Neuroprotective and neurorestorative effects of erythropoietin after brain injury

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PhD Thesis

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SUMMARY

Erythropoietin (EPO), a hematopoietic growth-factor, is produced by the kidney in response to hypoxia. EPO exerts its actions via EPO receptors (EPO-R) which are members of the cytokine type 1 receptor subfamily. Diverse cell types have been demonstrated to produce EPO and many cells express the EPO-R, including neurons. Besides its hematopoietic function, EPO exerts neuroprotective activity upon reduced oxygenation or ischemia of brain, retina, and spinal cord. The main focus of this study was to examine the mechanisms mediating EPO's neuroprotective activity in vivo. To this and we made use of our transgenic mouse line tg21 that constitutively expresses human EPO in brain without inducing excessive erythrocytosis. Our data show that human EPO is expressed in tg21 brains and retinas and that cortical, striatal neurons and retinal ganglion cells (RGCs) carry the EPO receptor.

After middle cerebral artery occlusion, human EPO potently protected brains of tg21 mice against ischemic injury, both when severe (90 min) and mild (30 min) ischemia was imposed. Histochemical studies revealed that EPO induced an activation of JAK-2, ERK-1/-2, and Akt pathways in the ischemic brain. This activation was associated with elevated Bcl-XL and decreased NO synthase-1 and -2 levels in neurons. Intracerebroventricular injections of selective inhibitors of ERK-1/-2 (PD98059) or Akt (wortmannin) pathways revealed that both ERK-1/-2 and Akt were required for EPO's neuroprotective function and antagonization of either pathway completely abolishing tissue protection. On the other hand, ERK-1/-2 and Akt blockade did not reverse the neuronal NO synthase-1/-2 inhibition, indicating that EPO down-regulates these NO

synthases in an ERK-1/-2 and Akt independent manner. Our data show that the dual activation of ERK-1/-2 and Akt is crucial for EPO's neuroprotective activity.

After retrograde degeneration of retinal ganglion cells (RGCs) following optic nerve transection in vivo, the RGCs of EPO transgenic tg21 mice were protected against degeneration, as compared with wild-type control animals. Histochemical analysis revealed decreased phosphorylation levels of STAT-5 and reduced expression of Bcl-XL in RGCs of axotomized tg21 animals, suggesting that the corresponding pathways are not crucial for EPO's neuroprotective activity. Increased phosphorylation levels of ERK-1/-2 and Akt, as well as decreased caspase-3 activity, however, were observed in injured tg21 retinae. Injection of selective inhibitors of ERK-1/-2 (PD98059) or Akt (Wortmannin) pathways into the vitreous space revealed that transgenic EPO protected the RGCs by a pathway involving ERK-1/-2 but not Akt.

In view that axotomy-induced degeneration of RGC occurs slowly, and considering the safety and efficacy of EPO in human stroke patients, we predict the clinical implementation of recombinant human EPO not only in patients with acute ischemic stroke, but also with more delayed degenerative neurological diseases.

Accordingly, we investigated how subacute intracerebroventricular delivery of EPO, starting 3 days after stroke onset, and continuing for 30 days (1 or 10 IU/day; via mini osmotic pump), influences neuronal survival and neurological function abnormalities in C57Bl6/j mice exposed to 30-minutes of focal cerebral ischemia. Six weeks after middle cerebral artery occlusion, human recombinant EPO was capable to protect the brain from ischemic injury, even when applied 3 days after stroke onset. Histochemical studies of ischemic striatum revealed that the number of surviving

neurons and capillaries were significantly higher in EPO treated animals as compared to non-treated animals. Cell survival was also associated with a long-lasting improvement of motor and coordination deficits, evaluated by the grip strength and RotaRod tests. Three days after ischemia, grip strength of paretic forepaw and motor coordination performance were both significantly lower than baseline measurements. EPO treatment improved grip performances 6 weeks after ischemia. We are convinced that the robust functional neurologic improvements, we found in mice, will encourage proof-of-concept studies with EPO in human stroke patients.

1.INTRODUCTION

Apart from its role in red blood cell propagation, the glycoprotein erythropoietin (EPO) exerts neuroprotective functions in various animal models in which neurons are lost (Gassmann et al., 2003; Grimm et al., 2005). As such, EPO has previously been shown to protect against ischemia and trauma of the brain, retina, and spinal cord (Brines et al., 2000, Siren et al., 2001; Celik et al., 2002; Grimm et al., 2002; 2004). Clinical studies have demonstrated that human EPO indeed reduces infarct size and also improves functional outcome in stroke patients, when administered up to 8 hours after stroke onset (Ehrenreich et al., 2002). In view of EPO being routinely administered in anemic patients with renal failure (Hudson et al., 2002; Macdougall, 2003), this hematopoietic factor is considered to be both very potent and safe.

The EPO receptor downstream signaling mechanisms responsible for EPO's neuroprotective functions in vivo are still largely unknown. Understanding these mechanisms, however, is crucial for future developments of neuroprotective compounds. To elucidate these pathways we investigated the effects of EPO on brain injuryusing a transgenic mouse line termed tg21. The tg21 mouse constitutively expresses human EPO in the brain without inducing erythrocytosis (Ruschitzka et al., 2000; Wiessner et al., 2001) and is therefore an attractive model for neuroprotection studies. Tg21 mice underwent middle cerebral artery (MCA) occlusions and in depth analysis of the effects of endogenously overexpressed EPO on brain injury and cell signaling was performed. By selectively blocking individual signaling pathways with specific signal transduction inhibitors in vivo, we characterized the signaling pathways mediating EPO's brain protective function.

1.1. Mechanisms of injury after ischemia

Cerebral ischemia may be either transient or permanent. A specific region of the brain may be affected, as occurs during an arterial or venous stroke, or the entire brain may become globally ischemic, as occurs during a cardiac arrest. In addition to such settings where ischemia is the primary insult, ischemia may also contribute secondarily to brain damage as in the case of mass lesions, hemorrhage, or trauma.

Within seconds of cerebral ischemia, local cortical activity as detected by electroencephalography ceases; if the ischemia is global, unconsciousness rapidly ensues (witness the Stokes-Adams attack). This massive shutdown of neural activity is induced by K^+ efflux from neurons, mediated initially by the opening of voltage-dependent K^+ channels and later by ATP-dependent K^+ channels, leading to transient plasma membrane hyperpolarization. A few minutes later, despite this energy sparing response, an abrupt and dramatic redistribution of ions occurs across the plasma membrane, associated with membrane depolarization (efflux of K^+ and influx of Na^+, Cl^-, and Ca^{2+}) (Siesjö, 1992). This “anoxic depolarization” results in the excessive release of neurotransmitters, in particular, glutamate, promoting further spatial spread of cellular depolarization, depletion of energy stores, and advancement of injury cascades (Choi 1988; 1992).

1.1.1. Excitotoxicity, NMDA and Ca^{2+} overload

During the past years, a considerable amount of experimental work has been devoted to the elucidation of glutamate-mediated mechanisms of ischemic brain injury. It is widely accepted that the release of this and other excitatory amino acids during the early phase of brain ischemia triggers a cascade of molecular events that lead to irreversible injury, not only in areas where oxygen supply is critically reduced but also in regions with seemingly undisturbed energy metabolism, e.g., the penumbra of focal ischemia or the selectively vulnerable regions after transient global ischemia (Hossmann, 1994). Glutamate is the main excitatory neurotransmitter throughout the CNS, and is found in high concentrations in CNS tissue, where it is stored in vesicles. When excess glutamate is released or reuptake is impaired, the extracellular concentration can rise significantly, leading to neuronal death (Choi, 1992). Olney and Sharpe created the term excitotoxicity to describe over-activation of glutamate receptors (Olney and Sharpe, 1969). Under ischemic conditions, the neurotransmitter glutamate can accumulate in the extracellular space (Benveniste et al., 1984). Unfortunately, such increased concentrations of glutamate are neurotoxic, and there is substantial evidence underlining the toxicity of glutamate in the pathogenesis of neuronal death after ischemia and other acute insults (Rothman and Olney, 1986). Extracellular glutamate accumulating under ischemic conditions overstimulates N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate-type glutamate receptors, promoting Na^+ influx and K^+ efflux through glutamate receptor-activated membrane channels. NMDA receptor-gated ion channels are in addition highly permeable to Ca^{2+} and mediate Ca^{2+} influx into neurons.

