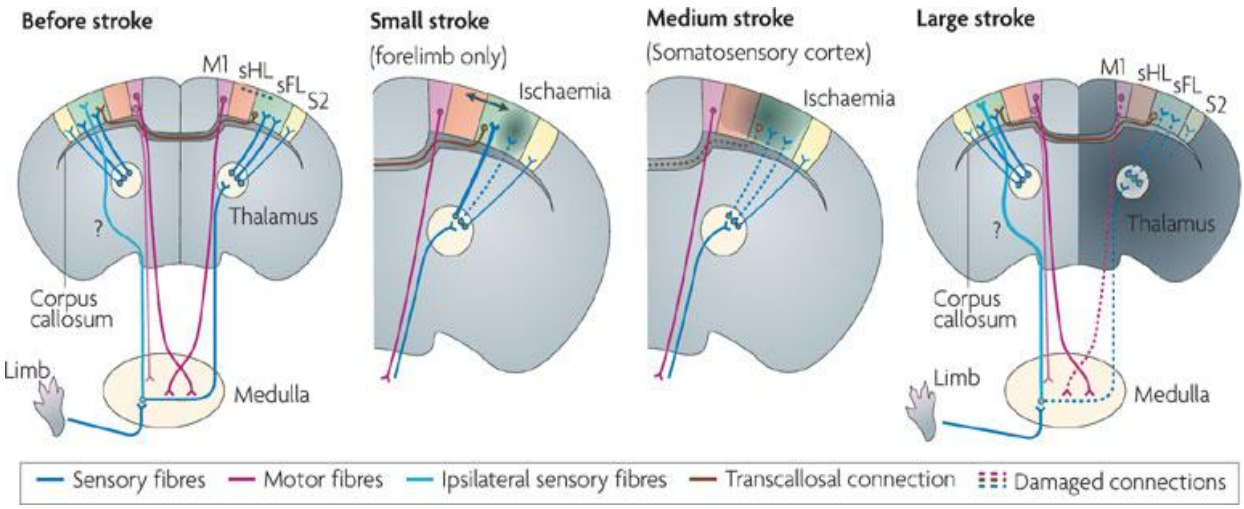

Figure 4. Axonal sprouting after stroke (figure copied from Murphy and Corbett, 2009). This figure explains the recovery of sensorimotor function after stroke. In case of small and medium stroke lesions the nearby tissue with similar sensorimotor function will contribute to the recovery process through a strengthening of difusse synaptic connections or by creation of new structural connections under the guidance of synaptic learning rules. In case of large ischemic lesions the recovery will be sustained by circuits coming from the contralateral hemisphere which are interconnected at subcortical level. The primary motor cortex (M1) and hindlimb (sHL), forelimb (sFL) and secondary somatosensory cortex (S2) are indicated.

Kreisel et al. (2006) proposed a timeline of recovery processes after stroke, differentiating between 5 distinct stages: (1) hyperacute phase from the stroke event up to 6 hours after; (2) acute phase lasting up to the fourth day after stroke, characterised by secondary events; (3) subacute phase from the second day up to 2-3 weeks, characterised by neurofunctional plasticity; (4) consolidation period lasting up to several months, characterised by neurofunctional alteration; (5) chronic phase characterised by the tendency of the events to become static.

1.3.4. Extracellular matrix as a trigger for axonal plasticity

The extracellular matrix (ECM) consists of: 1) Glucoseamineglycans (GAGs) which may be found either bound with proteins (proteoglycans) or unbound as hyaluronan; 2) fibrous proteins (e.g. collagens and elastin); 3) adhesive glycoproteins (e.g. fibronectin, laminin and tenascin) and 4) a wide variety of growth factors bound (inactive) or unbound (active) to matrix.

Besides its structural role, the ECM controls functional interactions between cells in different processes such as development, cell survival and tissue homeostasis (Oohira et al., 2000). After stroke, the composition of the ECM is altered (Charmichael, 2003). Among the regulated molecules, chondroitin sulphate proteoglycans (CSPGs), such as aggregan, versican and phosphacan, have been reported to be involved in stroke recovery processes (Charmichael, 2003).

1.3.5. Clinical evidence of corticospinal tract plasticity after stroke

Evidence from positron emission tomography (PET), functional magnetic resonance imaging (fMRI), transcranial magnetic stimulation (TMS) and magnetoencephalography (MEG) studies also supported the relevance of contralesional brain plasticity for human stroke recovery (Gerloff et al., 2006; van der Zijden et al., 2008; Ding et al., 2008). However, implications of contralesional activation were difficult to interpret as activation patterns in humans are strongly influenced by the severity and inhomogeneity of strokes. Whether the promotion of functional neurological recovery with growth factors influences contralesional reorganization processes was until not described.

1.4. Role of growth factors in stroke recovery

1.4.1. Erythropoietin (Epo)

The discovery of erythropoietin (Epo) and its receptor in neurons and astrocytes (Bernaudin et al., 2000) represented the beginning of a decade of follow-up studies which established Epo as a neuroprotective factor after stroke. Epo inhibits apoptosis in the tissue adjacent to the lesion (Tan et al., 1992) and modulates nitric oxide synthesis (Siren et al., 2001) and neurotransmitter release after stroke (Minnerup et al., 2009).

A small clinical study classified Epo as being “both safe and beneficial” for acute stroke (Ehrenreich et al, 2002). Based on these results a multicentre clinical trial was conducted with 522 patients in whom Epo (40,000 I.U./administration) was infused intravenously at 3 timepoints (6, 24 and 48 h) after acute ischemic stroke both in patients with and without concomitant rtPA administration. Surprisingly, the study showed an increased mortality in patients treated with both Epo and rtPA, whereas Epo alone showed a neuroprotective effect (Ehrenreich et al., 2009). Further preclinical investigations showed that Epo administered together with rtPA after stroke increases brain edema, thereby increasing mortality after stroke (Zechariah et al, 2010). This is why a thorough evaluation of experimental conditions is necessary, as improper testing puts not only patients at risk, but also endangers future studies aimed at investigating the therapeutic potential of Epo in stroke patients. Because of this interaction between therapy and rtPA in the first hours after stroke, a delayed administration may reduce this interaction potential and thus enable the innate regeneration capacity of the brain.

The post-stroke potential of Epo must still be analysed and understood at the basic research level. Epo was shown to have neuroregenerative potential in a rodent model of Parkinson’s disease, where it increased fiber outgrowth in the ventral mesencephalon (McLeod et al., 2006), by enhancing axonal sprouting in a model of optic nerve transection (King et al., 2007; Kilic et al., 2005b) and by enhancing white

matter reorganisation in rat focal ischemia (Li et al., 2009). The neuroregeneration potential of Epo after stroke, and particularly its effects on axonal plasticity and interhemispheric remodelling, have not been fully explored (Bernaudin et al., 1999). Therefore, the present study explored the effects of Epo after a MCA occlusion (stroke model) in mice.

The inflammatory response after stroke is receiving increasing attention in the field of brain tissue remodelling and reorganisation. Experimentally and clinically, stroke is followed by an acute and a chronic inflammatory response. Microglia cells seem to have a dual role in the orchestration of cellular recovery after stroke. Whereas microglia are detrimental for neurogenesis after stroke during the acute phase (mainly driven by Il-6 and TNFά synthesis) (Iosif et al., 2006; Koo and Duman, 2008), a chronic activation pattern of these cells was correlated both in vivo (Bonde et al., 2006) and in vitro (Walton et al., 2006) with long-term survival of newly formed neurons after stroke. Another important factor that mediates the cause-effect interrelationship between inflammation and regeneration is the activation of astrocytes with the formation of glial scar, a cellular-biochemical wall that inhibits axonal regeneration after stroke.

The present study evaluated the effect of delayed Epo administration upon local and distal tissue reorganisation, acute and chronic inflammation and functional recovery after stroke, presenting for the first time a complete picture of the temporo-spatial processes that culminate with an improved recovery post-stroke.

1.4.2. Vascular endogenous growth factor (VEGF)

Subsequent to a stroke, the brain tissue undergoes profound remodeling processes involving finely tuned cell-cell (Zhang and Chopp, 2009) and cell-extracellular matrix (ECM) (Zhao et al., 2006) interactions, aimed at the reconstitution of a functional neurovascular system. These events are sustained by ontogenic protein expression programs which are reactivated after stroke (Cramer and Chopp, 2000). These programs stimulate de novo expression of ECM proteins and their proteases (MMPs) in the peri-lesional tissue (Zhao et al., 2006). Matrix molecules orchestrate inter- and intracellular pathways, facilitating interactions of growth factors with the cellular surface (Yong, 2005).

VEGF is an endogenous growth factor which is activated in hypoxic conditions in the brain via activation of HIF-1α expression (Forsythe et al., 1996), which then stimulates the transcription of VEGF, VEGF receptors (flt-1 and neuropilin-1), and angiopoietin (Pugh and Ratcliffe, 2003). Because two additional anatomical disruptions occur in the acute phase after stroke, loss of vascular integrity and cell matrix degradation (Hermann and Zechariah, 2009), VEGF`s activation is potentiated. These two processes facilitate the activation of growth factors, their receptors and their guidance molecules, which are incorporated in the cellular matrix in a functionless state under normal conditions. Endogenous VEGF is activated by cellular matrix degradation, which activates angiogenesis and thereby induces endothelial cell proliferation and migration after stroke (Carmeliet, 2003).

The VEGF family comprises 5 related genes: VEGF-A, -B, -C, -D and PIGF (placenta induced growth factor). VEGF-A is a vascular permeability factor and has several isoforms (VEGF-A_{204}, -A_{189}, -A_{165}, -A_{145}, -A_{121}) which have different amino acid length and capacity of binding to heparin sulfates. VEGF-A_{165} binds heparan sulfate to some extent, which reduces its diffusibility but at the same time increases its ability to stimulate VEGF receptors (Simons, 2005).

Recent data in non-human primates subjected to circumscribed infarcts of the motor cortex showed that in addition to associating with neurons throughout the lesioned hemisphere, VEGF can also be found in injury-remote neurons of the

contralateral motor cortex (Stowe et al., 2007). The VEGF receptor VEGFR2 is *de novo* expressed on motor cortical neurons remote from the lesion site (Stowe et al., 2008). This observation raises questions about VEGF's role in the reorganization of ischemic brain tissue. Besides VEGF and its receptors, MMP9 is also upregulated in cerebral cortex surrounding a stroke lesion for up to 14 days (Zhao et al., 2006). Colocalization of MMP9 with markers of neurovascular remodeling suggested that MMP9-induced matrix degradation contributes to brain plasticity (Zhao et al., 2006). Recruitment of remote brain areas has been shown to contribute to neurological recovery after stroke in rodents (Papadopoulos et al., 2002; Wiessner et al., 2003; Reitmeir et al. 2011) and humans (Gerloff et al., 2006; van der Zijden et al., 2008). By means of anterograde tract-tracing techniques, pyramidal tract plasticity can be analyzed in rats (Papadopoulos et al., 2002; Wiessner et al., 2003) and mice (Reitmeir et al., 2011).

In view of the unique activities of VEGF in stimulating the survival and proliferation of endothelial cells and neurons, we sought to determine whether VEGF influences long distance axonal plasticity in the ischemic brain and, secondly, how angiogenesis and axonal plasticity are linked. For this purpose, a comprehensive analysis was performed to characterize the effects of post-acute VEGF delivery on neurological recovery, perilesional vascular remodeling, and pyramidal tract plasticity ipsilateral and contralateral to a stroke.

2. AIMS OF THE STUDY

2.1. Effect of delayed Epo administration on axonal plasticity modulation and functional recovery after stroke

Promising results from pre-clinical studies, in which Epo was found to be neuroprotective after ischemic brain injury, failed to be translated into clinical efficacy in the German Multicenter EPO Stroke Trial (Ehrenreich et al., 2009). The main reason for clinical failure was an unfavourable interaction between Epo and rt-PA, which resulted in an increase in death, intracerebral haemorrhage, brain edema and thromboembolic events (Ehrenreich et al., 2009; Zechariah et al., 2010). In view of these results we wondered if a delayed Epo administration 3 days after stroke would influence functional stroke recovery and axonal sprouting.

The inflammatory reactions in the acute phase after stroke are responsible for the boost of neurogenesis that occurs in the post-acute phase (Zhang et al., 2004; Jin et al., 2001). The role of chronic inflammation after stroke and its impact upon regeneration was until now poorly examined. One of the purposes of this study was to characterise in the post-acute phase how inflammatory processes are associated with stroke recovery

2.2. Effect of delayed VEGF administration on axonal plasticity modulation and functional recovery after stroke

The mutual interactions between vessels and neurons are a major challenge in neurobiology as they unravel basic principles of brain structure and function. During development, angiogenesis and neuronal sprouting are closely linked. It is widely assumed that new vessel formation and axonal sprouting mutually promote each other, forming a functional neurovascular unit driving reorganization processes in the injured brain (Hermann and Chopp, 2012). However, experimental evidence for this concept is scarce (Hermann and Zechariah, 2009). The relationship between angiogenesis and axonal plasticity has so far not yet been addressed in experimental studies.

Aiming to explain how the plasticity is coordinated between the two hemispheres after stroke, we examined effects of VEGF on pyramidal tract plasticity and extracellular matrix remodelling. By measuring the expression of different proteoglycans in different regions of the brain, we characterised molecular signals controlling plasticity responses in the ischemic brain.

3. MATERIALS AND METHODS

3.1. Animal groups

Experiments were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals with local government approval (Kantonales Veterinäramt Zürich, ZH169/2005; Bezirksregierung Düsseldorf, TSG966/08). Male C57Bl6/j mice (8-10 weeks; 23-25 g) were subjected to 30 min of left-sided middle cerebral artery occlusion (MCAO) (Kilic et al., 2005a, 2006, 2008). At 72 hours post-ischemia, mice received implantations of miniosmotic pumps into the left-sided lateral ventricle that were filled with 0.9% NaCl (vehicle) or Epo (1 or 10 I.U. / day diluted in 0.9% NaCl) or VEGF (0.004 µg or 0.02 µg / day diluted in 0.9% NaCl) and were left in place during the subsequent four weeks (Fig. 5 and Fig. 6).

In addition to the mice undergoing MCAO, sham-operated mice were also operated, in which vehicle filled pumps were implanted as specified. One set of mice was used for studies on functional neurological recovery and for analysis of axonal plasticity (n = 10 animals per group). For that purpose, mice received anterograde tract tracer injections (see below) in both frontal motor cortices at 42 days after the stroke was induced. Ten days later, these animals were sacrificed.

Additional male C57Bl6/j mice were also subjected to 30 min MCAO using the same protocol, followed by implantation of intraventricular pumps filled with vehicle or Epo (10 I.U. / day in 0.9% NaCl) (Fig. 5) or VEGF (0.004 µg or 0.02 µg / day diluted in 0.9% NaCl) (Fig. 6) three days later. These animals were sacrificed at 3, 14 and 30 days (used for reverse transcriptase-polymerase chain reaction [RT-PCR] studies) or at 14, 30 or 52 days (used for conventional immunohistochemical studies) after the stroke (n = 4 animals per group, survival time and series). For RT-PCR studies, additional sham-operated mice (n = 4) and control mice subjected to 30 min MCAO without pump implantation (n = 4) were also operated. The latter sham-operated and untreated mice were sacrificed at 3 days post-surgery.

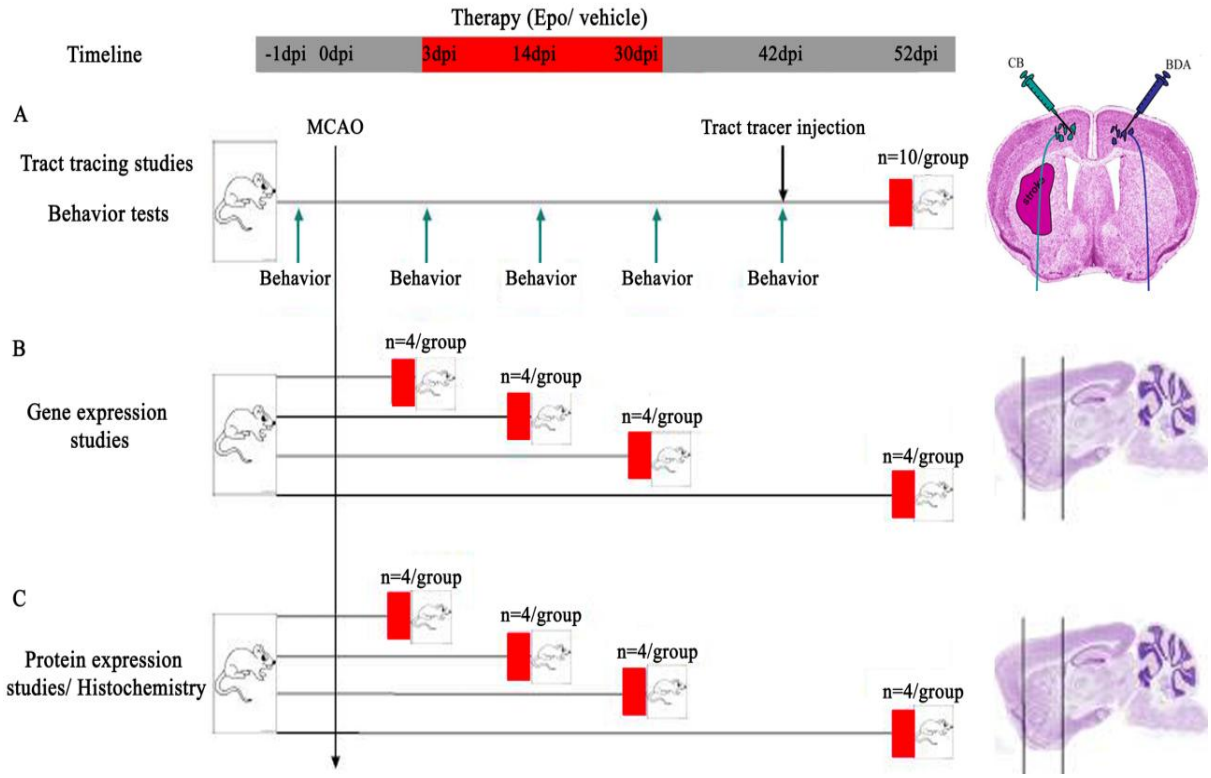

Figure 5. Experimental procedures and animal groups in Epo experiment.

Mice subjected to MCAO that were treated with Epo or vehicle from 3 – 30 days post ischemia (dpi) were used for (A) tract tracing studies and behavioural analysis, (B) gene expression studies (RT-PCR) and (C) protein expression studies (immunohistochemistry, Western blots) and conventional histochemical analysis. Numbers of animals evaluated for each group and time points of animal sacrifice are also shown. On the far right, the rostrocaudal level, from which brain sections and tissue samples were harvested, is also illustrated. In addition to the animals shown in this scheme, additional sham-operated animals and ischemic animals not receiving intraventricular pumps were generated as control groups in some of the studies, as outlined in the Materials and Methods section. CB (cascade blue), BDA (biotinylated dextran amine), dpi (days post ischemia).

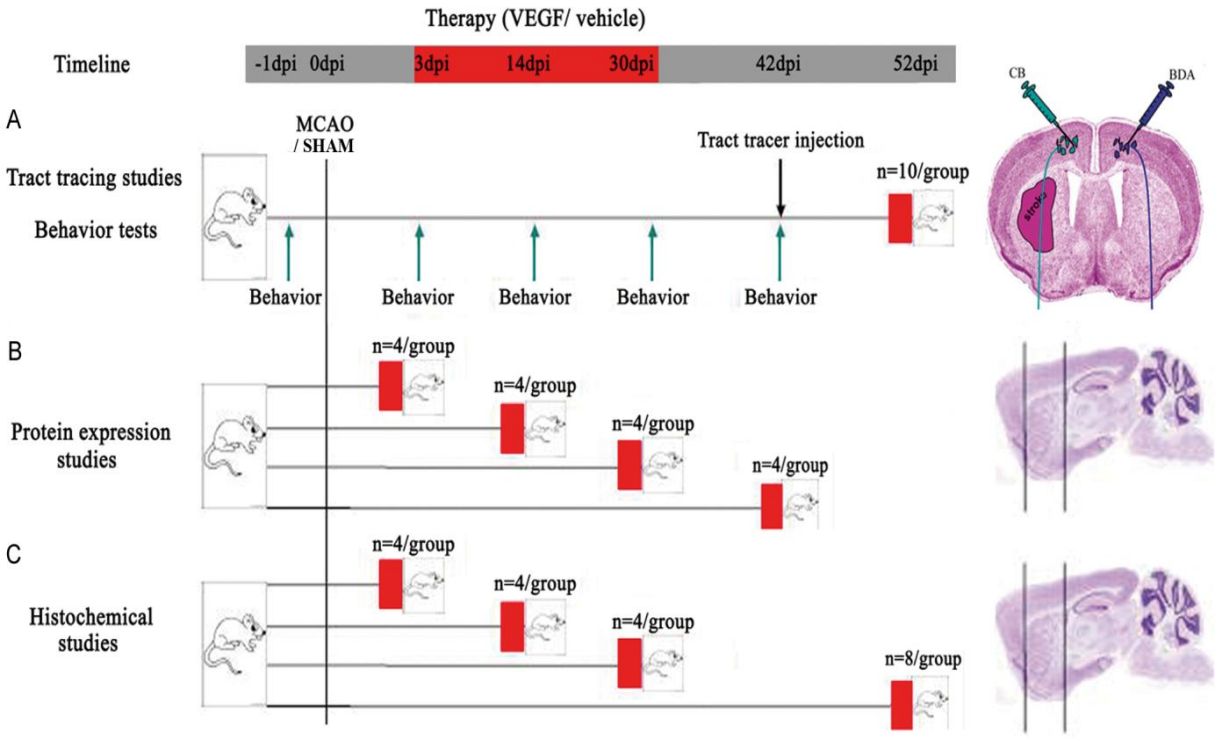

Figure 6. Experimental procedures and animal groups in VEGF experiment**.**

Mice subjected to MCAO that were treated with VEGF or vehicle from 3 – 30 dpi were used for (A) tract tracing studies and behavioral analysis, (B) protein analysis studies (Western blots, gelatin zymography) and (C) conventional histochemical and immunohistochemical studies. Numbers of animals evaluated for each group and time point of animal sacrifice are shown. On the far right, the rostrocaudal level, from which brain sections and tissue samples were harvested, is presented. In addition to the experiments summarized in this scheme, additional sham-operated animals and ischemic animals not receiving intraventricular pumps were analyzed as additional control groups in a variety of studies (see Materials and Methods section). CB (cascade blue), BDA (biotinylated dextran amine) dpi (days post ischemia).

3.2. Induction of focal cerebral ischemia

Animals were anesthetized with 1% isoflurane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0°C using a feedback-controlled heating system. Cerebral blood flow was analyzed by laser Doppler flow (LDF) recordings.

Focal cerebral ischemia was induced using an intraluminal filament technique (Kilic et al., 2008). Briefly, a midline neck incision was made, and the left common and external carotid arteries were isolated and ligated. A microvascular clip was temporarily placed on the internal carotid artery. A silicon resin coated nylon monofilament was introduced through a small incision into the common carotid artery and advanced to the carotid bifurcation for MCAO. Thirty minutes later, reperfusion was initiated by monofilament removal.

LDF changes were monitored for up to 30 min after reperfusion onset. In sham-operated animals, a surgical intervention was performed, in which the neck was opened and the common carotid artery was exposed, but left intact, while LDF recordings were performed (Fig. 7). After the surgery, wounds were carefully sutured, anaesthesia was discontinued and animals were placed back into their cages.

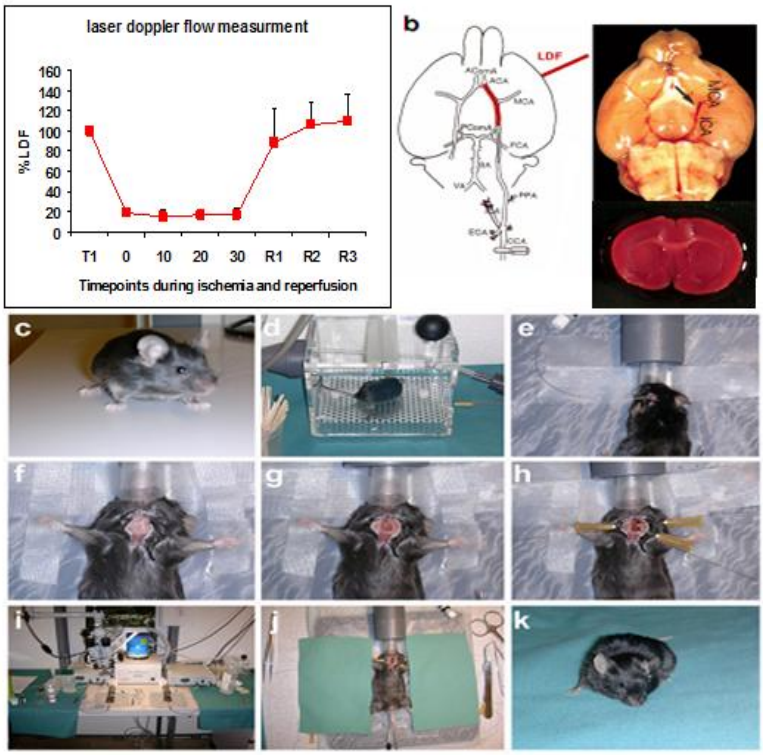

Figure 7. Middle cerebral artery occlusion and intraoperative LDF.

Presentation of operative procedure of MCAO and of the intraoperative monitoring with laser Doppler flow (LDF). (a) LDF measurement during the operation as a control of blood flow reduction during the filament occlusion and after removing the filament. (b) Middle cerebral artery occlusion (MCAO) technique with introduction of a silicon filament in the common carotid and the in the internal carotid, causing occlusion at the base of the middle cerebral artery. (c-k) Intraoperative steps from the anesthesia induction up to the postoperative management.

3.3. Intraventricular pump implantation

Three days after surgery, animals were reanesthetized with 1% isofluran (30% O_2, remainder N_2O) and a cannula (Brain infusion kit 3, Alzet, Cupertino, CA) linked to miniosmotic pumps (Alzet 2004 or 1002; Alzet) filled with 0.9% NaCl or Epo (NeoRecormon, Roche, Basel, Switzerland) (1 or 10 I.U./day in 0.9% NaCl) or VEGF (0.004 µg or 0.02 µg / day in 0.9% NaCl) were implanted into the left-sided lateral ventricle through a burr hole (Kilic et al., 2010) (Fig. 8). Both types of pump

administered the therapy continuously in a dose of 0.25µl/hour. These pumps were left in place for up to 14 days (Alzet 1002) or 30 days (Alzet 2004) and then removed.

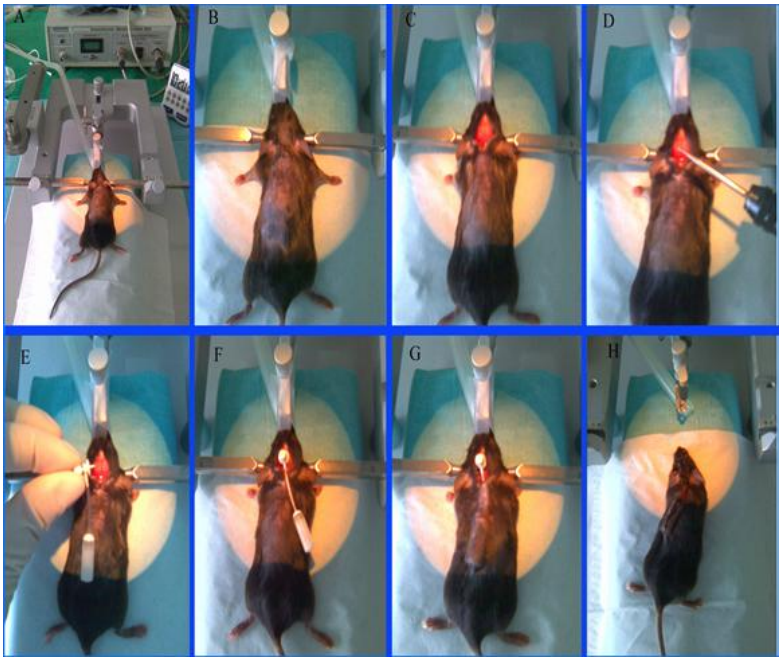

Figure 8. Intraventricular pump implantation technique

Fixation of the animal in the stereotactic frame afer the anesthetic procedures (A,B). Median incision of the skin fronto-occipital (C). Treppanation of 1mm diameter burr hole (0.5mm behind Bregma and and 1 mm left of the midline) (D). Intraventricular pump implantation (E,F). Subcutaneous internalisation of the pump (G). Suture of the skin and disinfection (H).

3.4. Functional neurological tests

Functional neurological recovery was assessed using a battery of tests at baseline and on days 3, 14 and 42 after MCAO.

Grip strength test. The grip strength test consists of a spring balance coupled with a Newtonmeter (Medio-Line Spring Scale, metric, 300g, Pesola AG, Switzerland) that is attached to a triangular steel wire, which the animal instinctively grasps. When pulled by the tail, the animal exerts force on the steel wire (Kilic et al., 2010). Grip strength was evaluated at the right paretic forepaw, the left non-paretic forepaw

being wrapped with adhesive tape. Grip strength was evaluated five times on each test occasion, and mean values were calculated. From these data, percentage values (post-ischemic vs. pre-ischemic) were computed. Pre-ischemic and pre-treatment results did not differ between groups.

RotaRod test. The RotaRod consists of a rotating drum with a speed accelerating from 6 to 40 rpm (Ugo Basile, model 47600, Comerio, Italy), which allows assessment of motor coordination skills (Kilic et al., 2010). Maximum speed is reached after 245 seconds, and the time at which the animal drops off the drum is evaluated (maximum testing time: 300 seconds). Measurements were performed five times each on the same occasion when grip strength was evaluated. For all five measurements, mean values were computed, from which percentage values (post-ischemic vs. pre-ischemic) were calculated. Pre-ischemic and pre-treatment data did not differ between groups.

Elevated O maze. The elevated O maze consists of a round 5.5 cm wide polyvinyl-chloride runway with an outer diameter of 46 cm, which is placed 40 cm above the floor and which detects spontaneous locomotor behaviour and correlates with fear and anxiety (Kilic et al., 2010). Two opposing 90° sectors are protected by 16 cm high inner and outer walls made of polyvinyl-chloride (closed sectors). The remaining two 90° sectors are not protected by walls (open sectors). Animals were released in one of the closed sectors and observed for 10 min. The total number of zone entries - as correlate of motor activity - and the time spent in the unprotected sector - as correlate of exploration behaviour, fear and anxiety - were registered whenever the animal moved into a sector with all four paws. Assessments took place at baseline and at two and six weeks post-stroke.

3.5. Delivery of Cascade Blue-labelled dextran amine (CB) and biotinylated dextran amine (BDA)

The anterograde tract tracer BDA has previously been used to evaluate pyramidal tract plasticity contralateral to the stroke in rats submitted to permanent focal cerebral ischemia (Wiessner et al., 2003). We adapted this method to mice, administering two different tracers, CB and BDA, in the motor cortex both ipsilateral (CB) and contralateral (BDA) to the stroke. For this purpose, cranial burr holes were drilled 0.5 mm rostral and 2.5 mm lateral to the bregma, via which deposits of 10% CB or 10% BDA (both 10,000 MW; Molecular Probes, diluted in 0.01 M phosphate-buffered saline [PBS] at pH 7.2) were placed into the motor cortex by means of microsyringe injections six weeks after MCAO. As such, a total volume of 2.1 µl tracer was administered to each animal, which was injected in three equal deposits located rostrally, medially and caudally of the burr hole inside the motor cortex. For this purpose, the syringe was inserted into the brain at angles of 45°, 90° and 135° against the midline at a depth of 1.5mm (Z'Graggen et al., 1998).

Ten days after the tracer injection, mice were transcardially perfused with 0.1 M PBS pH 7.4 containing 100,000 I.U. heparin and 0.25% $NaNO_2$ followed by 4% paraformaldehyde in 0.1 M PBS and 5% sucrose. Brains were removed and post-fixed over-night in 4% paraformaldehyde in 0.1 M PBS and 5% sucrose and cryoprotected in increasing concentrations of sucrose (5%, 10% and 30%) over 3 days. The tissue was then frozen with isopentan and cut into 20 µm and 40 µm thick coronal cryostat sections that were used for conventional and tract tracing histochemistry.

3.6. Immunohistochemistry

For conventional immunohistochemistry, four animals per each therapy group in both Epo and VEGF studies were transcardially perfused with 0.9% NaCl at 14, 30 and 52 days after the stroke. Brains were frozen on dry ice and cut on a cryostat into 20 µm coronal sections (Kilic et al., 2010). Brain sections from the level of the bregma (i.e. midstriatum) were fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS, rinsed, pre-treated for antigen retrieval with 0.01 M citrate buffer (pH 5.0), again rinsed and immersed for 1 h in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) and 10% normal donkey serum. Four brain sections were analysed per animal. The tissue was incubated overnight at 4°C with monoclonal mouse anti-NeuN (MAB377; Chemicon), monoclonal rat anti-CD31 (#557355; BD Biosciences), monoclonal mouse anti-GFAP (green fluorescence actinic protein) Alexa Fluor 555 conjugated (#3656; Cell Signaling) for both studies. In the Epo study further immunohistochemistry with monoclonal rat anti-CD45 antigen (#550539; BD Pharmingen) and polyclonal rabbit anti-ionized calcium binding adaptor protein (Iba)-1 (Wako Chemicals, Neuss, Germany) antibodies (diluted 1:100 in 0.1 M PBS) were performed. After detection with Cy3 or Cy2 conjugated secondary antibodies (Jackson ImmunoResearch, Suffolk, UK), sections were incubated with 4'-6-diamidino-2-phenylindole (DAPI) and coverslipped. Alternatively, in some experiments (CD45, Iba1) biotinylated secondary antibodies were used that were detected with avidin-biotin kit (Vector Laboratories, Burlingame, CA) followed by 3,3'-diaminobenzidine (DAB) (#D4418, Sigma, Missouri, USA) staining.

Sections were evaluated under a fluorescence microscope (Olympus BX 41) connected to a CCD camera (CC12; Olympus). Surviving neurons (NeuN+), microvascular profiles (CD31+), reactive astroglia (GFAP+), leukocytes (CD45+) and microglia (Iba1+) were analysed in a blinded way by counting numbers of cells or profiles in six defined regions of interests per striatum, both ipsi- and contralateral to the stroke (Kilic et al., 2005a, 2006). Mean values were calculated for all areas. With these data, neuronal survival, capillary density, astrogliosis, leukocyte infiltration and microglial activation were determined. In case of GFAP stainings, the overall area of scar tissue was outlined using the Soft imaging System Olympus Cell F Program.

Stereometric assessments of the degree of post-ischemic atrophy of the striatum and corpus callosum were done using modified Bielschowsky's silver stainings as previously described (Ding et al., 2008). The borders of both structures were outlined and their surface analysed (in case of corpus callosum up to 1mm lateral to midline, thus reflecting areas on coronal brain sections [Bacigaluppi et al., 2009]).

3.7. Immunohistochemistry for CB and BDA

Brain sections of animals that had been transcardially perfused with PFA were rinsed three times for 10 min each in 50 mM Tris-buffered saline (pH 8.0) containing 0.5% Triton X-100 (TBST). For detection of CB, sections were immersed overnight at 4°C with polyclonal rabbit anti-Cascade Blue antibody (A-5760; Molecular Probes, 1:100), diluted 1:100 in 50 mM TBST, followed by incubation for one hour at room temperature with a horseradish peroxidase (HRP)-labelled secondary anti-rabbit antibody (1:1000). For detection of BDA, sections were incubated overnight with avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA). Reactions were visualized with DAB containing 0.4% ammonium sulfate and 0.004% H_2O_2.

3.8. Analysis of corticorubral and corticobulbar projections

The location of tracer deposits was checked at the levels of the needle tracks, thus ensuring that the motor cortex had indeed been injected in all animals. To account for variabilities in tracer uptake in different mice, we first evaluated the number of tracer-stained fibres in the corticospinal tract (CST) both at the level of the red nucleus and facial nucleus. For this purpose, two consecutive sections were analysed, counting the number of fibres crossing the sections in four regions of interest of 2865 µm² each that had been selected in the dorsolateral, ventrolateral, dorsomedial and ventromedial portion of the CST. By measuring the total area of the CST using the Cell Software image system (Olympus) connected to an Olympus BX42 microscope, we calculated the overall number of labelled pyramidal tract fibres, as described previously (Z'Graggen et. al., 1998).

Analysis of corticorubral projections. Corticorubral projections were evaluated at the level of the parvocellular red nucleus (bregma –3.0 to –3.5 mm). A 500 µm long intersection line was superimposed on the brain midline. Along that line those fibres crossing into the contralateral hemisphere in direction of the red nucleus were quantified. For each animal, the total number of fibres counted was normalized with the total number of labelled fibres in the CST and multiplied by 100, resulting in percent values of fibres crossing the midline. For both tracers two consecutive sections were analysed, from which mean values were determined.

Analysis of corticobulbar projections. Corticobulbar projections were assessed at the level of the facial nucleus (bregma –5.8 to –6.3 mm). Two 500 µm long intersection lines were superimposed on the sections parallel to the midline, both representing tangents touching the most lateral extension of the pyramidal tract. Along both lines, those fibres crossing the lines in direction of the contralateral and ipsilateral facial nucleus were quantified. For each animal, the total number of fibres counted was normalized with the number of labelled fibres in the CST and multiplied by 100, resulting in percent values of fibres originating from the pyramidal tract. For both tracers two consecutive sections were evaluated, from which mean values were calculated.

3.9. Gene expression analysis by RT-PCR

For gene expression studies, mice were sacrificed at three different time-points, namely at 3, 14 and 30 days after stroke by transcardiac perfusion with cold sterile 0.1 M PBS containing 0.01 M ethylenediaminetetraacetic acid (EDTA) (pH 7.4). Brains were immediately removed and dissected on dry-ice. Blocks of tissue were cut from 2 mm rostral to 2 mm caudal to the bregma. From these blocks, samples were collected from six regions of interest: the motor cortex, the striatum and the parietal cortex both ipsilateral and contralateral to the stroke. All regions were processed in RNA-later RNA stabilization reagent (#76104; Qiagen, Hilden, Germany) and stored at -80° until RNA extraction. Brain tissue samples were homogenized and total RNA was isolated.