The gating of glutamate receptor activated channels effectively achieves membrane shunting, which spreads in waves (spreading depression) from the ischemic core out towards the margins of the ischemic zone (ischemic penumbra). Spreading depression increases metabolic demand and energy failure, thus further enhances glutamate release. Marked neuronal cell body swelling and dendrite swelling occur, hallmarks of necrotic death, as Na^+ and Ca^{2+} entry is joined by the influx of Cl^- and water. Elevations in neuronal intracellular free Ca^{2+} ($[Ca^{2+}]_i$), mediated both directly by NMDA receptors and indirectly via membrane depolarization-activated voltage gated Ca^{2+} channels and reverse operation of the Na^+-/Ca^{2+} exchanger, bear particular responsibility in promoting spreading depression and triggering deleterious cytotoxic cascades.

In neuronal cell cultures, selective NMDA receptor blockade prevents most of the Ca^{2+} influx and cell death induced by brief intense glutamate exposures (Choi, 1988). NMDA antagonists also markedly attenuated the death of cultured neurons induced by oxygen and/or glucose deprivation. Exposure to NMDA for as little as 3–5 minutes is sufficient to trigger widespread cultured cortical neuron death (“rapidly triggered excitotoxicity”), whereas exposure to even saturating concentrations of kainate typically requires hours to reach the same level of toxicity (“slowly triggered excitotoxicity”). This difference in time course fits with a higher rate of Ca^{2+} influx mediated directly by NMDA receptor-gated channels, compared with a slower rate of Ca^{2+} influx mediated by the voltage gated channel and exchanger routes activated by AMPA or kainate receptors. A number of experiments have shown reduction of cortical infarct size (Dirnagl et al., 1990) and improvement of neurological clinical outcome or survival (Zabramski et al., 1991) with various NMDA receptor-antagonists administered after induction of focal cerebral ischemia in animals. In this latter

setting, NMDA receptor–mediated excitotoxicity may be less prominent than AMPA receptor–facilitated Zn^{2+} entry in inducing lethal neuronal injury. Reasons for this shift in prominence are presently not well-defined, but a contributing factor may be extracellular acidity due to accumulation of lactic acid during global ischemia, an event which is less prominent in the penumbra of focal ischemia where, in contrast to the core of ischemic injury, perfusion is partially maintained. This acid shift selectively downregulates NMDA receptors and NMDA receptor-mediated excitotoxicity but enhances AMPA receptor-mediated excitotoxicity (Lee et al., 1999); it may also enhance toxic Zn^{2+} entry through voltage-gated Ca^{2+} channels (Kerchner et al., 2000).

1.1.2. Other signaling messengers and growth factors

In addition to glutamate, other neurotransmitters released to the extracellular space during ischemia can significantly influence resulting brain injury. Dopamine, which increases 500-fold in the extracellular space following global ischemia, may contribute to striatal neuronal death. Moreover, experimental reduction of dopamine release, which can be accomplished by creating lesions in the dopaminergic neurons projecting from the substantia nigra or by using the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine to deplete endogenous stores of dopamine, attenuates striatal injury in rodent global ischemia models (Obrenowitch and Richards, 1995). Enhance glutamate receptor currents may contribute to dopamine induced potentiation of ischemic injury. Neurotransmitters do not all act to promote injury; several, including serotonin, gamma-aminobutyric acid (GABA; see below), and adenosine, act neuroprotective. Adenosine, which accumulates rapidly during ischemia due to breakdown of ATP, has beneficial effects in many tissues. The activation of

adenosine A2α receptors on vascular smooth muscle cells and neutrophils enhances blood flow and decreases inflammation. Adenosine also has nervous system specific beneficial effects, mediated by the ability of neuronal adenosine A1-receptors to reduce neurotransmitter release and membrane excitability (Von Lubitz, 1999). In addition, the expression of several growth factors increases in ischemic tissues, likely as a protective response. Exogenous administration of growth factors has been shown to be therapeutically promising in several experimental models of organ ischemia, including liver, kidney, heart, and brain (Behrens et al., 1999). Examples of growth factors whose administration reduces brain damage in rats subjected to cerebral ischemia are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins 4/5 (NT-4/5), basic fibroblast growth factor (bFGF), and IGF-1, which apparently blocks neuronal apoptosis. Some growth factors may also enhance nerve fiber sprouting and synapse formation after ischemic injury, thereby promoting functional recovery. Despite their overall salutary effects, certain growth factors may also enhance neuronal vulnerability to excitotoxic and free radical-induced death. Acute exposure to BDNF, NT-3, or NT-4/5 reduces the vulnerability of cultured neocortical neurons to apoptosis, but exacerbates cellular necrosis of the same cells after oxygen-glucose deprivation or exposure to NMDA. The deleterious actions of neurotrophins as well as IGF-1 may be explained in part by enhanced NMDA receptor-mediated Ca^{2+}-influx, enhanced production of free radicals, or possibly acute proexcitatory effects that could increase excitotoxicity (Behrens et al., 1999; Han et al., 2000).

1.1.3. Zinc neurotoxicity

Recent work showed that Ca^{2+} may not be the only divalent cation whose toxic influx contributes to ischemic brain cell death. Zinc, the second most abundant transition metal in the human body, is present in all cells, for the most part tightly bound to proteins, such as metalloenzymes and transcription factors, where it serves catalytic and structural roles. In the brain, there is an additional substantial pool of chelatable Zn^{2+} localized to synaptic vesicles in excitatory (glutamatergic) nerve terminals, which is released in a Ca^{2+}-dependent fashion with depolarization and can alter the behavior of several transmitter receptors and voltage-gated channels (Frederickson, 1989). While the normal functional significance of this presumptive signaling Zn^{2+} pool is presently not well understood, growing evidence suggests that it contributes to nerve cell death under pathological conditions such as ischemia or seizures or following head trauma (Choi, 1998). In transient global cerebral ischemia and induction of kainate seizures, zinc accumulation was observed in degenerating neuronal cell bodies (Frederickson, 1989), and injection of an extracellular zinc chelator into cerebrospinal fluid markedly reduced both zinc accumulation and neuronal death (Kim et al., 1999). Furthermore, exposure to high micromolar concentrations of Zn^{2+} that are likely to occur in brain extracellular space after synchronous cellular depolarization is sufficient to kill cultured neurons, especially if the neurons are already depolarized, which facilitates entry of Zn^{2+} across the plasma membrane through voltage-gated Ca^{2+} channels (Choi and Koh, 1998).

1.1.4. Downstream mediators

The massive release of neurotransmitters and elevations in $[Ca^{2+}]_i$ induced by cerebral ischemia produces gross perturbations in intracellular signaling pathways that may contribute to the resultant injury or even cell death. Protein kinase C (PKC) is rapidly activated during ischemia as a common response in several organs including the brain, kidney, and heart, and it may enhance neuronal excitotoxicity by increasing vesicular glutamate release and neuronal excitability (Kaczmarek, 1987). Selective PKC inhibitors have, to my knowledge, not been tested to date in animal models of cerebral ischemia. However, pretreatment with broad spectrum protein kinase inhibitors, such as staurosporine or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), has provided some therapeutic potential by decreasing neuronal cell death in a global model of cerebral ischemia and attenuating the extracellular accumulation of glutamate induced by ischemia in rodent brains, respectively (Hara et al., 1990; Nakane et al., 1998).