One µg of cDNA synthesized from 2 µg of total RNA was used for RT-PCR using pre-designed TaqMan low density arrays (TLDA) as previously described (Pluchino et al., 2008). Briefly, for each TLDA, there were eight separate loading ports that distributed the cDNA into a total of 48 wells, for a total of 384 different wells per card. Each well contains a specific primer and probe, capable of detecting one single gene. The gene cards evaluated 46 different genes (see Table 1) together with two housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA. The latter is a mandatory control provided by the manufacturer. RT-PCR was processed with samples obtained from individual animals (avoiding pooling of the tissue) from each of the six regions of interest, each sample containing 10 µl cDNA (1 µg).

RT-PCR was performed using the Applied Biosystems 7900HT Fast-Real-Time PCR System. Gene cards were analysed using the threshold cycle (CT) relative quantification method. CT values were normalized for endogenous reference [ΔCT=CT (target gene) - CT (GAPDH)] and compared with a calibrator using the ΔΔCT formula [ΔΔCT= ΔCT (sample) – ΔCT (calibrator)]. In this study, we consistently used GAPDH as endogenous control. As calibrator sample we utilized a brain obtained from an untreated mouse of the same age, sex and strain. Data were presented using the logarithmic transformation of F.I. (fold induction) ratios between ischemic vehicle- and non-ischemic vehicle-treated mice (MCAO effect) and of ratios between ischemic Epo 10 I.U.- and ischemic vehicle-treated mice (Epo effect) or VEGF- and ischemic vehicle-treated mice (VEGF effect).

Abbreviation	Gene name	Assay ID
Inflammation genes		
IL-1b	interleukin-1b	Mm00434228_m1
IL-6	interleukin-6	Mm00446190_m1
Il4	interleukin-4	Mm00445259_m1
eNOS	nitric oxide synthase 3, endothelial	Mm00435204_m1
nNOS	nitric oxide synthase 1, neuronal	Mm00435175_m1
iNOS	nitric oxide synthase 2 , inducible	Mm00440485_m1
IFNγ	interferon-γ	Mm00801778_m1
TNFα	tumor necrosis factor-α	Mm00443258_m1
LIF	leukemia inhibiting factor	Mm00434761_m1
CXCR4	chemokine receptor type 4	Mm99999055_m1
GFAP	glial fibrillary acidic protein	Mm00546086_m1
TGF-β 1	transforming growth factor, beta 1	Mm03024053_m1
MMP-9	matrix metallopeptidase 9	Mm00442991_m1
Fgf2	fibroblast growth factor II	Mm00433287_m1
VCAM-1	vascular cell adhesion molecule-1	Mm00449197_m1
L1cam	L1 cell adhesion molecule	Mm00493049_m1
Ncam1	neural cell adhesion molecule	Mm03053534_s1
Pro-plasticity (and survival modulating) genes		
GAP43	growth associated protein 43	Mm00500404_m1
Basp1	brain abundant, membrane attached signal protein 1	Mm02344032_s1
MARCKS	myristoylated alanine rich protein kinase C substrate	Mm02524303_s1
SPRR1	small proline-rich protein 1A	Mm01962902_s1
Ptprz1	Protein tyrosine phosphatase, receptor type z	Mm00478484_m1
CNTF	ciliary neurotrophic factor	Mn00446373_m1
BDNF	brain-derived neurotrophic factor	Mm00432069_m1
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	Mm01303209_m1
NGF	neuronal growth factor	Mm00443039_m1
IGF1	insulin-like growth factor 1	Mm01233960_m1
KDR	vascular endothelial growth factor receptor 2	Mm00440111_m1
Nrp1	neuropilin 1	Mm00435372_m1
c-Jun	c-Jun oncogene	Mm00495062_s1
Pten	phosphatase and tensin homolog	Mm01212532_m1
Nefl	neurofilament light peptide	Mm01315666_m1
Bmp10	bone morphogenic protein 10	Mm01963768_s1
Caspase-3	caspase-3	Mm01195085_m1
Anti-plasticity genes		
sema 3a	semaphorin 3 a	Mm00436469_m1
Ephrin A5	Eph receptor A5	Mm00433074_m1
Ephrin B1	Eph receptor B1	Mm00557961_m1
Versican	versican	Mm00490179_m1
Ncan	neurocan	Mm00484007_m1
Ntn1	netrin1	Mm00478484_m1
Angiogenesis genes		
VEGF-B	vascular endothelial growth factor-B	Mm00442102_m1
VEGF-A	vascular endothelial growth factor-A	Mm00437306_m1
ANGP1	angiopoietin	Mm00456503_m1
Epo	erythropoietin	Mm01202754_g1
Epor	erythropoietin receptor	Mm00833882_m1
HIF-1α	hypoxia inducible factor, α-subunit	Mm00468869_m1

Table 1. List of genes whose expression pattern was investigated with regard to EPO or VEGF therapy after stroke.

3.10. Western blot analysis

At postoperative days 3, 14, and 30 (for Epo study) and 3, 14, 30 and 42 (for VEGF study) four animals at each time point received a lethal dose of anaesthetic; and a 2 mm rostral-caudal section centred on bregma (as shown in the Fig 5 and 6) was dissected. The regions corresponding to the motor cortex and perilesional cortex both ipsi- and contralateral to the ischemic lesion were separated. Tissues belonging to the same group and the same time-point were pooled and further homogenized on ice for 30 sec, ultrasonicated for 2 min and treated with protease inhibitor cocktail and phosphatase inhibitor cocktail. The protein estimation was done by the Bradford method and controlled by stripping the blots and reprobing with actin antibody. For the Epo study equal amounts of each protein were loaded on 12% electrophoresis gels for SPRR1A mouse antibody (a kind gift of Prof. Strittmater, Yale University, USA) whereas for the VEGF study the amounts of protein were loaded on 5% (NG2 proteoglycan), 7% (brevican) or 10% (ephrin B1, ephrin B2, c-Jun, activated caspase-3) gels. A sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, followed by protein transfer onto a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 5% non-fat milk in Tris-buffered saline Tween 0.1% (TBS-T) for 1 hour at room temperature, washed in TBS-T and incubated over night with mouse monoclonal SPRR1A (1:1000) in TBS-T (Epo study) or with monoclonal rabbit anti-total c-Jun (#9165; Cell Signaling), polyclonal rabbit anti-phospho-c-Jun (#9164S; Cell Signaling), polyclonal rabbit anti-activated caspase-3 (#9661; Cell Signaling), polyclonal rabbit anti-NG2 proteoglycan (H-300, sc-20162; Santa Cruz, Heidelberg, Germany), monoclonal mouse anti-brevican (610895; BD Biosciences), polyclonal rabbit anti-ephrin B1 (H70, sc-20723; Santa Cruz) and polyclonal goat anti-ephrin B2 (AF496; R&D Systems, Wiesbaden, Germany) antibody, diluted 1:1000 in TBS-Tw (VEGF study). The second day the membranes were washed and further incubated in blocking solution with peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Antibody binding was developed by a chemiluminescence kit according to the manufacturer's protocol. The intensity of each signal was measured by Image J Program on three separate trials. Protein loading was controlled by means of β-actin blots using a polyclonal rabbit antibody (#4967; Cell Signaling). The relative levels of proteins were

normalized to their control both ipsi- and contralesional, at 3 days after the therapy was administered. The mean of three normalised values was calculated and graphically represented.

3.11. MMP9 gelatin zymography

Tissue samples from the same animals were processed for gelatin zymography using a previously published protocol (Zechariah et al., 2010). Brain samples were homogenized and lysated in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, pH 8.0) containing 5% protease inhibitor cocktail. Protein concentration was estimated using the DC Protein Assay Kit using an iMark microplate reader. Samples containing 5 µg protein were subjected to SDS-PAGE using 8% bis-acrylamide gel containing 0.1% gelatin (Sigma, Deisenhofen, Germany). After electrophoresis, gels were incubated for 1 hour at room temperature in modified enzymatic activation buffer (MEAB) (50 mM Tris-HCl, 6 mM $CaCl_2$, 1.5 µM $ZnCl_2$, pH 7.4) containing 2.5% Triton X-100, followed by overnight incubation in MEAB. The next day, gels were stained in Coomassie brilliant blue R-250. Gels were dried and digitized. Three experiments were run for each tissue sample.

3.12. Statistical analysis

Behavioural tests were analysed by means of two-way repeated measurement analysis of variance (ANOVA; treatment vs time) at three different time-points starting at 3 days post-stroke, at the time of Epo adnminstration, and at the time of VEGF administration. For those tests in which significant treatment or treatment by time interaction effects were noticed (at 0.05 level), one-way ANOVAs were done for each time-point, using least significant differences (LSD) tests for post-hoc analyses. Tract tracer histological data were evaluated by one-way ANOVA (comparison between n ≥ 3 groups). Immunohistological data, gene and protein expression studies were analysed by two-way ANOVA (treatment vs time). Whenever a treatment effect or treatment by time interaction effect was present at the 0.05 level, two-tailed t-tests were performed for each time-point.

4. RESULTS

4.1. Post-acute delivery of Epo improves post-ischemic neurological recovery

To evaluate whether Epo influences neurological recovery in the post-acute stroke phase, mice subjected to 30 minutes of left-sided MCAO were intracerebroventricularly (i.c.v.) treated with vehicle or Epo (1 or 10 I.U./day) starting at 3 days post-ischemia (dpi). LDF measurements and body weight did not show any differences between groups (Fig. 9A, B). In all groups, LDF decreased to ~15-20% of baseline during MCAO, followed by a rapid restoration of blood flow after reperfusion (Fig. 9A). Except for a transient mild reduction in body weight at 3 dpi (< 10%) that was similar in all groups, no abnormalities in weight development were seen (Fig. 9B).

The surgical implantation of the pump and the pump itself did not create any serious complication during the whole period of treatment of the animals. 24 hours after implantation no differences were observed between the groups with regard to complications or acceptance of the pump by the animals. All the pumps were removed on day 30 post-ischemia, after testing of behaviour skills. Towards the last week of treatment a small irritation of the skin in the pump vicinity was observed, an irritation that appeared in all groups independent of treatment and that was treated by cleaning and desinfection with Betadine. Two animals removed their pumps before day 30, and were removed from the experiment.

Neurological recovery was investigated by grip strength (Fig. 9C) and Rotarod (Fig. 9D) tests, which assess motor force of the paretic right forelimb and motor coordination. Significant reductions in motor force (Fig. 9C) and coordination skills (Fig. 9D) occurred in animals submitted to 30 minutes MCAO. In vehicle-treated ischemic animals and in animals receiving Epo at the low dosage (1 I.U./day), grip strength and RotaRod performance remained largely unchanged over the entire 42-day observation period (Fig. 9C, D). In animals treated with Epo at the higher dosage

(10 I.U./day), progressive improvement of motor force and coordination were observed at 14 and 42 dpi (Fig. 9C, D). Elevated O maze tests did not show any differences in spontaneous locomotor activity and exploration behaviour between groups (Fig. 9E, F).

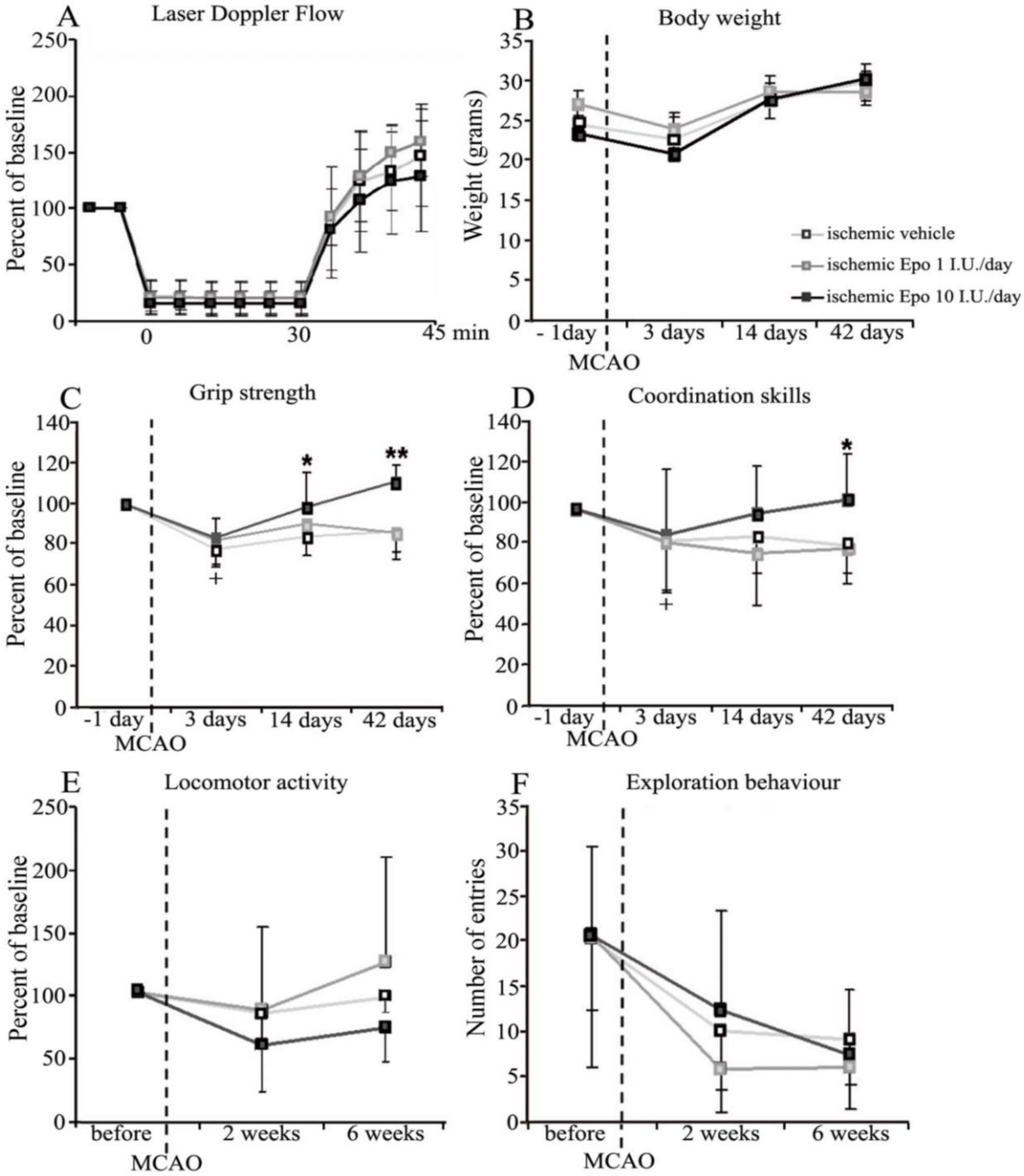

Figure 9. Delayed delivery of Epo at 10 I.U./day, but not 1 I.U./day promotes post-ischemic functional recovery.

(A) LDF recordings above the core of the MCA territory, **(B)** body weight, **(C)** grip strength of the lesion-contralateral right paretic forepaw, **(D)** coordination skills evaluated by RotaRod tests, **(E)** locomotor activity, and **(F)** exploration behaviour. Note that motor force **(C)** and coordination skills **(D)**, which were compromised by the stroke, did not exhibit any major improvements over time in vehicle-treated mice and mice receiving Epo at a dosage of 1 I.U./kg, but progressively improved over 14 to 42 days in animals treated with Epo at a 10 I.U./kg dosage. Spontaneous locomotor activity **(E)** and exploration behaviour **(F)** were not influenced by Epo. LDF recordings **(A)** and body weight **(B)** did not differ between groups. Data are mean values ± S.D. Data were analysed by two-way repeated measures ANOVA, followed by one-way ANOVA / LSD tests for each time-point. $^{+}P< 0.05$ compared with pre-ischemic baseline; *P< 0.05/ **P< 0.01 compared with vehicle-treated ischemic mice.

4.2. Post-acute delivery of Epo promotes peri-lesional tissue remodeling

In order to assess whether the post-acute delivery of Epo influences the remodeling of ischemic brain tissue, histochemical studies were performed. Immunohistochemical stainings for the neuronal marker NeuN revealed slowly progressive degeneration in the striatum of vehicle-treated ischemic mice, reflected by a continuous decline of surviving neurons (Fig. 10A) and striatal atrophy (Fig. 10B) that developed between 14 and 52 dpi. Notably, Epo delivered at the higher dosage (10 I.U./ day) significantly increased neuronal survival (Fig. 10A), at the same time preventing striatal shrinkage (Fig. 10B). The thickness of the corpus callosum was not changed by Epo treatment (Fig. 10C).

To define Epo's impact on angiogenesis, an accompaniment of successful neurovascular remodeling (Hermann and Zechariah, 2010), immunohistochemical stainings for the endothelial marker CD31 were assessed. Focal cerebral ischemia was followed by an increase in the density of CD31+ striatal capillaries to ~200% of baseline in vehicle-treated ischemic mice that persisted for up to 30 days after MCAO (Fig. 10D). Epo further increased the capillary density, which remained elevated until the end of the experiments, i.e. at 52 dpi (Fig. 10D).

To evaluate how Epo influences the astroglial responses to stroke, immunohistochemistry for the astroglial marker GFAP were analysed. In vehicle-

treated ischemic mice, focal cerebral ischemia corresponded with reactive astrocytes, which were dispersed throughout the middle cerebral artery territory and persisted over the observation period of 52 dpi (Fig. 10E). During Epo therapy, reactive astrogliosis was less pronounced (Fig. 10E). However, this glial-inhibitory effect disappeared after pump removal, i.e., at 52 dpi, when reactive astrogliosis again returned to levels similar to those of vehicle-treated ischemic animals (Fig. 10E).

In the most lateral portion of the striatum, a localized scar characterized by densely packed GFAP+ astrocytes developed beginning at 30 days, more clearly distinguishable at 52 dpi in vehicle-treated mice (Fig. 10F). Epo also reduced the size of this latter scar (Fig. 10F).

Looking at the relation between Epo and inflammatory reaction after stroke by means of leukocyte infiltration (Fig. 10G) and microglial activation (Fig. 10H) showed that Epo may reduce inflammation in the acute to post-acute phase after stroke, but on a long term (53 dpi) it causes a significant increase in leucocyte infiltration of the ischemic striatum.

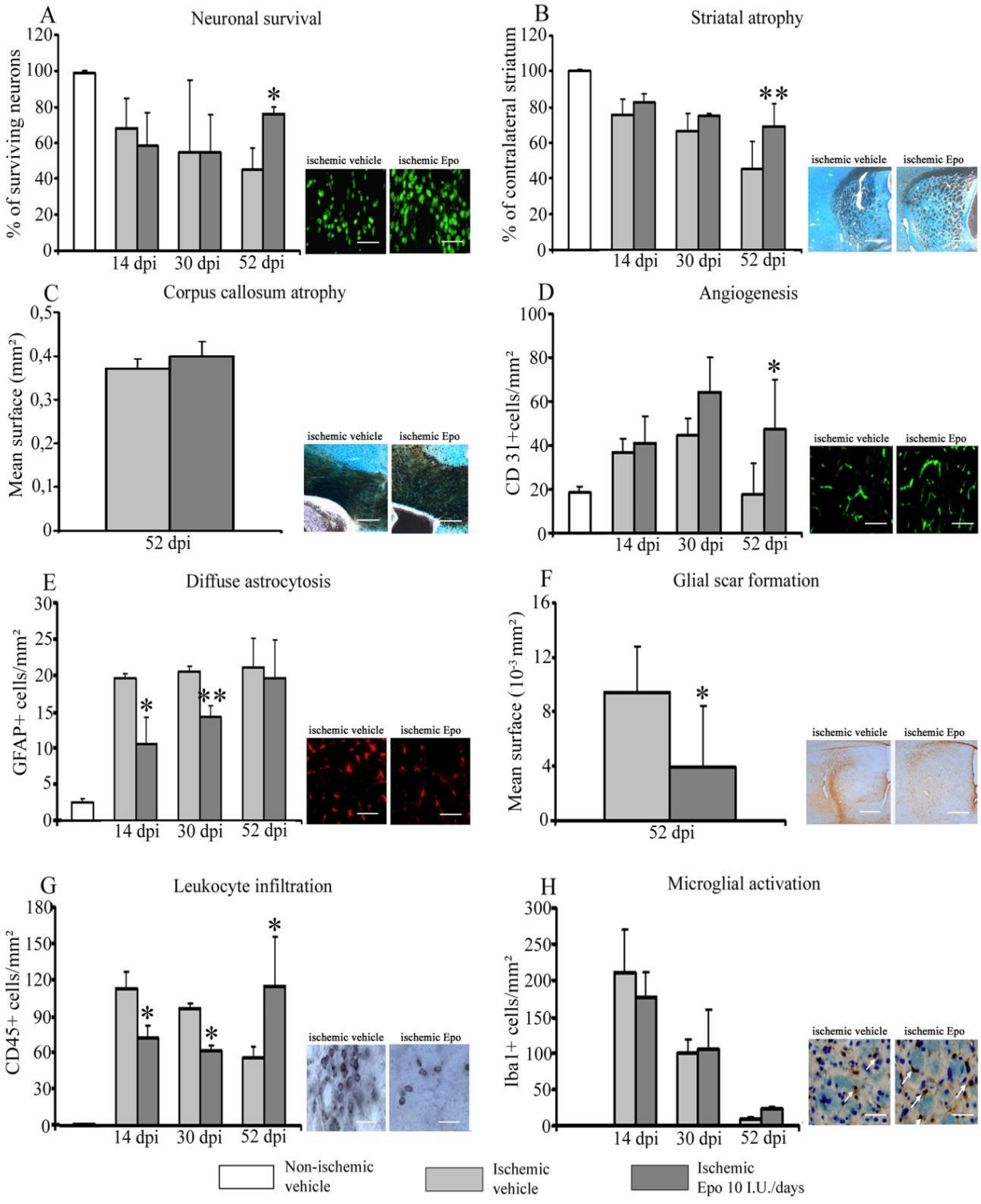

Figure 10. Epo promotes peri-lesional tissue remodelling and exerts anti-inflammatory actions

(A) Surviving neurons in ischemic striatum evaluated by NeuN immunohistochemistry, (B) striatal atrophy and (C) corpus callosum atrophy examined by Bielschowski stainings, (D) angiogenesis

assessed by CD31 immunohistochemistry, (E) diffuse astrocytosis at various time points and (F) circumscribed scar formation in the most lateral striatum at 52 dpi revealed by GFAP immunohistochemistry, as well as (G) leukocyte infiltration and (H) microglial activation analysed by CD45 and Iba1 immunohistochemistry. Note that Epo increased neuronal survival at 52 dpi (A), diminished progressive brain atrophy (B) without influencing corpus callosum thickness (C), promoted angiogenesis (D), reduced diffuse astrocytosis (E) and glial scar formation (F) and inhibited leukocyte infiltration (G), without affecting microglial activation (H). Photomicrographs are also shown that were taken at 52 dpi (A-D, F) or 14 dpi (E,G,H). Data are mean values ± S.D. Data were analysed by two-way ANOVA followed by two-tailed t-tests for individual time points. *P< 0.05/ **P< 0.01 compared with vehicle-treated ischemic mice. Bar, 200 µm (B, F) /50 µm (C) /20 µm (A, D, E, G, H).

4.3. Analysis of lesion-remote plasticity using anterograde tract-tracers

Because the pyramidal tract crosses the middle cerebral artery territory, which was affected by ischemia, we wanted to understand how Epo influences pyramidal tract degeneration and plasticity both ipsilateral and contralateral to the stroke. To this end, we administered two dextrane conjugates, CB and BDA, into both motor cortices. There were no relevant differences between groups in the locations of the injection sites. In all mice, the injection sites covered the more caudal forelimb area and rostral hindlimb area of the primary motor cortex without relevant spreading of tracer deposits into subcortical structures.

To analyse whether Epo influenced the survival of CST fibres distant to the stroke lesion, we counted CB-labelled fibres in the cerebral peduncle both at the level of the red nucleus and facial nucleus. This quantification did not reveal any differences between vehicle- and Epo-treated mice (red nucleus level: 44453±9944 vs. 44629±5509 fibres / facial nucleus level: 19188±7383 vs. 19943±1592 fibres, respectively), thus indicating that Epo did not influence the survival of descending pyramidal tract axons. Similar to CB-labelled fibres in the ipsilesional pyramidal tract, BDA-labelled fibres in the contralesional CST did not differ between vehicle- and Epo-treated mice. Similar to the total number of fibres, the overall size of the pyramidal tract, analysed on coronal sections at the bulbar level, was not changed by

Epo, neither ipsilesional (0.06±0.02 vs. 0.07±0.03 mm^2), nor contralesional (0.07±0.01 vs. 0.06±0.02 mm^2) to the stroke. As such, the corticospinal system was not affected by secondary degeneration.

4.4. Epo promotes contralesional, but not ipsilesional corticorubral plasticity

CB- and BDA-stained fibres originating from the cerebral peduncle converted dorsomedially at mesencephalic levels, terminating as previously described (Z'Graggen et al., 1998; Brown, 2007) in the parvocellular part of the ipsilateral red nucleus. At this level, we quantified the number of fibres crossing the midline towards the contralesional red nucleus. Our results revealed a moderate (though not significant) increase in the percentage of BDA-labelled midline crossing fibres derived from the ipsilesional CST upon MCAO. On the other hand, the percentage of BDA-labelled midline crossing fibres originating from the contralesional CST remained unchanged (Fig. 11). Importantly, Epo significantly promoted the outgrowth of midline crossing fibres from the contralesional CST, without influencing the plasticity of ipsilesional CST fibres (Fig. 11).

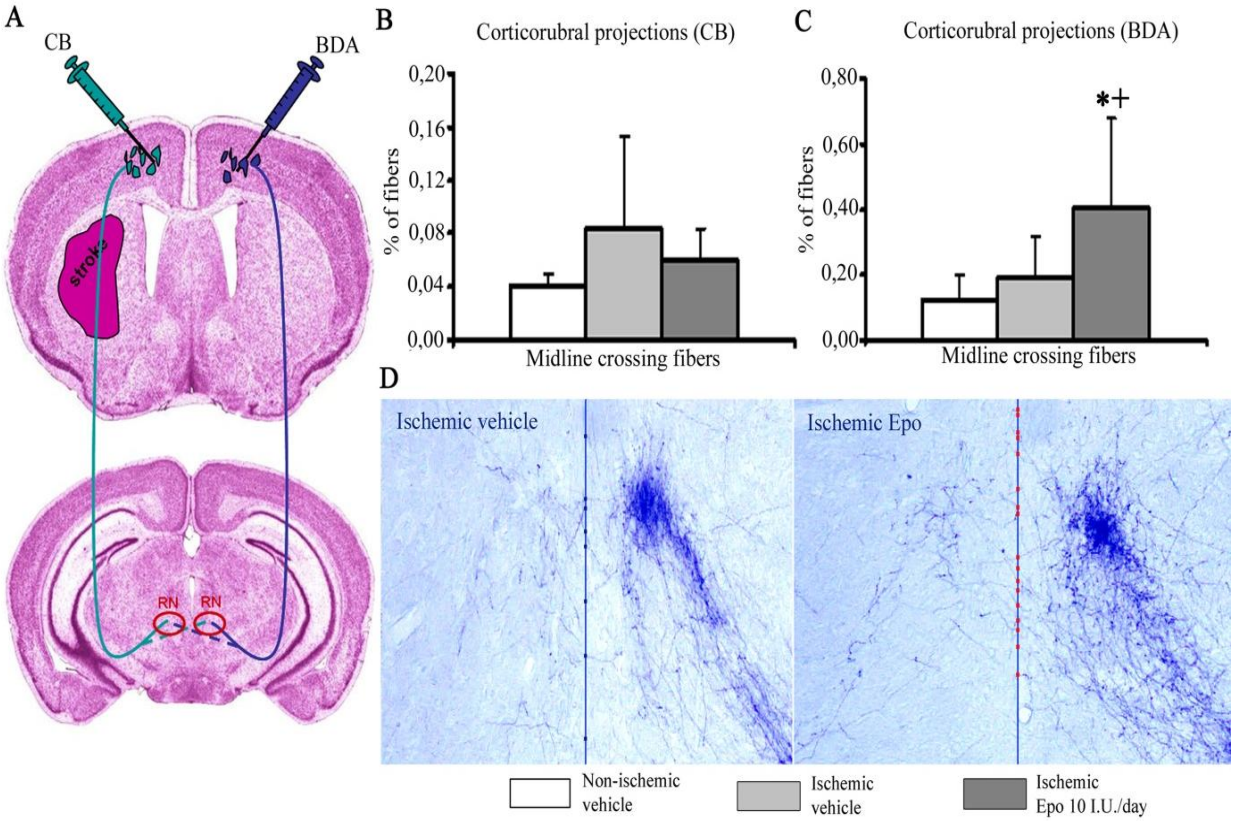

Figure 11. Epo promotes contralesional, but not ipsilesional corticorubral plasticity.
Epo promotes contralesional, but not ipsilesional corticorubral plasticity.

Tract tracing analysis of corticorubral projections ipsilateral and contralateral to the stroke in mice receiving Cascade Blue (CB) and biotinylated dextran amine (BDA) injections into the ipsilesional and contralesional motor cortex (for experimental procedures see **(A)**). Percent of midline crossing fibers to **(B)** the contralesional red nucleus (RN) traced by CB and **(C)** the ipsilesional, denervated RN traced by BDA. Note that the percentage of midline crossing fibres after ipsilesional CB injection moderately, but not significantly, increased in response to stroke **(B)**. Interestingly, Epo did not further elevate the percentage of midline-crossing fibres of the ipsilesional pyramidal tract **(B)**, but increased contralesional pyramidal tract sprouting across the midline, resulting in fibre outgrowth towards the denervated lesion-sided RN **(C)**. **(D)** Photomicrographs of representative ischemic vehicle-treated and Epo-treated mice illustrating BDA traced corticorubral fibers intersecting the midline between both RN. Note that the denervated RN receives more BDA traced fibers after Epo than after vehicle delivery. Data are means ± S.D. Data were analysed by one way ANOVA followed by LSD tests. $^{+}P< 0.05$ compared with vehicle-treated non-ischemic mice. *P< 0.05 compared with vehicle-treated ischemic mice.

4.5. Epo enhances contralesional, but not ipsilesional corticobulbar plasticity

At the midpontine level, two fibre bundles originated from the CST innervate the ipsilesional and contralesional facial nucleus. At this level, we counted the fibres leaving the CST in direction of both facial nuclei, evaluating fibres crossing two intersection lines. Our data showed that focal cerebral ischemia significantly increased the density of fibres originating from the CB-labelled ipsilesional CST innervating the ipsilesional facial nucleus, without affecting the density of fibres derived from the BDA-labelled contralesional CST (Fig. 12). Epo significantly increased the sprouting of BDA-labelled contralesional CST axons to the contralesional facial nucleus, at the same time mildly but non-significantly ($p=0.07$) reducing facial nucleus projections derived from the CB-labelled ipsilesional CST (Fig. 12).

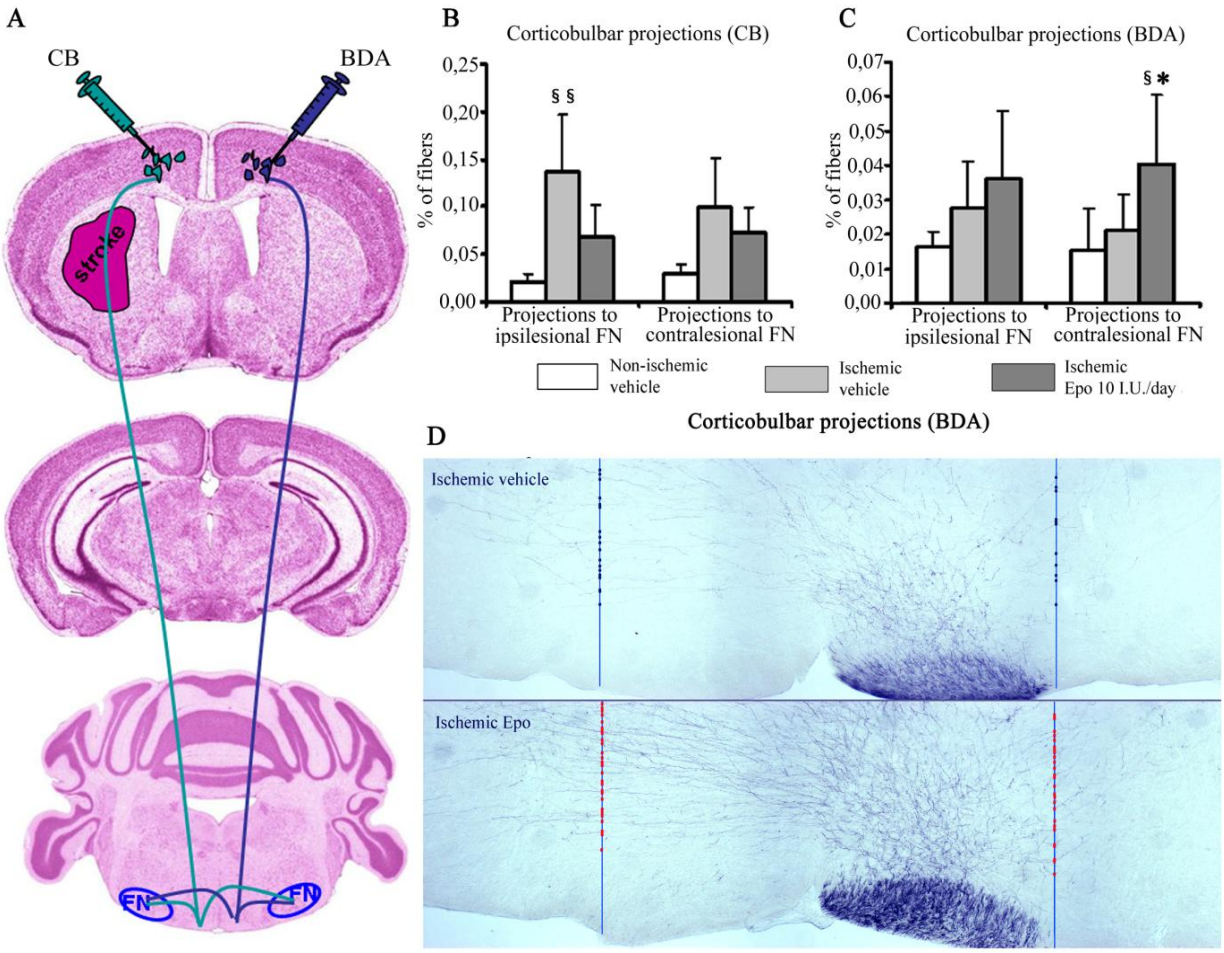

Figure 12. Epo increases contralesional corticobulbar plasticity without influencing ipsilesional corticobulbar plasticity that is increased by the stroke.

Tract tracing analysis of corticobulbar projections ipsilateral and contralateral to the stroke at the level of the facial nucleus (FN) in mice receiving CB and BDA injections into the ipsilesional and contralesional motor cortex (experimental procedures shown in **(A)**). Percent of fibers leaving the pyramidal tract in direction of the ipsilesional and contralesional FN traced by **(B)** CB and **(C)** BDA. Note that the percentage of fibres projecting to the ipsilesional FN after CB injection into the lesion-sided motor cortex significantly increased in response to stroke **(B)**. Interestingly, Epo did not further strengthen this ipsilesional projection, but rather reduced it **(B)**, at the same time increasing the percentage of BDA stained contralesional pyramidal tract fibres innervating the contralesional FN **(C)**. **(D)** Microphotographs of representative ischemic vehicle-treated and Epo-treated mice showing BDA-traced corticobulbar fibers crossing the intersection lines on both sides of the pyramidal tracts. Note that Epo increased fibre outgrowth towards the contralesional FN. Data are means ± S.D. Data were analysed by one way ANOVA followed by LSD tests. $^{+}P< 0.05$/ $^{++}P<$

0.01 compared with vehicle-treated non-ischemic mice. *P< 0.05 compared with vehicle-treated ischemic mice.

4.6. Post-ischemic remodelling by Epo involves anti-inflammatory effects

To elucidate the mechanisms underlying Epo's restorative actions, we performed semi-quantitative RT-PCR-based gene expression profiling, investigating a series of acute and chronic inflammation markers (for complete list see Table 1) in the striatum (Str), motor cortex (MCx) and parietal cortex (ParCx) of both hemispheres. As expected, stroke robustly increased several inflammation markers in the ischemic hemisphere, including interleukin (IL)-1β, tumour necrosis factor (TNF)-α, leukemia-inhibitory factor (LIF), transforming growth factor (TGF)-β, IL-6, GFAP and inducible NO synthase (iNOS) (Fig. 13). Epo significantly reduced all these mRNAs in the ipsilesional hemisphere, and to a lesser extent in the contralesional hemisphere (Fig. 13).

In order to identify immune cells involved in the anti-inflammatory action of Epo, immunostainings for the leukocyte marker CD45 and microglia marker Iba1 were performed. Epo significantly reduced the brain infiltration of CD45+ leukocytes (Fig. 10G), but not the presence of Iba1+ microglia (Fig. 10H) in the ischemic striatum. In the contralesional hemisphere, neither Iba1+ microglia, nor CD45+ leukocytes were seen. These data indicate that attenuation of leukocyte recruitment, besides reduced reactive astrogliosis, may contribute to Epo's anti-inflammatory actions.