The highly conserved mitogen-activated protein (MAP) kinases, including c-Jun NH2-terminal kinases (JNKs), p38 kinases, and extracellular signal regulated kinases (ERKs) are activated in many cells by stress and may modify processes relevant to cellular injury and programmed cell death (Cohen, 1997). In the brain, all three MAP kinase pathways may be activated following the induction of ischemia, and the p38 and ERK pathways have been implicated in enhancing ischemic neuronal death. Pretreatment with the selective p38 inhibitor SB203580 reduced both activity of the p38 pathway and neuronal death after transient global ischemia (Sugino et al., 2000). Another study showed that pretreatment with the ERK inhibitor PD98059, but not SB203580, reduced infarction after transient focal ischemia (Alessandrini et al.,

1999). ERK signaling has also been suggested to have neuroprotective effects, either by attenuating apoptosis, or by mediating the development of resistance to subsequent oxygen-glucose deprivation (Gonzalez-Zulueta et al., 2000). Reflecting limitations in current pharmacology, contributions of the JNK pathway have not yet been identified in cerebral ischemia studies, but the possibility of such a role is raised by the finding that mice lacking Jnk3, an isoform with restricted expression in the brain, heart, and testes, exhibit resistance to seizure induced neuronal death (Yang et al., 1997).

1.1.5. Free radicals and catabolic enzymes

Adding to the injury occurring during a given ischemic insult, postischemic reperfusion appears to induce even further tissue damage in virtually all organs, likely mediated by the accelerated formation of several reactive oxygen species including superoxide, hydroxyl, and nitric oxide (NO) radicals. One particularly damaging consequence of reactive oxygen species formation in several cell types may be single-strand DNA breakage, leading to activation of the repair enzyme poly(ADP-ribose) polymerase (PARP) and PARP-mediated depletion of cellular NAD^+ and energy stores (Szabo and Dawson, 1998). NO, that is generated by inducible NO synthase (iNOS or type II NOS), and is expressed in macrophages, neutrophils, and microglia following immunological challenge, may also contribute to late tissue injury. In contrast, a second isoform of NO synthase present in endothelial cells (eNOS or type III NOS) may play a protective role, relaxing vascular smooth muscle cells and helping to preserve blood flow (Andrew and Mayer, 1999).

In the central nervous system, free radical production is thought to be likely a specific downstream mediator of glutamate-induced neuronal death. Neurons have a special

ability to respond to increases in $[Ca^{2+}]_i$ with increased NO production by neuronal NO synthase (nNOS or type I NOS, a Ca^{2+}-calmodulin-dependent enzyme). Inhibiting nNOS either pharmacologically or genetically (via gene deletion) renders cultured neurons resistant to NMDA-induced death, and also reduces infarct volume in rodent models of transient focal ischemia (Samdani et al., 1997). NMDA receptor activation may also stimulate oxygen free radical production by uncoupling neuronal mitochondrial electron transport (Dugan et al., 1995). Another link between brain signaling and free radical generation in the ischemic brain may be neuronal Zn^{2+} overload (Kim YH, 1999).

Free radical-mediated cytotoxicity in the ischemic brain is likely augmented by damage that is mediated by the excessive activation of Ca^{2+}-dependent catabolic enzymes. Phospholipase A2 and C (PLA2 and PLC) are activated following NMDA receptor stimulation and promote membrane phospholipid breakdown (which itself enhances free radical formation and inflammation). The Ca^{2+}-activated proteases, or calpains, are likely to contribute to destruction of structural and regulatory proteins. Genetic ablation of the cytoplasmic form of PLA2 (Bonventre et al., 1997), or pharmacological inhibition of PLC (Umemura et al., 1992) or calpains, reduces brain injury in animal models of cerebral ischemia (Markgraf et al., 1998).

1.1.6 Inflammation

Ischemia and reperfusion in the brain, as in other organs, induce an inflammatory response which may exacerbate initial levels of tissue injury. Intracellular adhesion molecule-1 (ICAM-1) is expressed by vessels in the core of the infarction and at the border of an infarction (Yang et al., 1999). ICAM-1 mRNA and endothelial leukocyte adhesion molecule-1(ELAM-1) and selectin are induced 3 hours and 6 hours

respectively, after ischemia and peak at 6 to 12 hours (Feuerstein, 1997). ICAM-1 protein is expressed mainly within the core of the infarct on endothelial cells (Kim, 1996) and plays a role in neutrophil invasion of ischemic tissue. Cytokine-induced neutrophil chemo-attractant protein (CINC) is also induced mainly within an infarct and at its margins (Yamasaki et al., 1995). CD11 positive neutrophils appear within a day at the infarct site and are numerous by 3 days (Kato et al., 1996). Many studies that show that a reduction in inflammatory cells or inhibition of adhesion molecules reduce injury in experimental models of stroke suggest that adhesion molecules and inflammatory cells play a role in mediating focal ischemic brain injury (Chopp et al., 1996; Feuerstein et al., 1997).

Integrin αβ 3 is expressed primarily in the core of an infarct and is possibly related to vascular responses (Abumiya et al., 1999). Monocyte-chemoattractant protein-1 and macrophage inflammatory protein-1 alpha (MIP-1α) are induced primarily in the core and adjacent areas of ischemia (Kim et al., 1995), where the greatest damage occurs, and then in the regions adjacent to the infarction, (Takami et al., 1997) where damage is less severe and possibly where macrophages and microglia engulf single cells or small groups of cells that might die more slowly.

1.1.7. Apoptosis/Necrosis

Tissue ischemia is a defining example of a violent “environmental perturbation” capable of producing necrosis, fulminant cell death associated with plasma membrane failure, and swelling of cell body and internal organelles (Wyllie et al., 1980). In the nervous system, the notion that ischemic insults cause neurons to undergo necrosis is strengthened by the implication of excitotoxicity in ischemic neuronal death. As noted above, glutamate receptor overactivation typically leads to

prominent swelling of cell body and dendrites. Despite this intuitive link between ischemic insults and necrosis, growing evidence indicates that ischemia may additionally induce programmed cell death in many tissues, including the heart, kidney, and brain.

Apoptosis is a form of cell death characterized by a series of distinct morphological and biochemical alterations suggesting the presence of common execution machinery in different cells. Condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, a decrease in cell volume, and alterations to the plasma membrane are classically observed, resulting in the recognition and phagocytosis of apoptotic cells. The nuclear alterations are often associated with internucleosomal cleavage of DNA, recognized as DNA laddering on conventional agarose gel electrophoresis. Internucleosomal cleavage of DNA is a relatively late event in the apoptotic process, which in some models of neuronal cell death may be dissociated from early critical steps. In fact, apoptosis is not restricted to nucleated cells. Nevertheless, detecting DNA fragmentation is simple and often used as a criterion to determine whether or not a cell is dying by apoptosis (Schulz et al., 1999).