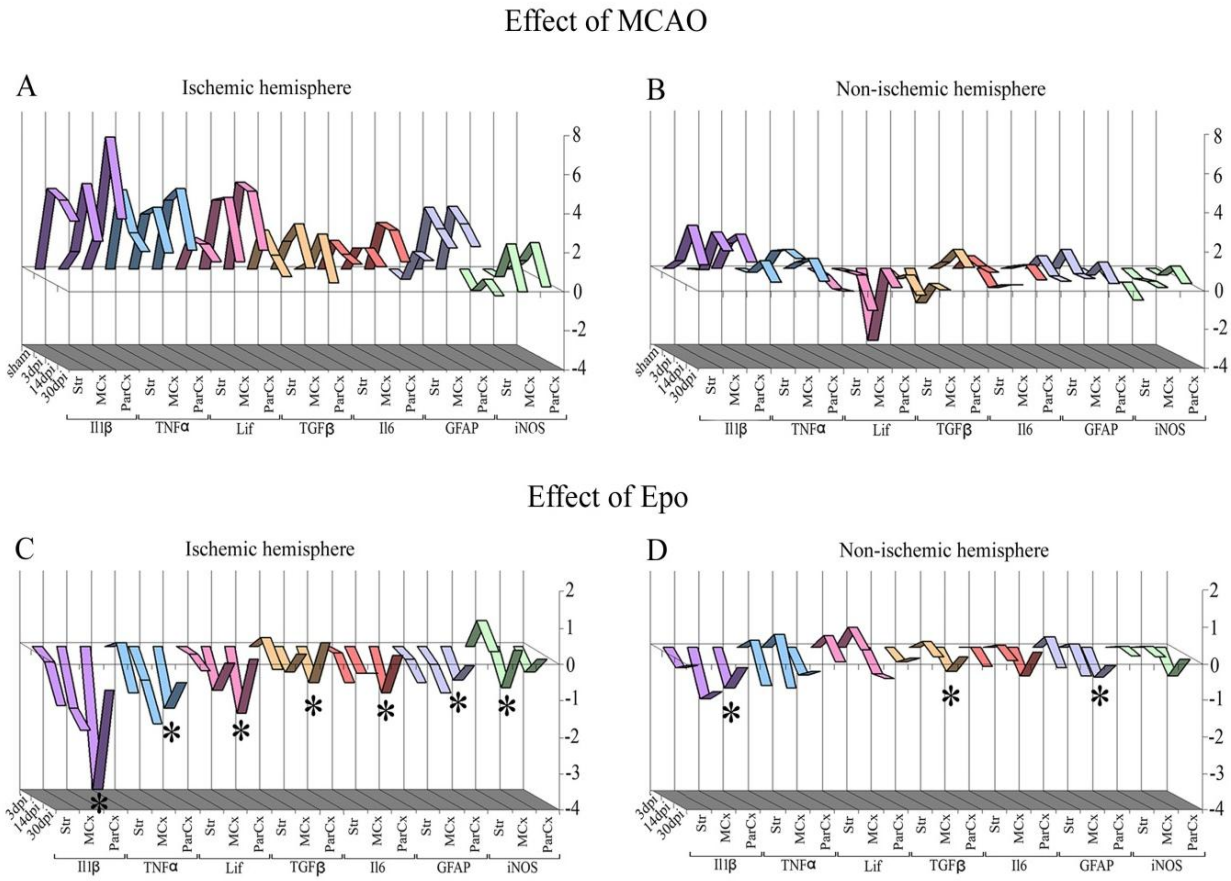

Figure 13. Temporospatial analysis of anti-inflammatory effects of Epo in the ipsilesional and contralesional hemispheres.

Semi-quantitative rt-PCR analysis summarizing the expression of inflammation-related genes in the striatum (Str), motor cortex (MCx) and parietal cortex (ParCx) at 3, 14 and 30 dpi. Gene expression changes induced by MCAO **(A,B)** and by Epo **(C,D)** are depicted. Throughout the time period examined, from 3 – 30 dpi, focal cerebral ischemia increased the mRNA levels of Il-1β, TNF-α, Lif, TGFβ, Il-6, GFAP and iNOS **(A,B)**. Epo attenuated the expression of all seven genes at 14 dpi, most strongly in the ischemic hemisphere **(C)** but this effect was less pronounced also in the contralesional hemisphere **(D)**. Data are logarithmic ratios of fold inductions after MCAO vs. sham surgery **(A,B)** and Epo vs. vehicle treatment **(C,D)**. Data were analysed by two-way ANOVA. *P<0.05 compared with vehicle-treated ischemic mice.

4.7. Modulation of pro-plasticity and anti-plasticity genes by Epo

To better understand how Epo influences brain plasticity both ipsilateral and contralateral to the stroke, additional RT-PCR studies were performed, using plasticity-promoting and -inhibitory genes that were previously described to be induced in the first days or weeks after stroke (Carmichael and Li, 2006; Table 1).

Induction of ischemia was accompanied by a robust upregulation of the mRNAs of the following pro-plasticity genes in the ischemic but not the contralesional hemisphere between 3 and 30 dpi: small proline-rich protein (SPRR)1, insulin-like growth factor (IGF)-1, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor receptor-2 (KDR) and myristoylated alanine-rich C-kinase substrate (MARCKS) (Fig. 14). On the other hand, no comparable unequivocal pattern in the regulation of anti-plasticity mRNAs was observed (Fig. 14).

Whereas Epo diminished the expression of different pro-plasticity genes (i.e., IGF-1 and KDR) in the ischemic hemisphere at 14 but not 30 dpi, in the non-ischemic hemisphere Epo downregulated the anti-plasticity mRNAs neurocan and ephrin B1 and upregulated the pro-plasticity SPRR1 mRNA at 14 and 30 dpi (Fig. 14).

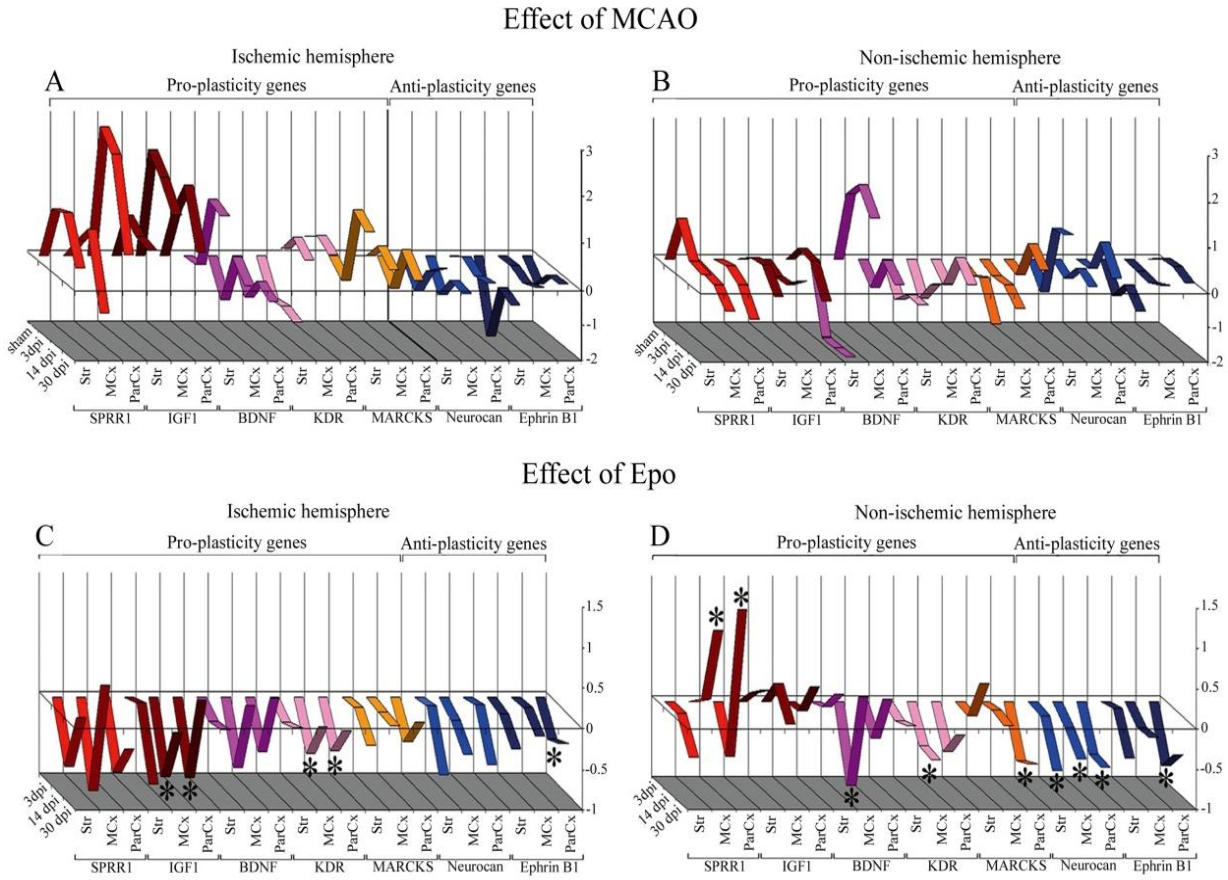

Figure 14. Temporospatial analysis of plasticity-promoting and -inhibiting effects of Epo in the ipsilesional and contralesional hemisphere. Semi-quantitative RT-PCR analysis**.**

The expression of pro-plasticity and anti-plasticity genes that were regulated by the stroke in the striatum (Str), motor cortex (MCx) and parietal cortex (ParCx) at 3, 14 and 30 dpi are summarized. Gene expression changes induced by MCAO **(A,B)** and by Epo **(C,D)** are summarized. Throughout the time period examined, focal cerebral ischemia elevated the mRNA levels of different pro-plasticity genes, namely SPRR1 and IGF-1, in the ischemic hemisphere **(A)**. Interestingly, Epo reduced the expression of SPRR1, IGF-1, BDNF and KDR at 14 dpi in the ischemic hemisphere **(A)**, at the same time downregulating the anti-plasticity genes neurocan and ephrin-B1 more clearly in the contralesional hemisphere **(B)** as compared to the ipsilesional hemisphere **(A)**. Data are logarithmic ratios of fold inductions after MCAO vs. sham surgery **(A,B)** and Epo vs. vehicle treatment **(C,D)**. Data were analysed by two-way ANOVA. *P< 0.05 compared with vehicle-treated ischemic mice.

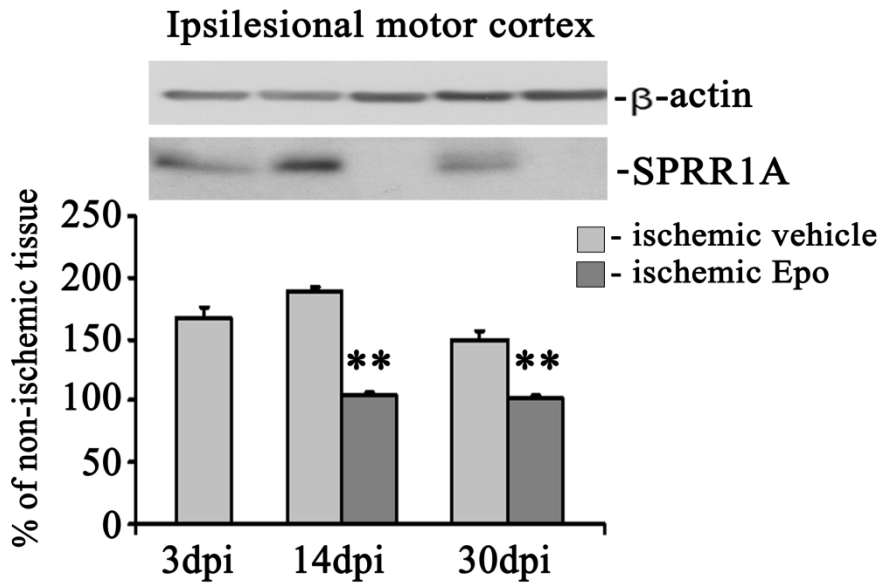

Figure 15. Epo downregulates SPRR1A protein in the ipsilesional motor cortex.

Western blot analysis shows reduced SPRR1A in Epo- as compared with vehicle-treated mice at 14 and 30 dpi. Protein loading was controlled by reprobing the blots with a β-actin antibody. SPRR1A levels were densitometrically analysed. Data are mean values ± S.D. Data were evaluated by two-way ANOVA followed by two-tailed t-tests for both time points. **P< 0.01 compared with vehicle-treated ischemic mice.

4.8. Neurological recovery is improved by post-acute VEGF delivery

In order to evaluate whether VEGF promotes neurological recovery when administered in the post-acute stroke phase, mice subjected to 30 min of left-sided MCAO were intracerebroventricularly (i.c.v.) treated with vehicle or VEGF (0.004 or 0.02 µg/day) starting at 3 dpi. LDF, which was recorded to evaluate changes in cerebral blood flow (Fig. 16A), and body weight (Fig. 16B) did not reveal any differences between groups. Reproducible ischemias were followed by stable reperfusions to levels slightly above baseline values (Fig. 16A). Only a mild reduction in body weight was noticed at 3 dpi in all groups (~10%), from which animals quickly recovered within 14 dpi (Fig. 16B).

Motor recovery was investigated by grip strength (Fig. 16C) and Rotarod (Fig. 16D) tests. Reductions in motor force of the contralesional right forelimb (Fig. 16C) and coordination skills (Fig. 16D) were noticed in animals subjected to 30 min MCAO. In vehicle-treated ischemic animals and animals receiving VEGF at low dosage (0.004 µg/day), deficits in motor force and coordination persisted over the observation period of 6 weeks (Fig. 16C, D)

In animals treated with 0.02 µg/day VEGF, on the other hand, grip strength and coordination skills progressively improved during that time interval, reaching levels significantly above vehicle-treated animals at 42 dpi (Fig. 16C, D). Spontaneous locomotor activity and exploration behavior, which were evaluated by elevated O maze tests, did not differ between groups (Fig. 16E, F).

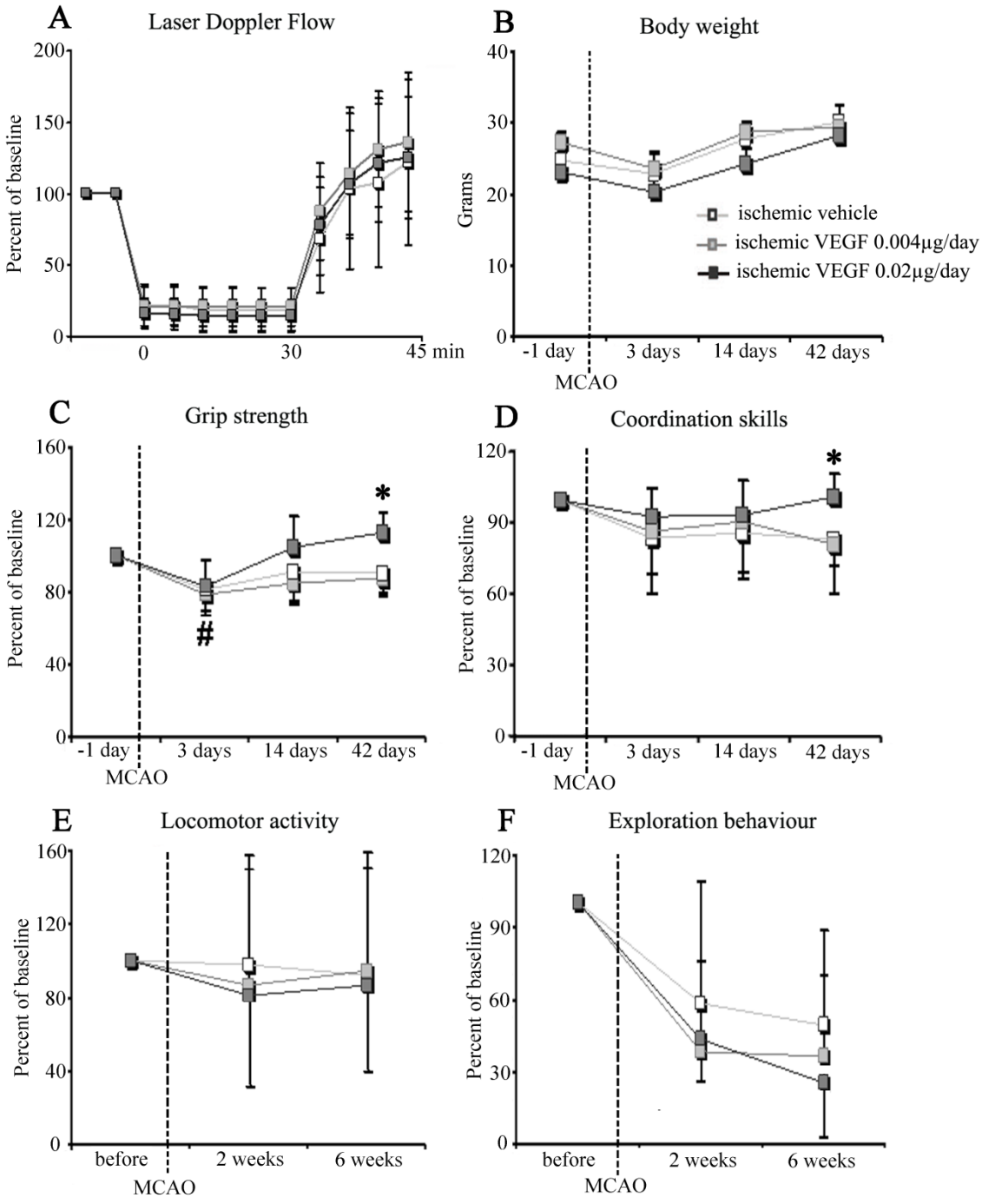

Figure 16. Delayed delivery of VEGF at 0.02 µg / day, but not 0.004 µg / day promotes post-ischemic neurological recovery.

(A) LDF recordings above the core of the MCA territory, **(B)** body weight, **(C)** grip strength of the lesion-contralateral right paretic forepaw, and **(D)** coordination skills evaluated by RotaRod tests. Note that grip strength **(C)** and coordination skills **(D)** did not exhibit any

major improvements over time in vehicle-treated mice and mice receiving VEGF at a dosage of 0.004 µg / day, but progressively increased over 14 to 42 days in animals treated with 0.02 µg VEGF / day. LDF recordings **(A)** and body weight **(B)** did not differ between the groups. There was no difference in between groups regarding locomotor activity **(E)** and exploration behavior **(F)**. Data are mean values ± S.D. (*n*=10 animals per group). Data were analyzed by two-way repeated measures ANOVA, followed by one-way ANOVA / LSD tests for each time-point. $^{\#}p< 0.05$ compared with pre-ischemic baseline; $*p< 0.05$ compared with vehicle-treated ischemic mice.

4.9. VEGF promotes angiogenesis predominantly in the ischemic hemisphere, at the same time preventing secondary neuronal degeneration

To elucidate how VEGF influences new vessel formation, the density of brain capillaries was analyzed by immunohistochemistry for CD31, a marker for endothelial cells. Whereas brain capillary density did not change in the perilesional tissue of vehicle-treated mice, the density of cerebral microvessels increased to ~200% of baseline within 52 dpi in mice receiving VEGF at the higher dosage (0.02 µg/day) (Fig. 17A). Notably, capillary density in the contralesional striatum was only mildly influenced by VEGF (Fig. 17B).

To assess how VEGF affects neuronal survival, which closely accompanies angiogenesis in the perilesional brain tissue (Sun et al., 2003; Hermann and Zechariah, 2009), immunohistochemistry analyses for the neuronal marker NeuN were analyzed. Slowly progressive degeneration was noticed in the striatum of vehicle-treated mice, reflected by a loss of surviving neurons (Fig. 17C) and progressive striatal atrophy (Fig. 17D). Notably, VEGF significantly increased neuronal survival at 14 dpi (Fig. 17C), translating into reduced shrinkage of the ischemic striatum at 52 dpi (Fig. 17D).