1.1.7.1. Apoptosis in animal stroke models

The molecular link to the pathogenesis of apoptosis was identified first in the invertebrate Caenorhabditis elegans (C. elegans). Three cell death genes were identified, the pro-apoptotic (death promoting) genes *ced-3* and *ced-4* and the anti-apoptotic gene *ced-9*. Mammalian homologues are the caspase family, the apoptosis protein activating factor-1 (Apaf-1), and the Bcl-2 family, respectively. A family of at least 14 related cysteine proteases is known, termed caspase-1 through caspase-14, named by order of discovery. The family includes two murine homologues (caspase-

11 and caspase-12) that have no known human counterparts yet. These proteins are characterized by almost absolute specificity for aspartic acid on the N-terminal side of the substrate cleavage site and each one contains a specific pentapeptide sequence in the catalytic site (QACXG) of the enzyme. Caspases are synthesized and stored as inactive proenzymes. They contain an N-terminal prodomain together with one large (p17 to p20) and one small (p10 to p12) subunit. The activation of caspases requires cleavage (usually by other caspases) to liberate one large and one small subunit, which associate into a heterotetramer containing two small and two large subunits. Caspases may be divided into two classes: caspases that target and regulate activation of other caspases (caspases 1, 2, 4, 5, 8, 9, and 10) and caspases involved in the downstream execution phase (caspases 3, 6, 7, and 14). Evidence is accumulating that the interleukin-1 converting enzyme (ICE) subfamily (caspases 1, 4, and 5) plays a predominant role in inflammation, an important component of cerebral ischemia, whereas members of the Ced-3 subfamily (Caspases 2, 3, 7, 10, and 14) are largely involved in apoptosis (Schulz et al., 1999).

Pro-apoptotic and anti-apoptotic proteins such as the caspases, the Bcl-2 family, and inhibitor of apoptosis protein (IAP) family members are constitutively expressed or induced in ischemic tissue. It has also been shown that caspase inhibition (Loddick et al., 1996) or enhanced expression of Bcl-2 and NAIP (Neuronal apoptosis inhibitory protein) reduce injury in experimental stroke. Moreover, *in situ* DNA nick-end labelling (terminal dUTP nick-end labelling or TUNEL) and laddering on DNA gel electrophoresis have been reported in global and focal experimental ischemia (Schulz et al., 1999).

1.1.7.2. Molecular Ordering of the Cell Death Pathway

As already predicted from studies in C.elegans, expression of Ced-9/Bcl-2 controls the activation of Ced-3/caspases. Recent results have shown that they may do so by several different and complementary mechanisms. One key link is cytochrome C, an essential cofactor for activation of initiator caspases (e.g. caspase-9) by apoptotic protease activating factor-1 (Apaf-1). It is the current view that in mammalian cells Bcl-2 regulates the mitochondrial release of cytocrome C, the ubiquitous protein involved in the mitochondrial electron transport chain. In the cytosol, the released cytochrome C physically interacts with Apaf-1 inducing it to associate with initiator procaspases. In addition, the Bcl-2 family of proteins might also directly interact with Apaf-1. Apaf-1 can form a complex with Bcl-2 / Bcl-XL and caspases with long prodomains. Although Bcl-2 is presumed to inhibit caspase activation by acting upstream of caspases, Bcl-2 and also Bcl-XL may themselves be cleaved by caspases. Cleavage of Bcl-2 or Bcl-X_L may further activate downstream caspases and contribute to amplification of the caspase cascade (Schulz et al, 1999).

1.2. Mechanisms of injury after Optic Nerve Transection

In the first study presented here, we investigated the in vivo action of EPO in delayed neuronal degeneration. To this and we made use of a transgenic mouse line (termed tg21) constitutively expressing human EPO in the central nervous system (CNS) under the control of the human platelet-derived growth factor (PDGF) B-chain promoter (Wiessner et al., 2001) that we submitted to optic nerve transection. Note that the plasma EPO concentration and the hematocrit were identical in wild-type and tg21 animals (Wiessner et al., 2001). Optic nerve transection is a very reproducible in vivo model of CNS neuronal degeneration (Garcia-Valenzuela et al., 1994; Isenmann

et al., 1997). Ontogenetically, retinal ganglion cells (RGCs) are CNS-derived neurons that due to their accessibility outside the cranial cavity are an almost exemplary cell type to study cell death mechanisms and neuroprotection in mice (Kilic et al., 2002; 2004; 2006; 2008b).

1.2.1. The anatomy of the retinofugal system and the pattern of programmed cellular death (PCD)

The retina develops as an evagination of the telencephalic vesicles. It remains connected to the developing brain by the optic stalk that is later replaced by the optic nerve. The retina is thus a part of the CNS, and the optic nerve is a central tract myelinated by oligodendrocytes.

The RGCs represent the only projection neurons in the retina. They receive excitatory glutamatergic input from bipolar and excitatory (cholinergic) and inhibitory (GABAergic and glycinergic) input from amacrine cells. All RGCs send their axon to the optic nerve; therefore, counting optic nerve axons provides an accurate way of estimating RGC numbers. RGCs project to the primary visual centers in the brain, which are located in the thalamus and midbrain. The pattern of retinal projections varies considerably from species to species; in the rat, the vast majority of RGCs project to the superior colliculus and pretectal nuclei, with about 30% of them sending collaterals to the lateral geniculate nucleus in the thalamus (Fig. 1) (Cellerino et al., 2000).

In the past few years, much research has been devoted to the investigation of cell death mechanisms and pathways. Recent findings in the retina indicate that RGCs die by apoptosis, and developmental cell death might be regulated by the same

pathways as secondary apoptotic RGC death following optic nerve (ON) lesions (Bahr, 2000)

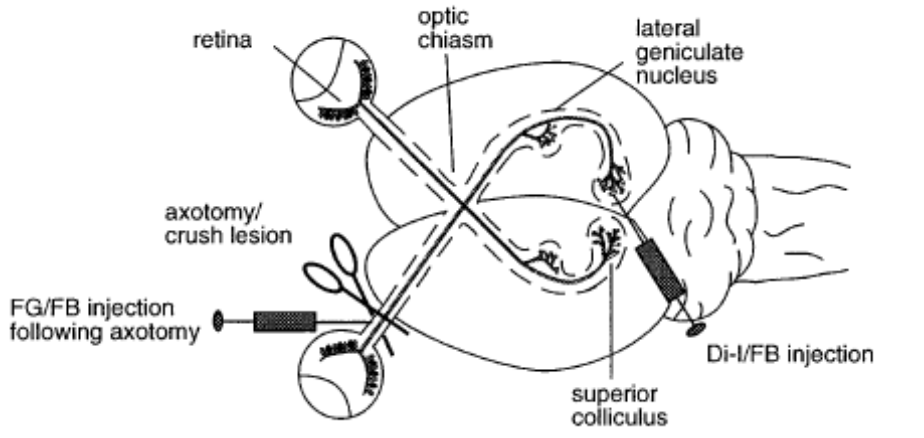

Fig.1. The retinotectal projection of the rat: anatomy, labelling techniques, and lesion paradigms. dorsal view on the rat brain. The eyes are located to the left, and the brainstem is located to the right. Retinal ganglion cells (RGCs) project through the optic nerve (ON) to the superior colliculus (SC), with some 30% of RGCs sending collaterals to the lateral geniculate nucleus. In the rat, more than 98% of RGC axons cross the midline at the optic chiasm to the contralateral side. Scissors indicate the location of ON transection or crush. RGCs can be retrogradely labeled with fluorescent dyes either from the SC or, following ON transection, from the ON stump (syringes) (FG fluorogold, FB fast blue, Di-I 1,1`-dioctadecyl-3,3,3,3`tetramethylindocarbocyanidperchlorate) (Adapted from Cellerino et al., 2000)

1.3. Erythropoietin (EPO) and its receptor (Epo-R)

Erythropoietin (EPO), a member of the cytokine type I superfamily (Ghezzi and Brines, 2004) acts primarily to stimulate erythroid cell proliferation by supporting the survival, proliferation and differentiation of erythroid progenitor cells. It`s role in the regulation of erythroid cell proliferation was first described by Carnot and Deflandre in 1906 (Marti, 2004). Its gene is located on chromosome 7 and it is a 34-kDa glycoprotein (Boron, 2003; Marti, 2004).

Under physiological conditions, endogenous EPO levels change with O_2 tension. In the presence of EPO, bone marrow erythroid precursor cells proliferate and differentiate into red blood cells (RBCs). In its absence, these cells undergo apoptosis (Kelley et al., 1994; Koury and Bondurant, 1988)

Besides local hypoxia, several hormones and other agents stimulate EPO production. For example, prostaglandin E2 and adenosine appear to stimulate EPO synthesis by increasing intracellular levels of cyclic adenosine monophosphate (cAMP). Norepinephrine and thyroid hormone also stimulate EPO release. Finally, androgens promote- whereas estrogens inhibit- EPO synthesis (Boron, 2003).

The human EPO gene was cloned in 1983 (Lin et al., 1985), allowing the clinical development of recombinant human EPO (rhEPO), a biotechnological advance that revolutionized anemia treatment. Endogenous EPO and rhEPO share the same amino acid sequence, with slight differences in the sugar profile (Skibeli et al., 2001). In clinical practice, rhEPO is typically administered as a bolus injection, and the dose is titrated to give the desired effect.

EPO acts through a tyrosine kinase-associated receptor to stimulate the production of erythroid cells in the bone marrow, as well as the development of red cells from their progenitor cells (Boron, 2003). In addition to hematopoietic cells, expression of the EPO receptor (EPO-R) and EPO response are observed in other cell types including endothelial and neural cells (Anagnostou et al., 1990, 1994; Morishita et al., 1997; Assandri et al. 1999; Jelkmann and Wagner, 2004).

EPO-R is a type I cytokine receptor with a single transmembrane domain that lacks an intrinsic tyrosine kinase domain. In the absence of EPO, EPO-R can form a

symmetric inactive homodimer complex on the cell surface mediated largely by the EPO-R transmembrane domains while the cytoplasmic domains can associate with the Janus family tyrosine kinase protein JAK2 (Livnah et al., 1999). EPO acts by binding to the EPO-R homodimer. Upon binding, EPO changes the conformation of the EPO-R, activates phosphorylation activity of JAK2 and phosphorylation of JAK2 and EPO-R, leading to signal transduction involving STAT5, PI3 kinase, MAPK and other signaling molecules (Quelle et al., 1996; Zhao et al., 2006). Neural cells exhibit activation of the JAK2/STAT5 and other signal transduction pathways as well as activation of NF- κB signaling pathways (Digicaylioglu and Lipton, 2001; Chong et al., 2002). The multi-tissue distribution of EPO-R and EPO response suggesst that rather than being instructive for erythroid differentiation, EPO may function more generally to provide proliferative and protective survival activity during erythropoiesis and contribute to other select tissue development, maintenance and/or repair.

Mice with targeted deletion of EPO or EPO-R lack definitive erythropoiesis and mature erythrocytes, and die in utero (Wu et al., 1995; Lin et al., 1996). These mice also exhibit increased apoptosis in brain prior to the severe anemia, raising the possibility that beyond EPO requirement for production of mature red blood cells, EPO signaling may contribute to normal brain development (Yu et al., 2002; Tsai et al., 2006). In addition to EPO production in fetal liver and adult kidney, EPO is produced in brain, in astrocytes and neurons (Masuda et al., 1994; Digicaylioglu 1995; Marti et al., 1996; Bernaudin et al., 1999).

Expression of EPO-R in brain has been observed during development and adulthood in mammals, including human (Digicaylioglu et al., 1995; Marti et al., 1996; Liu et al., 1997; Juul et al., 1998; Knabe et al., 2004; David et al., 2002). In the developing

mouse brain, EPO-R localizes to the neural tube in the neuroepithelium that contains proliferating neuroprecursors (Tsai et al., 2006; Liu et al., 1997). The high level of EPO-R expression in the neural tube at E10.5 is comparable to that of adult hematopoietic tissue. Brain expression then progressively decreases with intrauterine development and persists at low levels through adulthood (Liu et al., 1997). Direct binding of I^{125} labeled EPO localized EPO binding sites to specific adult brain regions including the hippocampus, cortex and midbrain in mouse, and EPO-R expression in adult brain was detected in human in analogous regions and in monkey (Marti et al., 1996; Digicaylioglu et al., 1995). EPO-R expression in brain localizes to various cell types such as mesenchephalic (midbrain), hippocampal and cortical neurons, astrocytes, oligodendrocytes and brain capillary endothelial cells (Morishita et al., 1997; Nagai et al., 2001; Yamaji et al., 1996). Expression of EPO-R in brain particularly in the embryonic stage suggests a role for EPO in brain development and tissue maintenance.

EPO expression in fetal liver and adult kidney is hypoxia inducible and is regulated *via* the hypoxia response element in the 3` region of the EPO gene, and reporter genes exhibit transactivation by the dimeric hypoxia-inducible factor-1 (HIF-1) (Fandrey et al., 2006). EPO expression is detected in human embryonic brain and biopsies from human adult hippocampus, amygdala and temporal cortex, and in various areas in brain of monkey. Hypoxia induction of endogenous brain EPO is also observed in monkey and mouse exposed to reduced pO_2 (8% O_2) (Marti et al., 1996; Prass et al., 2003). Hypoxia induction of EPO in brain persists for up to 24 h or more (Chikuma et al., 2000). Analogous to its activity in erythroid progenitor cells, EPO can stimulate the survival and proliferation of neural progenitor cells (Shingo et al., 2001) EPO neuroprotection in animal models of brain ischemia/trauma raise the possibility for EPO therapy in brain injury/disease.EPO-R is expressed in progenitor

cells from hematopoietic, endothelial, skeletal muscle and neural tissues (Anagnostou et al., 1994; Jelkmann et al., 2004; Marti et al., 2004; Shingo et al., 2001). While EPO-R expression is down regulated during differentiation of erythroid cells and myoblasts and is not expressed on mature red blood cells or skeletal muscle, EPO-R expression persists in select vascular endothelial cells and mature neurons (Noguchi et al., 2007). Increasing evidence from *in vitro* cell studies and from *in vivo* animal models suggests that EPO stimulation of progenitor cell proliferation and/or prevention of apoptosis may be useful in selective ischemic or stress events in other tissues or neurodegenerative diseases (Jelkmann et al 2004; Marti et al., 2004).

1.4. Erythropoietin: a neuroprotective molecule

Besides its role in red blood cell propagation, the glycoprotein erythropoietin (EPO) exerts neuroprotective functions in various animal models, in which neurons are lost (Gassmann et al., 2003). As such, several rodent studies have shown that EPO protects against focal cerebral ischemia and concussive brain trauma (Brines et al., 2000; Siren et al., 2001), as well as against light-induced photoreceptor degeneration (Grimm et al., 2005). EPO is an interesting candidate not only for stroke treatment, but also under more delayed degenerative neurological conditions, such as amyotrophic lateral sclerosis, Parkinson's and Alzheimer's disease. In view that EPO is routinely administered in anaemic patients with renal failure (Hudson et al., 2002; Macdougall et al., 2003), this drug is considered to be safe in humans (Grimm et al., 2005).

The molecular mechanisms underlying EPO-mediated inhibition of apoptosis have been the subject of intense study. In vitro studies using primary motor neurons and

neuronal-glial cultures submitted to serum withdrawal or treated with kainate provided further insight into the neuroprotective action of EPO. In vitro, EPO activates Janus-tyrosine kinase (JAK)-2, which is linked with the EPO receptor via its Box-1 motif (for review, see ref. Chong et al., 2002) (Fig. 2). JAK-2 in turn phosphorylates and thereby activates downstream signaling factors, such as the mitogen-activated protein (MAP) kinases extracellular-regulated kinase (ERK)-1/-2 (Siren et al., 2001), phosphatidyl inositol 3 kinase (PI3K)/Akt (Siren et al., 2001, Chong et al., 2003), Jun kinase (JNK) (Nagata et al., 1998; Jacobs-Helber et al., 2000), and signal transducers and activators of transcription (STAT)-5 (Siren et al., 2001; Ihle, 1995) (Fig. 2). STAT-5 then stimulates mitochondrial anti-apoptotic proteins, such as Bcl-XL (Chong et al., 2003), and consecutively inhibits cytochrome C-dependent caspases (Chong et al., 2003a, b). Cultures of cerebrocortical neurons show that EPO activation of JAK2 also results in IκB (inhibitor of NF-κB) phosphorylation and translocation of NF-κB (p65 subunit) to the nucleus allowing activation of NF-κB dependent neuroprotective genes, and that suppression of IκB blocks EPO neuroprotection (Digicaylioglu et al., 2001). As in erythroid progenitor cells, EPO binding to its receptor on neuronal cells stimulates JAK2 and activates a number of signal transduction pathways that promote cell survival (Fig. 2). In addition, it has been shown that the antiapoptotic genes XIAP, c-IAP2, and Bcl-XL are upregulated in the brain in animal studies of EPO infusion (Digicaylioglu and Lipton 2001; Wen et al., 2002; Elliott et al., 2003) (Fig. 2).