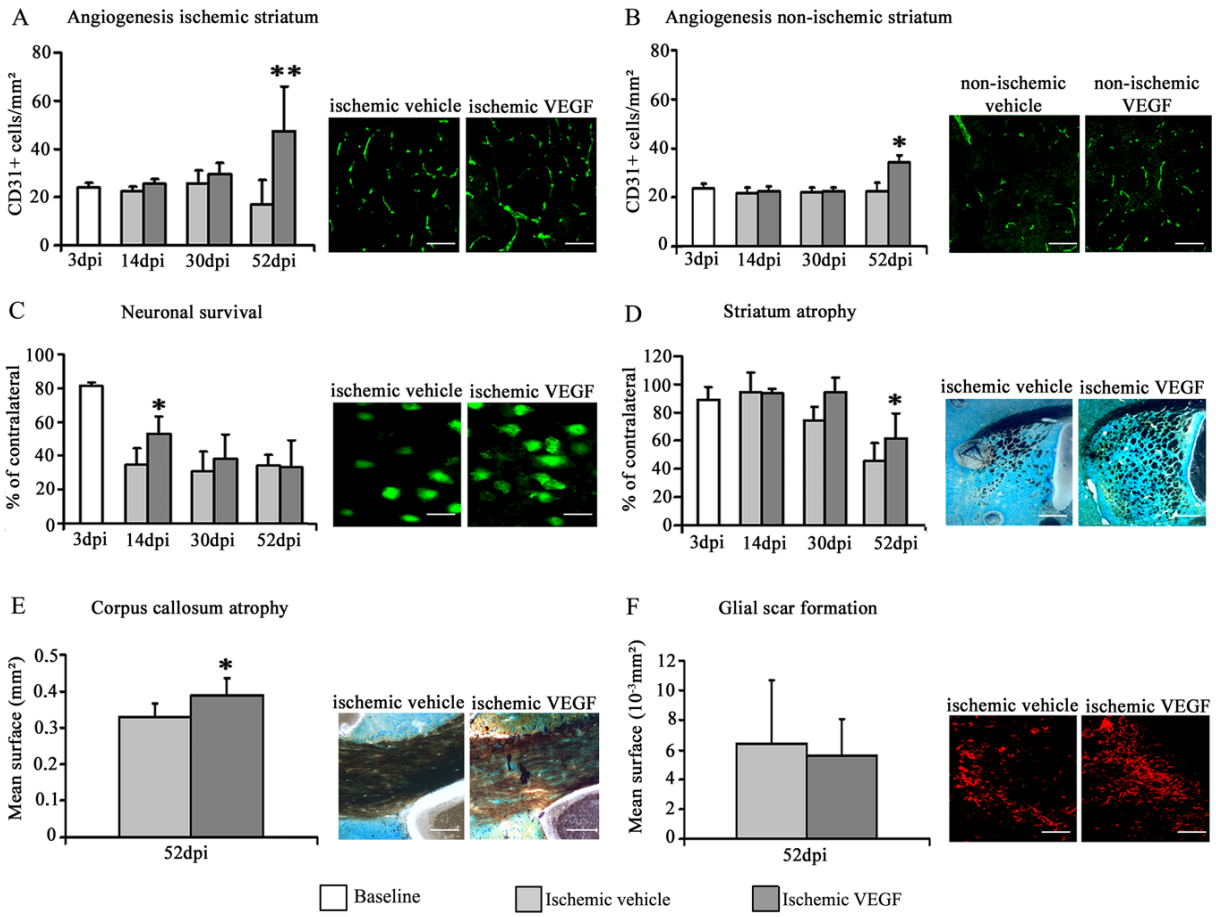

Figure 17. VEGF prevents secondary neurodegeneration and induces angiogenesis.

(A) capillary density in ischemic striatum assessed by CD31 immunohistochemistry and **(B)** in non-ischemic striatum. **(C)** Density of surviving neurons in ischemic striatum evaluated by NeuN immunohistochemistry, **(D)** striatal shrinkage examined by Bielschowsky staining, **(E)** corpus callosum atrophy evaluated by Bielschowsky staining **(F)** Glial scar formation. Note that VEGF increases neuronal survival at 14 dpi **(C)**, translating into reduced brain atrophy at 52 dpi **(D)**. In addition, VEGF promotes angiogenesis (**A**) and mildly increases corpus callosum thickness **(E)**. Photomicrographs were also taken at 14 dpi **(C)** or 52 dpi **(A, B, D, E, F)**. Data are mean values ± S.D. (*n*=4-8 animals per group). Data were analyzed by two-way ANOVA followed by two-tailed t-tests for individual time points or two-tailed t-tests, as appropriate. *p< 0.05/ **p< 0.01 compared with vehicle-treated ischemic mice. Bar, 200 µm **(D)**/ 50 µm **(E, F)**/ 20 µm **(C)**.

4.10. VEGF promotes contralesional, but not ipsilesional pyramidal tract plasticity

To analyze how VEGF influences pyramidal tract remodeling both ipsilateral and contralateral to the stroke, two dextran conjugates, CB and BDA, were administered into both motor cortices. Injection sites covered the more caudal forelimb area and rostral hindlimb area of the primary motor cortex. The number of anterogradely labeled axons in the pyramidal tracts did not differ between vehicle-treated and VEGF-treated mice, neither ipsilateral nor contralateral to the stroke (level of red nucleus: 44,453±19,944 vs. 44,554±12,393 fibers; level of facial nucleus: 19,188±7,383 vs. 25,408±16,306 fibers [on lesioned side] for vehicle and VEGF, respectively). The area covered by the pyramidal tract was also similar in both groups (level of facial nucleus: 0.05±0.02 vs. 0.07±0.04 mm^2 [on lesioned side]; 0.06±0.01 vs. 0.07±0.02 mm^2 [contralateral to stroke] for vehicle and VEGF, respectively), indicating that neither ischemia nor VEGF influenced the survival of corticospinal axons.

Importantly, VEGF promoted the sprouting of labeled fibers branching off the contralesional, but not ipsilesional pyramidal tract at the level of the red and facial nuclei. As such, the number of labeled midline-crossing fibers originating from the BDA-labeled contralesional pyramidal tract was significantly increased at the levels of the red and facial nucleus, as was the number of fibers branching off the contralesional pyramidal tract in direction of the contralesional facial nucleus (Figs. 18, 19). Interestingly, VEGF did not affect the sprouting of CB-labeled ipsilesional pyramidal tract fibers that were increased in ischemic as compared to sham-operated non-ischemic mice, neither at the rubral nor the bulbar level (Figs. 18, 19).

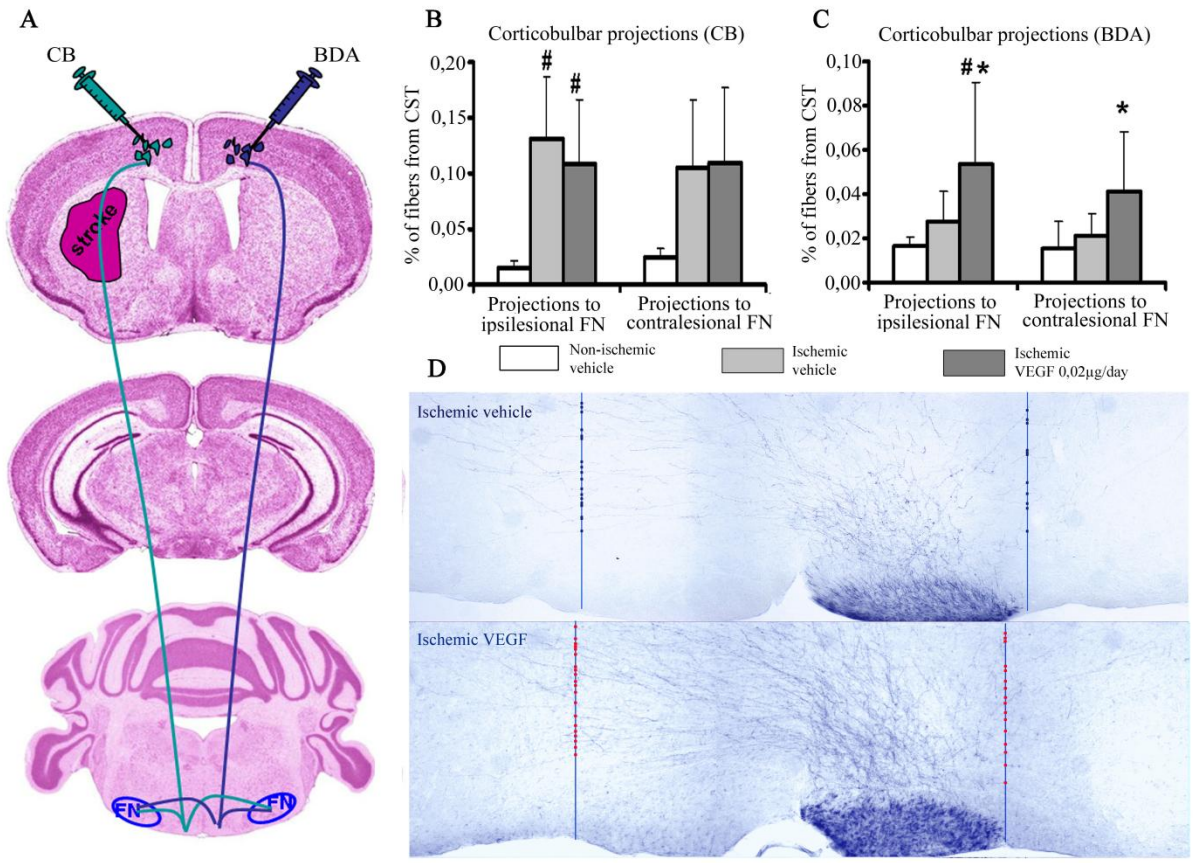

Figure 18. VEGF promotes contralesional, but not ipsilesional corticobulbar plasticity.

Tract tracing analysis at the level of the facial nucleus (FN) in mice receiving Cascade Blue (CB) and biotinylated dextran amine (BDA) injections into the ipsilesional and contralesional motor cortex (for placement of tracer injections see **(A)**). Percent of fibers leaving the pyramidal tract in direction to the ipsilesional and contralesional FN traced by **(B)** CB and **(C)** BDA. Note that the percentage of fibers projecting to the ipsilesional FN after CB injection into the lesion-sided motor cortex significantly increases in response to stroke **(B)**. Interestingly, VEGF does not further strengthen this ipsilesional projection **(B)**, but increases the percentage of BDA stained contralesional pyramidal tract fibers innervating the ipsilesional and contralesional FN **(C)**. **(D)** Microphotographs of representative ischemic vehicle-treated and VEGF-treated mice showing BDA-traced corticobulbar fibers crossing the intersection lines (superimposed in blue) on both sides of the brain. Note that VEGF increases axonal sprouting towards both FN (intersecting fibers labeled with dots). Data are means ± S.D. (*n*=10 animals per group [ischemic vehicle, ischemic VEGF] / *n*=4 animals per group [non-ischemic vehicle]). Data were analyzed by one way ANOVA followed by LSD tests. $^{\#}p< 0.05$ compared with vehicle-treated non-ischemic mice. *p< 0.05 compared with vehicle-treated ischemic mice.

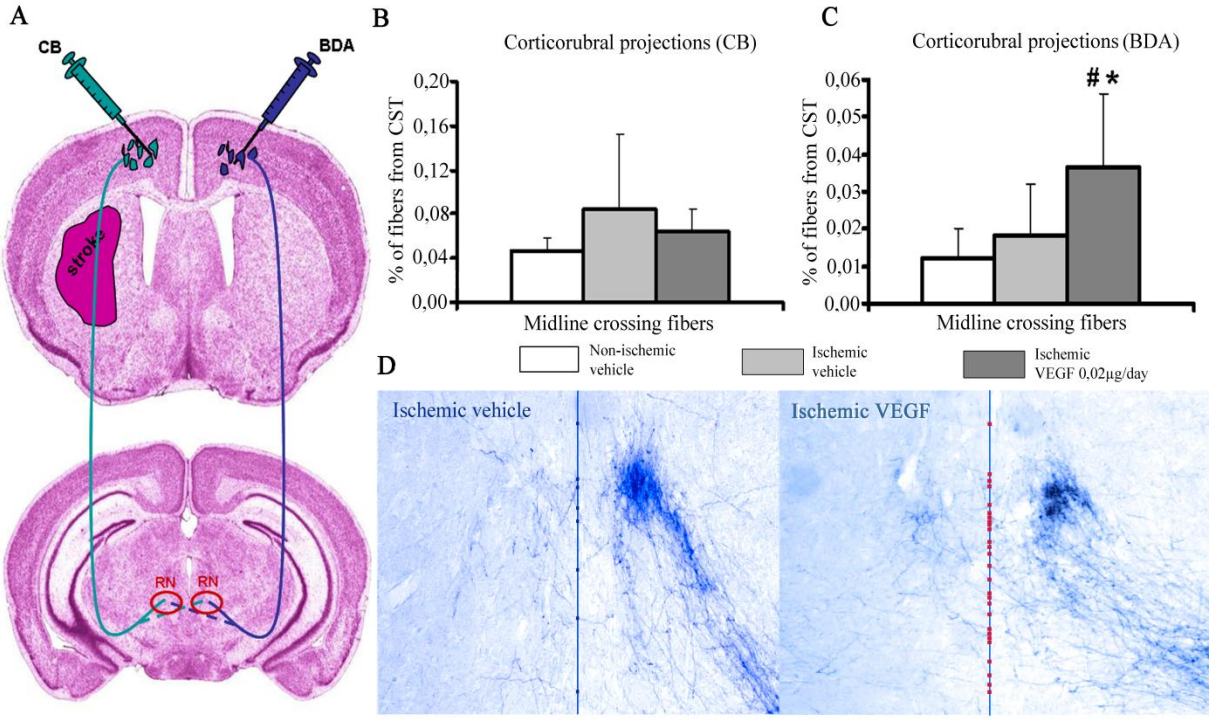

Figure 19. VEGF stimulates corticorubral plasticity.

VEGF promotes contralesional, but not ipsilesional corticorubral plasticity. Tract tracing analysis of corticorubral projections ipsilateral and contralateral to the stroke in mice receiving Cascade Blue (CB) and biotinylated dextran amine (BDA) injections into the ipsilesional and contralesional motor cortex (for experimental procedures see **(A)**). Percent of midline crossing fibers to **(B)** the contralesional red nucleus (RN) traced by CB and **(C)** the ipsilesional, denervated RN traced by BDA. Note that the percentage of midline crossing fibres after ipsilesional CB injection increased moderately, though not significantly, in response to stroke **(B)**. **(D)** Photomicrographs of representative ischemic vehicle-treated and VEGF-treated mice illustrating BDA traced corticorubral fibers intersecting the midline between the two RNs. Note that the denervated RN receives more BDA traced fibers after VEGF than after vehicle delivery. Data are means ± S.D. Data were analysed by one way ANOVA followed by LSD tests. $^{+}P< 0.05$ compared with vehicle-treated non-ischemic mice. *P< 0.05 compared with vehicle-treated ischemic mice.

4.11. Brain reorganization involves deactivation of matrix metalloproteinase MMP9 in the ischemic, but not contralesional hemisphere

To elucidate mechanisms underlying the reorganization of the brain tissue both ipsilateral and contralateral to the stroke, we examined the activation of MMP9 by gelatin zymography. Activation of MMP9 was noticed throughout the ischemic hemisphere (i.e., in striatum, parietal cortex and motor cortex) over the observation period of 42 days (shown for striatum in Fig. 20A). VEGF significantly attenuated MMP9 activity (Fig. 20A). Contralateral to the stroke, a mild activation of MMP9 was found at 3 dpi, but not at later time points (not shown). To clarify whether regulation of MMP9 takes place at the gene expression level, RT-PCR for *mmp9* was analyzed. Significantly reduced mRNA levels for *mmp9* were observed throughout the brain, both ipsilateral and contralateral to the stroke (Fig. 23), indicating that VEGF-induced MMP9 inhibition involves some transcription effects.

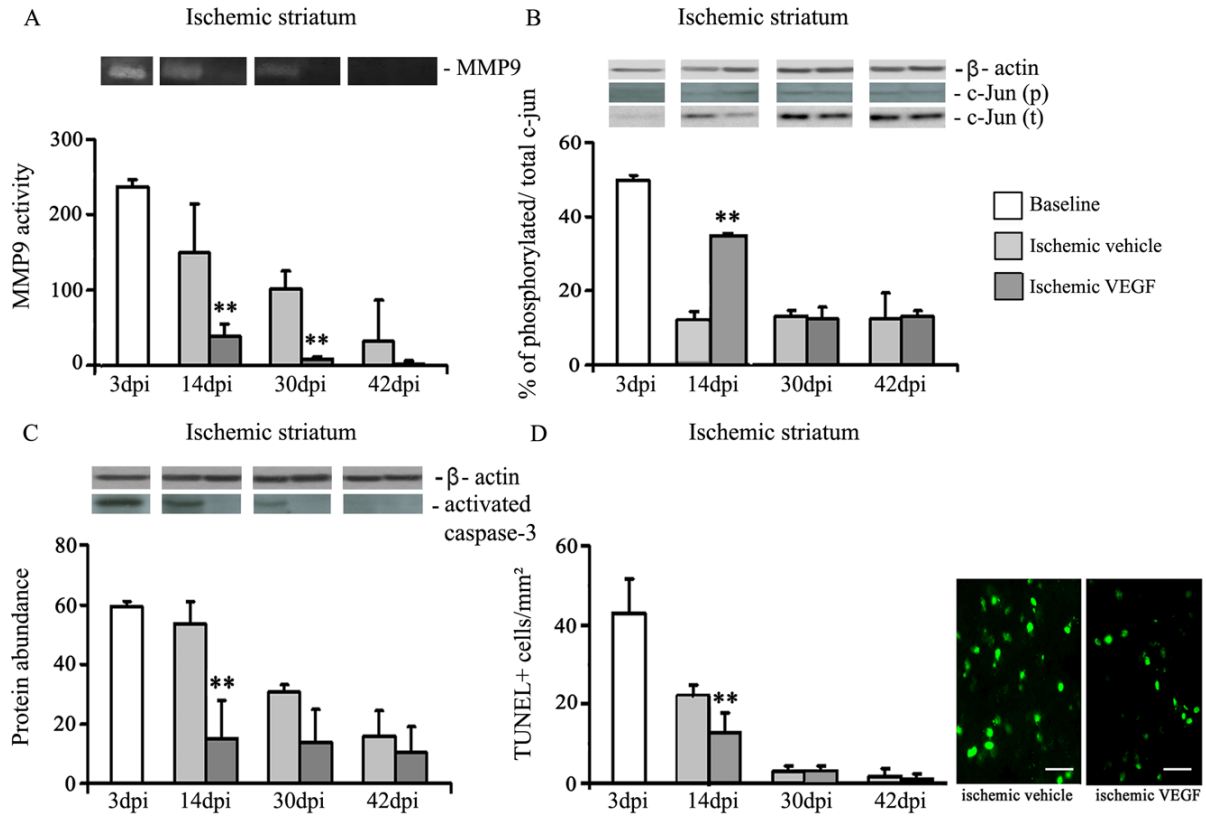

Figure 20. VEGF deactivates MMP9, activates c-Jun and prevents caspase-3-dependent apoptotic cell death.

Gelatin zymography for MMP9 **(A)**, Western blot analysis using antibodies detecting total and phosphorylated c-Jun **(B)** and cleaved (i.e., activated) caspase-3 **(C)**, as well as DNA fragmentation studies using TUNEL **(D)**. For **A-C**, tissue samples from the striatum were used. In **(B)** and **(C)**, data are presented as optical densities. Protein loading was controlled with a β-actin antibody. Representative zymographies, blots and TUNEL stainings are shown. Data are means ± SD (*n*=3 zymographies and Western blots **[A-C]** / *n*=4 animals per group). Data were analyzed by two-way ANOVA followed by two-tailed t-tests for individual time-points. **p< 0.01 compared with vehicle-treated ischemic mice. Bar, 45 µm.

4.12. VEGF modulates the transcription factor c-Jun and inhibits apoptotic cell death

To characterize mechanisms underlying perilesional tissue remodeling, Western blots for the transcription factor c-Jun and the executioner caspase-3 were evaluated using antibodies detecting both total and phosphorylated c-Jun as well as cleaved (i.e., activated) caspase-3 using tissue samples obtained from the ipsilesional and contralesional striatum. c-Jun was abundant in ischemic brain tissue (Fig. 20B). Although the expression of total (i.e., phosphorylated and non-phosphorylated) c-Jun was downregulated by VEGF in the ischemic (Fig. 20B), but not non-ischemic (not shown) striatum at 14 dpi, VEGF increased the percentage of phosphorylated c-Jun in the ipsilesional brain tissue (Fig. 20B).