In in vivo studies of middle cerebral artery occlusion in rat, it was observed that EPO posttreatment significantly reduced infarct volume as well as reduced TUNEL-positive cells within the ischemic penumbra (Siren et al., 2001). The role of EPO as an antiapoptotic agent in the CNS was further elucidated by studies reporting that

administration of EPO to rats, in both pretreatment and posttreatment paradigms of models of transient global retinal ischemia yielded functional improvement over controls by electroretinography (Junk et al., 2002). Histopathologically, preservation of retinal histoarchitecture as well as a statistically significant decrease in TUNEL positivity was observed in EPO-treated animals (Junk et al., 2002). In addition to its role in inhibiting apoptosis, EPO also seems to play a role in the central nervous system in promoting neural progenitor cells and in promoting ischemic preconditioning (Noguchi et al., 2007).

One of the most important controversies that have arisen, when considering the role of EPO in the treatment of neurologic disease, is the hematopoietic effects that will elevate the hematocrit and thus, will potentially put the patients to cardiovascular risk. Researchers have attempted to address this issue with the development of EPO-like cytokines, which lack erythropoietic potential. Asialoerythropoietin, which was developed by enzymatic desialylation of rhEPO, demonstrated neuroprotective properties in models of cerebral ischemia and spinal cord injury, without causing an increase in hematocrit (Erbayraktar et al., 2003). Carbamylerythropoietin is another EPO derivative shown to be effective in stroke (Villa et al., 2007).

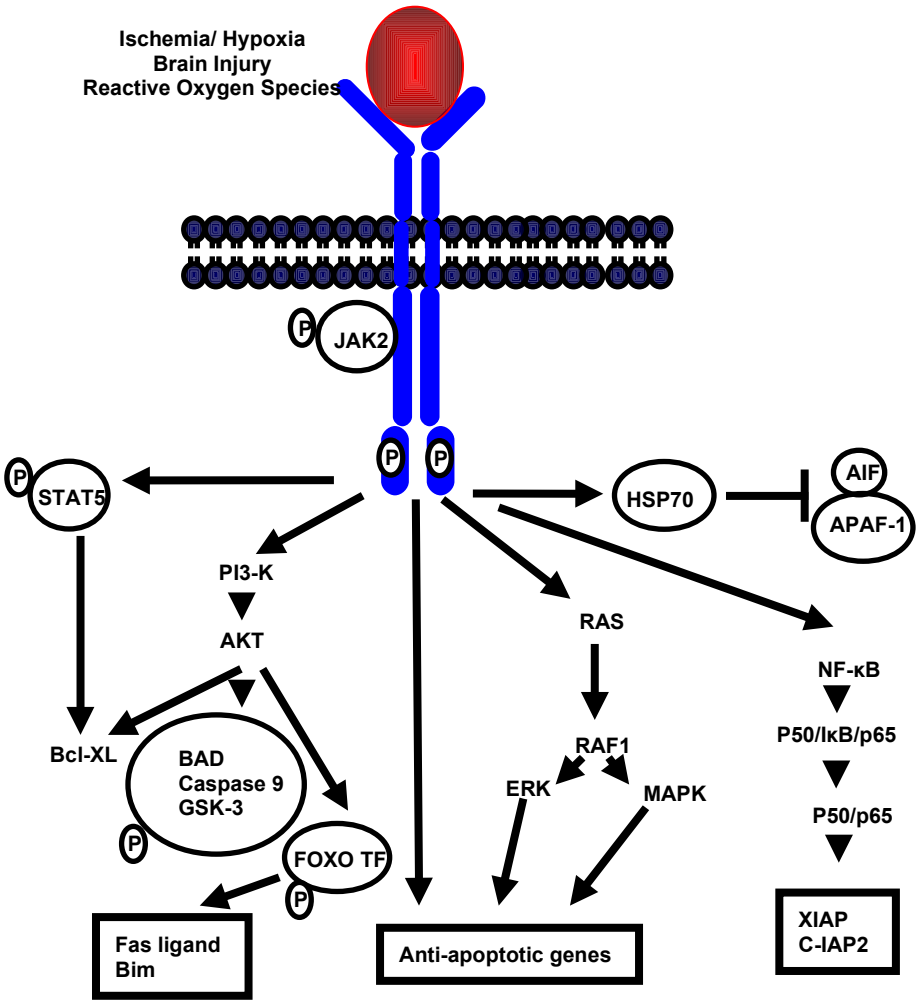

Fig.2. Signal transduction pathways of the erythropoietin receptor. Binding of erythropoietin (EPO) causes conformational changes to the EPO receptor, transphosphorylation of associated JAK2 molecules, phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor, and phosphorylation or activation of signaling molecules. Phosphorylation of signal transducers and activators of transcription (STAT) 5 transcription factor (TF) causes homodimerization, translocation to the nucleus, and activation of genes for antiapoptotic molecules. Phosphorylated phosphatidylinositol 3-kinase (PI-3 kinase) phosphorylates protein kinase B (PKB)/Akt. PKB/Akt: 1) phosphorylates and inactivates proapoptotic molecules (Bad, caspase 9 or glycogen synthase kinase-3b [GSK-3b]); 2) phosphorylates FOXO TF, inhibiting translocation to the nucleus and activation of target genes (Fas ligand, Bim); and 3) phosphorylates IkB, allowing the release of the transcription factor nuclear factor (NF)- κB that then translocates into the nucleus and activates target genes encoding antiapoptotic molecules (XIAP, c-IAP2). Binding of EPO to its receptor also activates Hsp70, which binds to and inactivates proapoptotic molecules (apoptosis protease-activating factor-1 [Apaf-1], apoptosis-inducing factor [AIF]). Activated extracellular signal regulated kinases (ERK) and mitogen-activated protein kinases (MAPK) pathways activate anti-apoptotic gene expressions (Adapted from Elliott et al., 2003).

2. MATERIALS AND METHODS

The following sections reflect three manuscripts, two of them already being accepted in the FASEB journal and one being submitted.

2.1. Project 1: Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways (Kilic E, Kilic U, Soliz U, Gassmann M, Bassetti CL, Hermann DM. (2005). FASEB J 19:2026-2028)

2.1.1. Animal surgery

Experiments were performed in accordance to the Swiss guidelines for the care and use of laboratory animals and approved by local government authorities (Kantonales Veterinäramt Zürich). Male tg21 mice, aged 8–10 weeks, expressing human EPO under control of the human PDGF B-chain promoter (*Hind*III–*Xmn*I fragment; (Ruschitzka et al., 2000) or wild-type control animals were anesthetized with 1% halothane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0°C using a feedback-controlled heating system. Focal ischemia was induced using an intraluminal technique, as described previously (Kilic et al., 2002; Wang et al., 2005), using a 8-0 silicon-coated (Xantopren: Bayer Dental, Osaka, Japan) nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany). During the experiments, laser Doppler flow (LDF) was monitored with a flexible fiber optic probe (Perimed, Stockholm, Sweden) attached to the intact skull overlying the MCA territory. LDF changes were measured during ischemia and up to 30 min after reperfusion onset. At that time, anesthesia was discontinued. After 24 h (90 min MCA occlusion; *n*=5 animals/group; Kilic et al., 2002) or 72 h (30 min ischemia; *n*=6 animals/group; Hermann et al., 2001a) of reperfusion, neurological deficits were

evaluated using a five-point neurological deficit score ranging from 0 = normal function to 4 = absence of spontaneous motor activity. Animals were then deeply anesthetized and decapitated. Brains were quickly removed and frozen on dry ice. Coronal 18 µm sections were prepared on a cryostat.

2.1.2. Analysis of histological injury

Cresyl violet staining

Brain sections from five equidistant rostrocaudal brain levels, 2 mm apart, were stained. Sections were digitized, and the border between infarcted and noninfarcted tissue was outlined using an image analysis system (Image J; National Institute of Health, Bethesda, MD). The area of infarction, the infarct volume, and brain swelling on these sections were analyzed.

Terminal transferase biotinylated-dUTP nick end labeling (TUNEL)

Adjacent sections were fixed with 4% paraformaldehyde/0.1 *M* phosphate-buffered saline (PBS). After labeling with terminal desoxynucleotidyl transferase (TDT) mix, containing 12.5 mg/ml TDT (Boehringer-Mannheim, Mannheim, Germany) and 25 mg/ml biotinylated dUTP (Boehringer-Mannheim), sections were incubated with streptavidin-fluorescein isothiocyanate (FITC), counterstained with 4',6-diaminido-2-phenylidole (DAPI) and coverslipped. Sections were evaluated by one of the examiners blinded for the experimental condition by counting DNA-fragmented cells in a total of six regions of interest of the striatum, each measuring 62,500 μm^2, as described previously (Hermann et al., 2001b). Mean values were calculated for all regions.

2.1.3. Brain angiograms

In additional animals (*n*=3/mouse line), brain angiograms were prepared in anesthetized mice by injecting black latex (38°C) through a PE10 catheter into the animals' aorta (0.3 ml/animal over 20 s) (Wang et al., 2005; Maeda et al., 1999). Ten minutes later, animals were decapitated and brains were removed. Photographs were taken of the dorsal brain surfaces, which allowed localization of the anastomotic lines between the MCA and anterior cerebral artery territories, as previously reported (Wang et al., 2005). The distance from midline to the line of anastomosis was measured at the levels of the striatum and thalamus (i.e., 4 and 6 mm from frontal brain pole). This procedure allowed us to evaluate the size of the MCA territory.

2.1.4. Radio immunoassay of tissue EPO levels

Further animals (*n*=3/mouse line) were used for determination of EPO brain levels using an ^{125}IEPO- based radioimmunoassay (RIA) (Amersham, Zurich, Switzerland), according to previously published protocols (Wenger et al., 1998). Shortly, tissue samples from the animals' striatum and overlying cortex were obtained from the MCA territory, which were homogenized in a lysis buffer (containing 150 mM NaCl; 10 mM EDTA; 50 mM Tris-HCl; 0.25% Triton; 0.1% NP-40 and protein inhibitors) and centrifuged at 15,000 rpm for 25 min. Total protein concentrations were calculated using a bicinchoninic acid (BCA) protein assay (pierce) reagent. The lower detection limit of our RIA was 4 U/l, the intrassay/interassay variances <2% and <6%. All EPO measurements were performed in duplicate. Mean values were calculated for both measurements.

2.1.5. Immunohistochemistry for EPO receptor, NO synthase (NOS)-1/-2, and activated caspase-3

Brain sections were fixed in 4% paraformaldehyde/0.1 *M* PBS, washed, and immersed for 1 h in 0.1 *M* PBS containing 0.3% triton (PBS-T)/10% normal goat serum (NGS). Sections were incubated overnight at 4°C with polyclonal rabbit anti-EPO receptor (H-194, sc-5624; Santa Cruz, Nunningen, Switzerland), anti-NOS-1 (nNOS, sc-1025; Santa Cruz), anti-NOS-2 (iNOS, sc-650; Santa Cruz) or antiactivated caspase-3 (CM-1; BD Biosciences, Basel, Switzerland) antibodies, diluted 1:100 in PBS-T, and subsequently stained with a Cy-3 labeled secondary antibody. Counterstainings were performed with a mouse antibody directed against the neuronal marker NeuN (MAB377, Chemikon, Lucerne, Switzerland; 1:500) that was detected with FITC. Sections were finally incubated with DAPI and coverslipped. Brain sections were evaluated under a fluorescence microscope using appropriate filters. Expression levels were also analyzed in a blinded manner by counting immunopositive cells in six regions of the striatum, as described above (see TUNEL staining), as well as, in case of EPO receptor, in additional four regions of the parietal cortex (size of sampled areas: 62,500 μm^2). Mean values were calculated for brain regions.

2.1.6. Western blots for cell signaling factors and Bcl-XL

Tissue samples harvested from the ipsilateral (ischemic) and contralateral (nonischemic) striatum of wild-type and tg21 mice subjected to 30 min of MCA occlusion were complemented with lysis buffer, homogenized, and centrifuged. Supernatants were used for sodium dodecylsulfate- PAGE (SDS-PAGE). Before processing, samples from animals belonging to the same experimental group were

pooled (*n*=6/group). After SDS-PAGE, proteins were transferred onto PVDF membranes. Membranes were dried overnight, incubated in blocking solution and immersed with rabbit anti-phospho-JAK-2 (3771; Cell Signaling, Allschwil, Switzerland), rabbit anti-total (=detecting both the phosphorylated and unphosphorylated forms) Akt (9272; Cell Signaling), rabbit anti-phospho-Akt (9271; Cell Signaling), rabbit anti-total extracellularregulated kinase (ERK)-1/-2 (9102; Cell Signaling), mouse anti-phospho-ERK-1/-2 (M8159; Sigma, Deisenhofen, Germany), rabbit anti-total Jun kinase (JNK)-1/-2 (JNK-2, sc-572; Santa Cruz), rabbit anti-phospho-JNK-1/-2 (9251; Cell Signaling) or rabbit anti-Bcl-XL (610212; BD Biosciences) antibody, each diluted 1:500 in 0.1% Tween 20/ 0.1 *M* Tris-buffered saline (TBS). Membranes were rinsed, incubated in peroxidase-coupled secondary antibodies, diluted 1:2000 in 0.1% Tween 20/0.1 M TBS, washed, immersed in enhanced chemoluminescence (ECL) solution and exposed to ECL-Hyperfilm (Amersham, Braunschweig, Germany). Protein loading was controlled using a monoclonal mouse antibody against anti-β-actin (A5316; Sigma). Blots were performed at least 3 times in order to confirm data reproducibility. Protein levels were analyzed densitometrically, corrected with values determined on anti-β-actin blots and expressed as relative values compared with wild-type mice.

2.1.7. Inhibition of ERK-1/-2 and phosphatidyl inositol-3 kinase (PI3K)/Akt pathways

By means of a glass microelectrode with a tip outer diameter of 50 µm, 2 µl of *1*) 100% DMSO (DMSO), *2*) the mitogen activated protein (MAP) kinase/ERK kinase-1 (MEK-1) inhibitor PD98059 (0.2 mM; New England Biolabs GmbH, Schwalbach, Germany), dissolved in 100% DMSO or *3*) the PI3K/Akt inhibitor wortmannin (0.1

mM; Sigma, St. Louis, MO), dissolved in 100% DMSO, was carefully injected into the intracerebroventricular space (*n*=6 tg21 animals/ group). Thirty minutes later, animals were submitted to MCA occlusions for 30 min, according to the same procedure as described above. Seventy-two hours later, animals were deeply reanesthetized and killed. Brains were removed, frozen on dry ice, and cut on a cryostat into 18- µm sections. TUNEL and immunohistochemical stainings for NOS-1 and -2 were then prepared.