In accordance with the promotion of neuronal survival, caspase-3 activity was reduced by VEGF in the ischemic striatum at 14 and 30 dpi (Fig. 20C), as was DNA-fragmentation evaluated by TUNEL at 14 dpi (Fig. 20D). To evaluate whether cell death-associated proteins were regulated on the transcriptional level, RT-PCR was performed. Similar to *mmp9*, the mRNAs for c-*jun*, the cell cycle gene *cdkn1*, and *caspase-3* were downregulated both ipsilateral and contralateral to the stroke (Fig. 21), indicating that transcriptional regulation of cell death genes reflects a bilateral response of the brain, whereas protein translation and activation is regionally confined to perilesional areas.

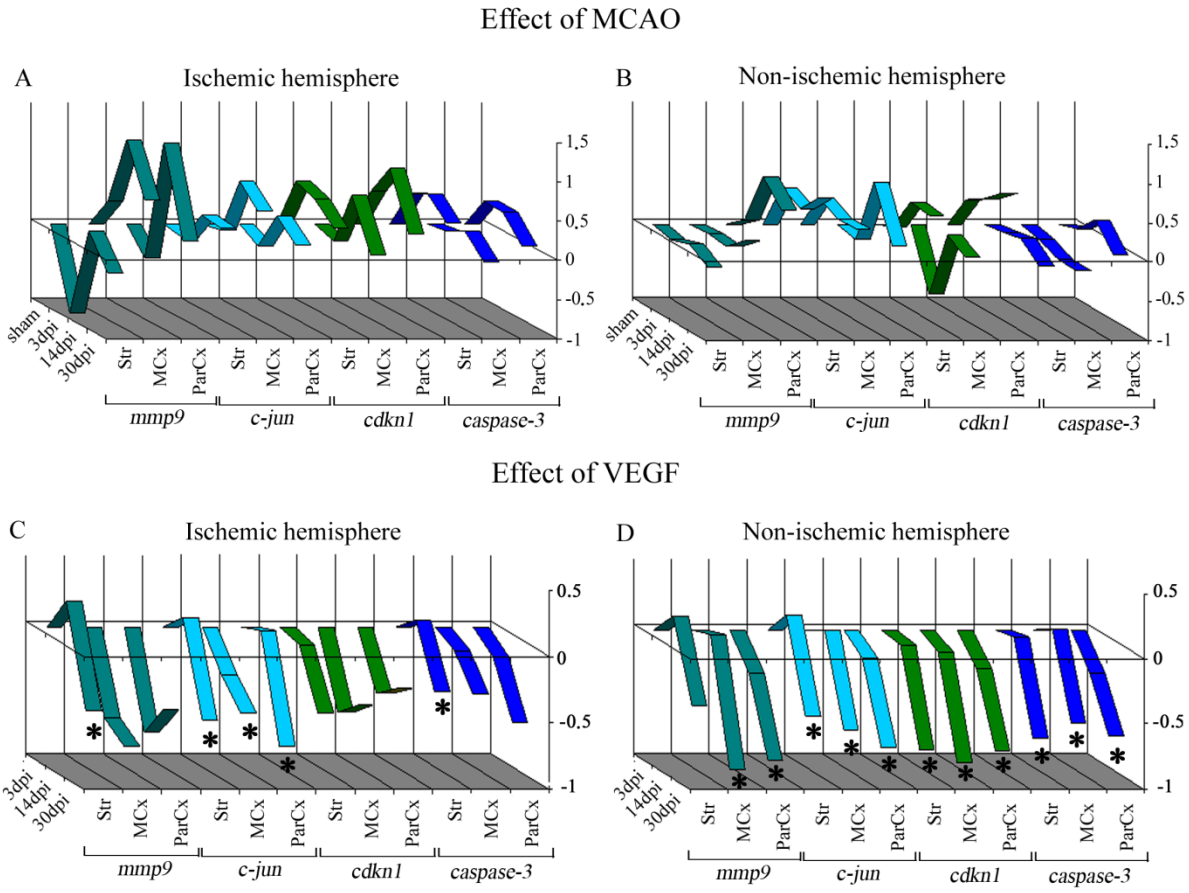

Figure 21. Effect of VEGF upon gene expression of mmp9, c-jun, cdkn1 and caspase 3. Semi-quantitative RT-PCR analysis.

Summarizing the expression of mmp9, c-jun, cdkn1 and caspase 3 genes that were regulated by the stroke in the striatum (Str), motor cortex (MCx) and parietal cortex (ParCx) at 3, 14 and 30 dpi. Both gene expression changes induced by MCAO **(A,B)** and by VEGF **(C,D)** are summarized. Throughout the time period examined, focal cerebral ischemia elevated the mRNA levels of mmp9, c-jun, cdkn1 and caspase 3 **(A)**. VEGF reduced the expression of mmp9, c-jun, cdkn1 and caspase 3 at 30 dpi in the ischemic hemisphere **(C)** and non-ischemic hemisphere (D). Data are logarithmic ratios of fold inductions after MCAO vs. sham surgery **(A,B)** and VEGF vs. vehicle treatment **(C,D)**. Data were analysed by two-way ANOVA. *P< 0.05 compared with vehicle-treated ischemic mice.

4.13. VEGF-induced angiogenesis and axonal plasticity involves differential responses of growth inhibitory proteoglycans and guidance molecules in both hemispheres

To clarify why VEGF influences angiogenesis and axonal plasticity in the two hemispheres in different ways, Western blots for the axonal growth inhibitory proteoglycans NG2 and brevican and for the vascular and axonal guidance proteins ephrin B1 and ephrin B2 were analyzed using tissue samples obtained from the ipsilesional and contralesional striatum.

In general, the abundance of these four proteins was upregulated in the subacute phase compared with acute phase of the stroke, particularly at 14 and 30 dpi (Fig. 22). Intriguingly, VEGF administration did not influence the expression of these proteins in the ipsilesional striatum, which is traversed by descending pyramidal tract axons, but reduced the abundance of NG2, brevican and ephrin B2 in the contralesional striatum at 14 dpi (Fig. 22). Gene expression studies by RT-PCR revealed that VEGF downregulated the mRNAs of the proteoglycan *neurocan* (*ncan*), the guidance molecules *ephrin B1* (*eph B1*) and *netrin 1* (*ntn1*), and the *neural cell adhesion molecule* (*ncam*) in both hemispheres (Fig. 23). These data indicate that the fine-tuned regulation of new vessel formation and axonal growth takes place on the protein and not on the mRNA level.

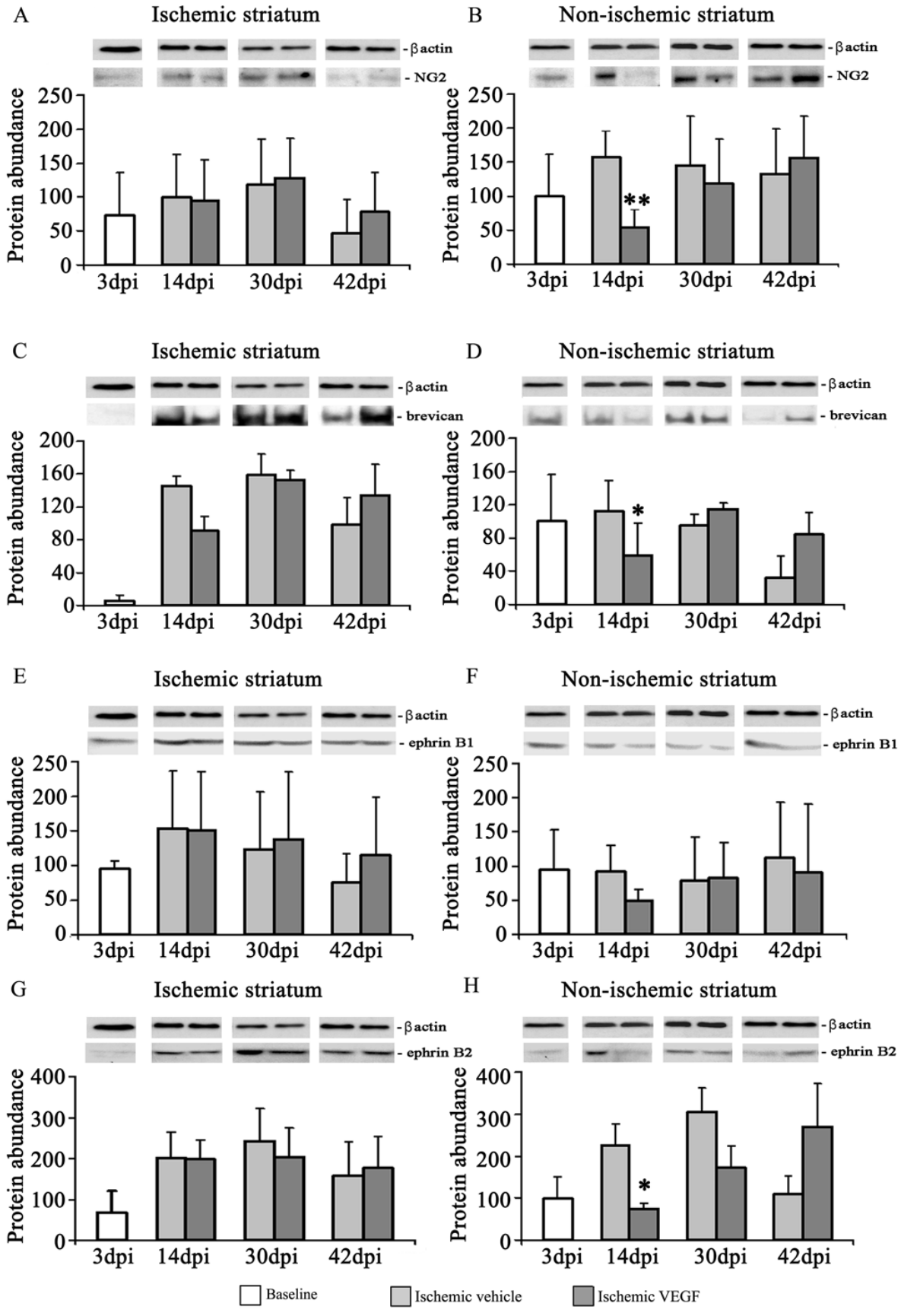
A
Ischemic striatum
B
Non-ischemic striatum
-βactin
- NG2
Protein abundance
250
200
150
100
50
0
3dpi
14dpi
30dpi
42dpi
**
C
Ischemic striatum
D
Non-ischemic striatum
- brevican
200
160
120
80
40
*
E
Ischemic striatum
F
Non-ischemic striatum
- ephrin B1
G
Ischemic striatum
H
Non-ischemic striatum
- ephrin B2
400
300
Baseline
Ischemic vehicle
Ischemic VEGF

Figure 22. VEGF downregulates growth inhibitory proteoglycans and guidance molecules in the contralesional hemisphere.

Western blot analysis using antibodies detecting total NG2 proteoglycan (A, B), brevican (C, D), ephrin B1 (E, F) and ephrin B2 (G, H), both for the ipsilesional and contralesional striatum. Note the downregulation of the proteoglycans and guidance molecules in the contralesional non-ischemic striatum at 14 dpi. Data were normalized with values determined in the contralateral striatum of animals submitted to focal cerebral ischemia followed by 3 days reperfusion. Protein loading was controlled with a β-actin antibody. Representative blots are shown. Data are means ± SD (n=3 Western blots). Data were analyzed by two-way ANOVA followed by two-tailed t-tests. *p< 0.05/ **p< 0.01 compared with non-ischemic vehicle.

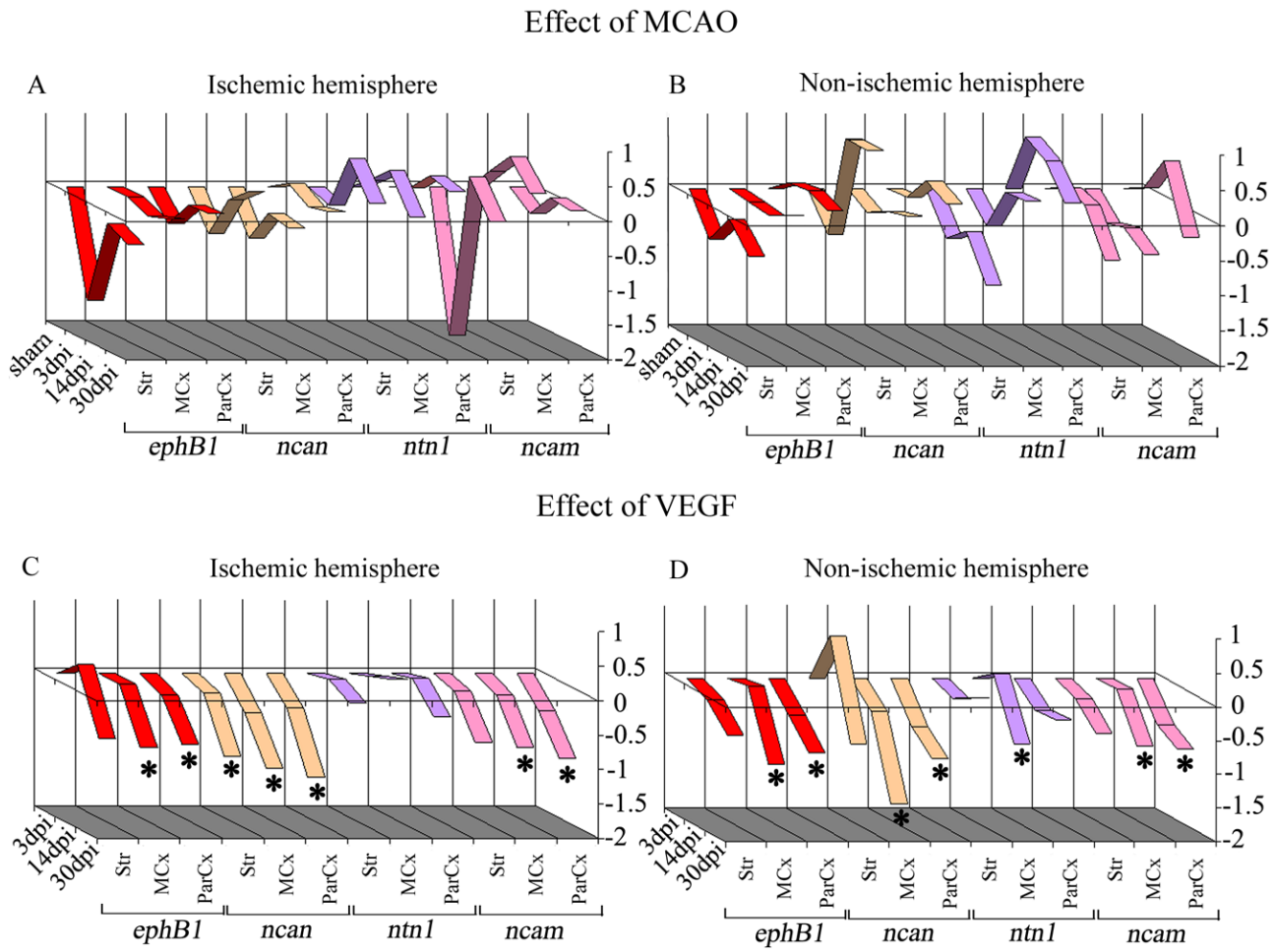

Figure 23. The effect of VEGF on plasticity gene expression ipsi- and contralateral to stroke.

Semi-quantitative RT-PCR analysis. Throughout the time period examined, focal cerebral ischemia did not modified the expression of ephB1, ncan, ntn1, ncam through time and regions after ischemia.

5. DISCUSSION

5.1. General aspects regarding post-acute therapy with Epo and VEGF after stroke

Using a comprehensive analysis of (i) motor and coordination deficits, (ii) reorganization processes of the peri-ischemic tissue, and (iii) pyramidal tract plasticity both ipsilateral and contralateral to the stroke, we found that post-acute delivery of the growth factors Epo and VEGF, initiated up to 72 hours after focal cerebral ischemia, promoted functional neurological recovery in mice submitted to transient intraluminal MCAO. Functional recovery was mediated by mechanisms involving perilesional tissue remodeling and promotion of contralateral pyramidal tract plasticity.

An overlooked aspect regarding stroke therapy until now was the acceptance of the strong self-recovery post stroke phase that takes place physiologically after stroke and lasts for up to 72 hours. Trying to treat the injury within this phase by the means of different neuroprotective therapies proved to be ineffective in the clinic, one of the explanations being the possible interference with the innate recovery capacity of the brain. This self-recovery phase was the subject of many intensive studies in preclinical research, leading to the conclusion that stroke reactivates the dormant ontogenetic program in the brain (Cramer and Chopp, 2000). This reactivation takes place on different levels, from new activation of genetic programmes, new synthesis of proteins, and new production of cells: neurogenesis (Li and Chopp, 1999), angiogenesis (Li et al., 1997), synaptogenesis (Albensi, 2001) up to macroscopic restructuration of anatomical pathways and cortical remapping (Dancause et al., 2005; Dijkhuizen et al., 2003).

In the present studies, growth factors were administered via miniosmotic pumps starting 3 dpi after stroke until 30 dpi. Our main outcome parameters were functional recovery after stroke, together with anatomical modulation of axonal regeneration due to tract-tracing studies.

Both Epo and VEGF promote neurological recovery beyond the acute stroke phase, opening new perspectives for post-acute therapies. In contrast to acute neuroprotection, which aims at preserving ischemic neurons from apoptotic or necrotic injury and which has so far proven impossible beyond a time-window of 3 to 6 hours after the stroke (Minnerup et al., 2009), post-acute therapies aim at the reorganization of the brain, both in the vicinity of the stroke lesion (Cramer and Chopp, 2000) and in remote areas (Wiessner et al., 2003). Slowly progressing secondary degeneration takes place in the striatum of vehicle-treated mice submitted to transient intraluminal MCAO, reflected by a continued loss of neurons and secondary tissue shrinkage, which in our study were both reduced by Epo and VEGF. The possibility that secondary neurodegeneration in the vicinity of the lesion may be relevant for stroke recovery has also been proposed by Taguchi et al. (2007), who reported deterioration of neurological deficits due to enhanced brain atrophy in granulocyte-colony stimulating factor (G-CSF)-treated mice submitted to distal MCAO.

Besides preventing delayed neurodegeneration, Epo and VEGF induced angiogenesis and inhibited reactive astrogliosis, at the same time preventing scar formation of ischemic tissue. Our data are in agreement with studies by Li et al. (2009), who made MRI-based observation of peri-lesional white matter remodeling in MCAO rats treated with Epo within 24 hours of reperfusion. Inhibition of glial scar formation was reported in studies evaluating cell based therapies, namely bone marrow-derived stem cells (Li et al., 2005) and adult neural precursor cells (Bacigaluppi et al., 2009), indicating that Epo and VEGF share common mechanisms of action with these cells. In contrast to Epo and VEGF, adult neural stem/ precursor cells did not stimulate angiogenesis (Bacigaluppi et al., 2009). An advantage of Epo as compared to cell-based therapies, namely with neural precursor cells, is the lack of malignant tumor growth, which still remains a risk for the latter that cannot entirely be ruled out (Amariglio et al., 2009).

The interhemispheric interaction after stroke is misbalanced; many pre-clinical and clinical studies reported an inhibition of the ipsilesional hemisphere by the contralesional hemisphere, inhibition that might be useful for improving cortical output

(Nair et al., 2007). It was also reported that after stroke the bilateral motor control from childhood is reactivated, meaning that the contralesional corticospinal tract may take over functional innervation of the hemiplegic body region (Nelles et al., 1999). Schwab and his co-authors successfully established anterograde neuronal tract tracing of the contralesional corticospinal tract as a tool for proving neuronal plasticity after stroke (Papadopoulus et al., 2002; Seymour et al., 2005). In our study we go a step further by applying an anterograde tract tracer in the ipsilesional motor cortex in addition to the contralesional one, which enables analysis of the impact of high dose Epo therapy on bilateral corticospinal tracts after stroke injury.

In the present studies, induction of focal cerebral ischemia significantly increased axonal plasticity ipsilateral to the stroke, namely at the level of the facial nucleus. Remodelling of the pyramidal tract investigated by means of manganese-enhanced MRI in rats from 4-10 weeks after 90 min MCAO showed significantly increased manganese transport in contralesional brain structures after ipsilesional motor cortex injections, suggesting enhanced interhemispheric axonal growth (van der Zijden et al., 2008). Unlike dextran amines, manganese is a combined anterograde (Canals et al., 2008) and retrograde (Matsuda et al., 2010) tracer, which is transsynaptically transported. Histochemical studies showed that focal cerebral ischemia induces the growth of short distance fibres (Carmichael et al., 2001) and promotes dendritic spine formation (Brown et al., 2007) in the perilesional tissue ipsilateral to the stroke, proving a short distance remodeling after stroke. The present data confirm once again that plasticity is indeed induced after stroke and that it not only affects short distance but also long distance projections.

In view of the enhanced plasticity ipsilateral to the stroke, which we observed in vehicle-treated ischemic mice, it is noteworthy that Epo and VEGF did not further enhance ipsilesional pyramidal tract plasticity but rather showed a tendency to reduce it ($p=0.07$ at the level of the facial nucleus for Epo and $p>0.1$ for VEGF). Instead, both growth factors significantly increased the projections from the contralesional motor cortex towards the denervated red and facial nuclei. Contralesional sprouting was shown to correlate with functional neurological recovery after antagonization of the neurite growth inhibitor NogoA (Papadopoulos et al.,

2002; Wiessner et al., 2003) and bone marrow-derived stem cell therapy (Andrews et al., 2008). In case of neutralizing NogoA antibodies, close correlations between the degree of functional neurological recovery and contralateral sprouting have been reported (Papadoupolus et al., 2002; Wiessner et al., 2003), demonstrating that cerebral connectivity represents a useful marker for the recovery processes. The present data confirm that stroke induces plasticity in the lesioned hemisphere and that Epo and VEGF shift plasticity towards the contralesional hemisphere, with a beneficial effect on functional recovery.

5.2. Specific aspects regarding Epo therapy after stroke

In the present study, Epo exhibited pronounced anti-inflammatory actions, which coincided with a reduction of leukocyte infiltration that persisted as long as Epo was infused. It has already been shown that Epo and its non-erythropoietic derivatives exhibit anti-inflammatory actions when delivered in the acute stroke phase. The resulting reduced leukocyte infiltration (Villa et al., 2003, 2007) decreased release of TNF-α, IL-6 and monocyte chemoattractant protein-1 (Villa et al., 2003) and diminished expression of iNOS (Kilic et al., 2005a). In cell culture, astrocytes but not leukocytes were shown to be responsive to Epo administration (Villa et al., 2003), suggesting that reduced release of inflammatory signals may be the trigger for the reduced invasion of blood-borne cells into the brain.

By means of RT-PCR we revealed that Epo downregulated the pro-inflammatory genes for IL-1β, TNF-α, LIF, TGF-β, IL-6, GFAP and iNOS, indicating that anti-inflammation may represent a mode of action, via which Epo enables brain reorganisation. Interestingly, pump removal in our study resulted in a rebound of leukocyte infiltration in Epo-treated mice, which however did not result in the reemergence of neurological deficits. Our data argue in favour of a critical time window, in which inflammatory responses impair recovery processes in the ischemic brain.

In addition, RT-PCR-based profiling of pro-plasticity genes showed that Epo diminished the expression of the pro-plasticity mRNAs SPRR1, IGF-1 and KDR in the

ischemic hemisphere, which were increased by the stroke. At the same time, Epo downregulated the anti-plasticity mRNAs neurocan and ephrin B1 and upregulated the pro-plasticity SPRR1 in the non-ischemic hemisphere. In case of SPRR1A, downregulation was also demonstrated on the protein level by means of Western blots. Upregulation of pro-plasticity markers in the lesion border zone has previously been described after permanent cortical ischemia for SPRR1, MARCKS, GAP 43 and synaptophysin, as well as delayed upregulation of anti-plasticity markers for neurocan and ephrin B1 (Carmichael and Li, 2006; Stroemer et al., 1995).

In our study, Epo regulated several pro- and anti-plasticity genes, especially in the non-ischemic hemisphere at 30 days post stroke, thus providing evidence for a specific plasticity modulating action of this growth factor. This is in accordance with previous studies on plasticity-promoting actions of Epo in rodent models of Parkinson's disease (McLeod et al., 2006) and following optic nerve transaction (King et al., 2007). The present study shows that Epo's plasticity effects are relevant for the stroke recovery processes.

After the German multicenter Epo trial, which recently showed unfavourable effects of Epo in patients undergoing thrombolysis (Ehrenreich et al., 2009), presumably due to exacerbation of extracellular matrix degradation by combined tissue-plasminogen activator / Epo treatment (Zechariah et al., 2010), further neuroprotection studies with Epo in the acute stroke phase are rather unlikely. Our present study offers a basis for the prolongation of time windows far into the sub-acute stroke phase, thus paving the way for plasticity-promoting trials with Epo, its non-erythropoietic derivatives (Leist et al., 2004) or with small molecule agonists of Epo receptors (Pankratova et al., 2010). The latter compounds, for which the utility for systemic delivery still has to be shown, may be specifically tailored to bind to Epo's CNS receptors. Non-erythropoietic derivatives of Epo do not induce hematoglobin or coagulation changes, and thus do not bear an elevated risk of thrombembolic events, which has recently been discussed in the context of chronic kidney disease trials (Pfeffer et al., 2009). With these considerations, proof-of-concept studies in human patients have interesting potential.

5.3. Specific aspects regarding VEGF therapy after stroke

Despite the fact that we used a delayed delivery protocol, with VEGF applied starting only 72 hours after the stroke, VEGF potently induced neuronal survival in the ischemic striatum, which accompanied the enhanced angiogenesis in the perilesional tissue. VEGF protected the brain against delayed neuronal death, inhibiting caspase-3-dependent apoptotic injury, and thus preventing post-acute tissue shrinkage. This observation highlights the importance of preventing degenerative processes in the ischemic brain, which continue to progress over up to two to six weeks (Bacigaluppi et al., 2009; Reitmeir et al., 2011; 2012). Perilesional angiogenesis and neuroprotection have repeatedly been described after acute VEGF delivery (Zhang et al., 2000; Sun et al., 2003; Wang et al., 2005). The neuroprotective effect of VEGF involves the release of neurotrophic growth factors from cerebral microvascular cells (Zhang and Chopp, 2009) as well as direct neurotrophic effects of VEGF (Wick et al., 2002; Kilic et al., 2006).

Because VEGF promoted angiogenesis and neuronal survival, the question arose whether this enhanced neurovascular remodeling is accompanied by long distance axonal plasticity. Congrous responses of vessels and axons to VEGF exposure could be expected based on observations during ontogeny, where angiogenesis and neuronal sprouting are closely linked. Throughout the periphery of the body, blood vessels and peripheral nerves share nerve and vessel sheaths, and navigate by very similar and partly overlapping growth and guidance cues (Giger et al., 2010). Examples are members of the slit and semaphorin families that are involved in blood vessel and nerve guidance (Autiero et al., 2005). Because angiogenesis and axonal sprouting are not jointly regulated after stroke, like angiogenesis and neuronal survival, but are instead spatially segregated from each other indicates that the responses of the adult CNS to injury differs from peripheral nerve-vessel systems.

In the induction of post-ischemic angiogenesis, MMPs, namely MMP9, plays a crucial role. MMP9 contributes to the breakdown of the ECM, which prepares the stage for vascular growth. Following acute VEGF delivery, MMP9 activity increases in

the perilesional ischemic tissue, closely in line with leaky vessels that are formed (Valable et al., 2005). The brain response to chronic VEGF differs from acute VEGF delivery, as shown in this report, since MMP9 was inhibited instead of activated by the growth factor. The downregulation of VEGF may reflect the maturation of newly formed brain vessels. MMP9 has not only been involved in angiogenesis, but also in axonal remodeling and repair. By cleaving growth inhibitory proteins, such as the proteoglycan NG2, MMP9 was suggested to contribute to axonal myelination processes. Impaired remyelination was observed in mice lacking MMP9 after lysolecithin-induced demyelination, which was interpreted as a consequence of the lack of proteoglycan proteolysis (Larsen et al., 2003).

In the ischemic brain, a link between MMP9 and neurovascular remodeling was shown by Zhao et al. (2006), who reported delayed expression of MMPs in peri-lesional cortex in brain areas exhibiting enhanced activation signals by means of functional MRI. Inhibition of MMP abolished this response, and it was concluded that MMP9 promotes both vascular remodeling and brain plasticity (Zhao et al., 2006). MMPs cleave the proteoglycan NG2, which plays an important role as inhibitor of oligodendrocyte maturation, and cleave proteins and glycoproteins acting as axonal growth inhibitors, namely NogoA and myelin-associated glycoprotein (Yong, 2005). In the present study VEGF selectively downregulated the proteoglycans NG2 and brevican in the contralesional, but not ipsilesional brain tissue, which may in part reflect a consequence of the deactivation of MMP9 in the ischemic hemisphere. The differential regulation of axonal growth inhibitors was noticed on the protein level, but not mRNA level, suggesting that the abundance of growth inhibitors following stroke may be controlled by degradation rather than *de novo* expression processes.

Clues for the spatial dissociation of angiogenesis and axonal plasticity may be derived not only from responses of axonal growth inhibitors, but also from the guidance molecules ephrin B1 and ephrin B2, which were, similar to NG2 and brevican, differentially regulated by VEGF in both hemispheres. Ephrins, namely ephrin B2, are required for developmental as well as tumor-associated angiogenesis, promoting the cellular internalization of VEGF receptors, namely of VEGF receptor-2 that mediates angiogenesis (Sawamiphak et al., 2010; Wang et al., 2010).

Internalization of VEGFR2 is necessary for activation and downstream signaling of the receptor and is needed for the VEGF-induced extension of the cells, which sets the stage for vessel sprouting (Sawamiphak et al., 2010). Intriguingly, the effects of ephrins on axons differ from those on vessels. During development, ephrins may have attractant and repellent properties, depending on their site of action and the coexpression of ephrin receptors (Hindges et al., 2002). In contrast, in the adult brain, the primary role of ephrins in regeneration is repulsion (Giger et al., 2010). Ephrins are expressed on reactive astrocytes, namely on scar tissue (ephrin B2), and myelin (ephrin B1) following CNS injury (Fabes et al., 2007). Inhibition of EphA4, using a peptide antagonist or genetic deletion, increases the sprouting of corticospinal axons *in vivo* (Fabes et al., 2007) and promotes the outgrowth of neurites from cultured neurons (Goldshmit et al., 2004). From this perspective, the differential regulation of ephrin B2 and ephrin B1, i.e., their downregulation contralateral but not ipsilateral to the stroke, may have created a favorable environment in which pyramidal tract plasticity became possible.

Our data show a dissociation of VEGF-induced angiogenesis and axonal plasticity between the ipsilesional and contralesional hemispheres that accompanies neurological recovery. This dissociation could be attributed to differential responses of extracellular matrix and guidance molecules in both hemispheres.

6. CONCLUSIONS

The differential regulation of matrix proteinases, axonal growth inhibitors and guidance molecules opens fascinating perspectives for translational studies and for delineating targets via which the efficacy of therapeutics might be predicted. This provides opportunities for the development of neurorestorative therapies. However, our understanding of successful stroke recovery is still evolving. With a proper understanding of the mechanisms that guide new vessel formation and axonal plasticity, clinical proof-of-principle concept studies may show promising results.

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8. PERMISSIONS AND IMAGE SOURCES

Parts of the Material and Methods, Results and Discussions are based on the accepted versions of the two publications published in 2011 and 2012.

Citation: Reitmeir R, Kilic E, Kilic U, Bacigaluppi M, ElAli A, Salani G, Pluchino S, Gassmann M, Hermann D. Post-acute delivery of erythropoietin induces stroke recovery by promoting perilesional tissue remodelling and contralesional pyramidal tract plasticity. Brain (2011) 134:84

Reitmeir R, Kilic E, Reinboth B., Elali A., Zechariah A., Kilic U, Hermann D. Vascular endothelial growth factor promotes functional recovery after focal cerebral ischemia: Role of extracellular matrix and guidance molecules in enhancing axonal plasticity. Acta Neuropathol. (2012) 132(2): 273-84

Figure 1.
Link:http://www.google.ch/url?q=http://neuro4students.wordpress.com/pathophysiology/&ei=8ckgUdGwDIWM4ATS8YCQDg&sa=X&oi=unauthorizedredirect&ct=targetlink&ust=1361105145204557&usg=AFQjCNF_HsFCIDb_oKQcERDZjEAm0FmTwQ (Date 12.6.2013)

Figure 2:
Drinagl U., Iadecola C., Moskowitz M.A..1999 Pathobiology of ischemic stroke: an integrated view. Trends. Neurosci. 22(9):391-7
Licence Number 2959530648148

Figure 3:
Duan Y., Gross R.A., Sheu S-S. 2007. Ca2+-dependent generation of mitochondrial reactive oxygen species serves as a signal for poly(ADP-ribose) polymerase-1 activation during glutamate excitotoxicity. J Physiol 741-758.
Licence Number: 2959531500950

Figure 4:
Murphy T.M. and Corbett D. 2009. Plasticity during stroke recovery: from synapse to behaviour. Nature Reviews Neuroscience 10: 861-872
Licence Number: 2959540728939

9. CURRICULUM VITAE

Professional Research Profile:

The main research focus is based on identifying the key molecules responsible for activation of ontogenetic program meant to enhance axonal plasticity after focal cerebral ischemia increasing functional recovery after stroke.

Education, Funding and Prizes

Residency in Neurosurgery 2010 – today

MEBEKO Recognition of medical studies in Switzerland in 2009

Department of Neurosurgery, Inselspital Bern, Switzerland, Prof. Raabe, 2011 until today

Department of Surgery, Limmattal Hospital Zürich, Switzerland, Prof. Schöb, 2010-2011

Supervisor: Prof. Dr. A. Raabe

MD-PhD Program 2007-2010

Department of Neurology; University Hospital Zürich (USZ), Switzerland (2007-2008)

Department of Vascular Neurology; University Duisburg-Essen, Germany (2008-2010)

Funding: Dr. Werner Jäckstädt-Stiftung

Supervisor: Prof. Dr. D.M. Hermann

Research Exchange Experience 2009 (2 months)

Department of experimental Neurology, San Raffaele Scientific Institute, Milano, Italy

Supervisor: Prof. Dr. Gianvito Martino / Prof. Dr. D. M. Hermann

Master of Science in Medical Biology 2007-2009

University Duisburg-Essen, Germany

Thesis Title (grade A): The influence of cholesterol diet and Rosuvastatin on neurological recovery after stroke

Supervisor: Prof. Dr. D.M. Hermann / Prof. Dr. Fandrey

Doctor of Medicine (Dr. med. univ.) at the Medical University of Vienna Austria 2007

Doctor Thesis (grade A): Neuroprotection and Regeneration in Spinal Cord Injury: Studies on Wlds knock-out Rat

Supervisors: Prof. Lubec / Dr. med. Mark Kotter

Prize for the best Presentation „Young academics NeuroVision 5“ 2010
Bochum University; Germany; Prize 1000 EUR

Erasmus Fellowship at the Medical University Vienna, Austria 2005-2007

IFMSA–„Standing Comittee on Professional Exchange“ Visiting Student 2004
Medical University Vienna, Austria

Romanian State Fellowship 2001-2005
Award for the first admission place at the Medical University University “luliu Hatieganu”, Cluj-Napoca, Romania 2001

School leaving examination (A-Level) Grade 9.78 (A)

Teaching Experience:

Supervisor of Master Thesis in “Biomedical Sciences” University of Bern 2012:
Thesis Name: Characterisation of spatio-temporal differentiation of molecular markers in the process of axonogenesis after vascular growth factor (VEGF) Therapy in the ischemic brain

Supervisor of Master Thesis at the Medical University of Bern 2012:
Thesis Name: Effect of early lumbal drainage application in the prevention of vasospasm after subarachnoidal bleeding.

Supervisor of Master Thesis in “Biological Sciences” University of Bochum 2010:
Thesis Name: Modulation of inflammatory processes after stroke by growth factor administration VEGF and EPO

Tutorial activity at the Medial University of Bern since 2011

Publications

1. **Reitmeir R**, Kilic E, Reinboth B., Elali A., Zechariah A. "Vascular endothelial growth factor promotes functional recovery after focal cerebral ischemia: Role of extracellular matrix and guidance molecules in enhancing axonal plasticity. Acta Neuropathol. (2012) 132(2): 273-84

2. **Reitmeir R**, Kilic E, Kilic U, Bacigaluppi M, ElAli A, Salani G, Pluchino S, Gassmann M, Hermann D. Post-acute delivery of erythropoietin induces stroke recovery by promoting perilesional tissue remodelling and contralesional pyramidal tract plasticity. Brain (2011) 134:84

3. **Reitmeir R** und Hermann D. M. Allgemeine Grundlagen: Pathophysiologie vaskulär-neurologischer Erkrankungen. In: „Vaskuläre Neurologie“ (Herausgeber: D. Hermann, T. Steiner, H.C. Diener), 1. Auflage, 2010.

4. Herz J, **Reitmeir R**, Hagen SI, Reinboth BS, Guo Z, Zechariah A, ElAli A, Doeppner TR, Bacigaluppi M, Pluchino S, Kilic U, Kilic E, Hermann DM. Intracerebroventricularly delivered VEGF promotes contralesional corticorubral plasticity after focal cerebral ischemia via mechanisms involving anti-inflammatory actions. Neurobiol Dis. (2012) 45(3):1077-85.

5. Baer A., Syed Y, SU K., Miterreger D, **Vig R**, Charles french-Constant, Franklin R., Lubec G, Kotter M. Myelin mediated inhibition of oligodendrocyte precursor differentiation can be overcome by pharmacological modulation of Fyn-RhoA and PKC signalling. Brain (2009) 132:465-81.

6. Kilic E., Spudich A., Kilic U, Rentsch K. M., **Vig R,** Matter C. M., Wunderli-Allenspach H., Fritschy J-M, Bassetti C. L. and Hermann D. M. ABCC1: a gateway for pharmacological compounds to the ischaemic brain. Brain (2008) 131:2679-2689.

10. ERKLÄRUNG

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Promoting stroke recovery using delayed growth factor delivery: Contribution of ipsilesional versus contralesional pyramidal tract plasticity“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (Dr. med. Raluca Reitmeir) befürworte.

Essen, den _____ _____ _____

Name des wissenschaftl. Betreuers/Mitglieds der Universität Duisburg-Essen	Unterschrift d. wissenschaftl. Betreuers/ Mitglieds der Universität Duisburg- Essen

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den _____ _____

Unterschrift des/r Doktoranden/in

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den _____ _____

Unterschrift des/r Doktoranden/in