2.1.8. Data analysis and statistics

Data were analyzed by two-tailed *t* tests (comparisons between 2 groups), one-way ANOVA, followed by LSD tests (comparisons between ≥3 groups) using SPSS for Windows 10.1. Results are presented as means ± SD values; *n* values indicate the number of different samples analyzed. *P* values less than 0.05 were considered significant.

2.2. Project 2: Erythropoietin protects from axotomy-induced degeneration of retinal ganglion cells by activating ERK-1/-2 (Kilic U, Kilic E, Soliz J, Gassmann M, Bassetti CL, Hermann DM. (2005). FASEB J 19: 249-251)

2.2.1. Retrograde labeling of RGCs

Experiments were performed in accordance to the Swiss guidelines for the care and use of laboratory animals and approved by local government authorities (Kantonales Veterinäramt Zürich). Male tg21 mice, aged 12 wk, expressing human EPO under control of a human PDGF B chain promoter (*Hin*dIII–*Xmn*I fragment; see refs. Wiessner et al., 2001; Kilic et al., 2002) or wild-type control animals were anesthetized with 1% halothane (30% O_2, remainder N_2O) and placed in a stereotactic frame. A bur hole was drilled into the pericranium overlying the superior colliculi (0.7 mm lateral to sagittal suture, 3 mm posterior to bregma). For retrograde labeling of RGCs, a Hamilton syringe was inserted 2.0 mm beneath the brain surface, and 0.7 µl of fluorogold (Fluorochrome, Denver, CO) were injected stereotactically into both superior colliculi (infusion rate 0.7 µl/min). After infusion, the injection needle remained inside the brain for 2 min to prevent fluorogold diffusion along the needle track. The syringe was then withdrawn, and wounds were carefully closed with a suture.

2.2.2. Optic nerve transection

Three days after axotomy, mice were reanesthetized with 1% halothane. After skin incision close to the superior orbital rim, the right orbita was opened, leaving the supraorbital vein intact, and the lacrimal gland was resected subtotally. After spreading the superior extraocular muscles, the right optic nerve was transected

under microscopical control ~0.5 mm distant to the posterior pole of the eye, taking care not to damage retinal blood vessels. The wounds were sutured, and retinal blood supply was verified by fundoscopy.

2.2.3. Tissue processing

Fourteen days after axotomy, mice were killed by an overdose of chloral hydrate, and both eyes were removed. Retinae were dissected, flat-mounted on glass slides, and fixed in 4% paraformaldehyde (PFA)/0.1 M phosphate buffered saline (PBS) for 30 min (*n*=5–6 animals/strain). Retinal flat mounts were evaluated by one of the investigators blinded for the experimental conditions by fluorescence microscopy using appropriate filters. RGC densities were determined by counting tracer-labeled RGCs in 12 distinct areas of 62,500 μm^2 each (three areas per retinal quadrant at 1/6, 3/6, and 5/6 of the retinal radius, respectively) both on the lesioned and on the contralateral nonlesioned side, subsequently calculating mean values over the three eccentricities analyzed as well as the whole retina. Additional retinae were used for Western blots (*n*=5 animals/strain).

2.2.4. Western blots

Tissue samples were dissected, complemented with lysis buffer, homogenized, and centrifuged, and supernatants were used for sodium dodecylsulfate-PAGE (SDS-PAGE). Blots were carried out with protein samples pooled from all animals belonging to the same group. Equal amounts of protein were diluted in 6X sample buffer, boiled, and loaded on polyacrylamide gels. Proteins were then transferred onto PVDF membranes. Membranes were dried overnight, incubated in blocking solution, and immersed with monoclonal mouse anti-diphospho-ERK-1/-2 (M8159;

Sigma, St. Louis, MO), polyclonal rabbit anti-phospho Akt (No. 9271; Cell Signaling, Allschwil, Switzerland), monoclonal mouse anti-diphospho-JNK (J4750; Sigma), polyclonal rabbit anti- JAK-2 (C20, sc-294; Santa Cruz, Nunningen, Switzerland), polyclonal rabbit anti-phospho- STAT-5 (No. 9351; Cell Signaling), polyclonal rabbit anti-Bcl-X (No. 610212; BD Biosciences, Basle, Switzerland), or polyclonal rabbit anti-activated caspase-3 (CM1; BD Biosciences), each diluted 1:500 in 0.1% Tween-20 in 0.1 M Tris-buffered saline (TBS). Membranes were rinsed, incubated in peroxidase-coupled, secondary antibodies (Santa Cruz), diluted 1:2000 in 0.1% Tween-20 in 0.1 M TBS, washed, immersed in enhanced chemoluminescence (ECL) solution, and exposed to ECL-Hyperfilm (Amersham, Braunschweig, Germany). Protein loading was controlled in additional blots using a monoclonal mouse antibody against anti-β-actin (A5316; Sigma). All blots were performed at least three times in order to confirm the reproducibility of the data. Protein levels were analyzed densitometrically, corrected with values determined on anti-β-actin blots, and finally expressed as relative values compared with nonlesioned retinae of wild-type mice.

2.2.5. Inhibition of ERK-1/2 and phosphatidyl inositol-3′ kinase (PI3K)/Akt pathways

By means of a glass microelectrode with a tip outer diameter of 50 µm, 0.5 µl of either 1) 100% DMSO; 2) the MAPK/ERK kinase-1 (MEK-1) inhibitor PD98059 (50 mM; New England Biolabs GmbH, Schwalbach, Germany), dissolved in 100% DMSO; or 3) the phosphatidyl inositol-3′ kinase (PI3K)/Akt inhibitor Wortmannin (0.1 mM; Sigma), dissolved in 100% DMSO, was carefully injected into the vitreous space, puncturing the eye at the cornea-sclera junction (n=3–4 tg21 animals/group). The injections were done at days 0, 4, 7, and 10 after optic nerve transection

following exactly the same protocol as described above. This procedure has previously been used successfully with the same signal inhibitors at identical concentrations in order to characterize signal tranduction pathways mediating neuroprotection in RGCs (Wenger et al., 1998; Digicaylioglu et al., 1995). Animals were killed 14 days after axotomy, and their retinae were removed, flat-mounted, and evaluated microscopically. In control experiments, repeated injections of 0.5 µl of 1) 100% DMSO; 2) 50 mM PD98059, dissolved in 100% DMSO; or 3) 0.1 mM Wortmannin, dissolved in 100% DMSO, were made into the vitreous space of nonlesioned (wild-type) mice according to the same protocol (injections at 0, 4, 7, and 10 days; *n*=2 animals/group). Animals were killed at 14 days after the first injection. RGC density was determined as above. These control studies revealed that the manipulations and chemicals used had no effect on RGC survival (RGC density in untreated control animals: 4152±162 RGCs/mm^2; in DMSO-treated animals: 4269±297/mm^2; in DMSO/PD98059-treated animals: 4090±381/mm^2; in DMSO/Wortmannin-treated animals: 4096±272/mm^2).

2.2.6. Radioimmunoassay (RIA) of retinal EPO levels

Additional animals (*n*=3 each for wild-type and tg21 mice) were used for EPO measurements using an ^{125}IEPO-based RIA (DiaSorin, Stillwater, MN), according to previously published protocols (Marti et al., 1996). Briefly, whole retinae were homogenized in a lysis buffer (containing 150 mM NaCl; 10 mM EDTA; 50 mM Tris-HCl; 0.25% Triton; 0.1% NP-40 and protein inhibitors) and centrifuged at 15,000 rpm for 25 min. Total protein concentrations were calculated using a bicinchoninic acid (BCA) protein assay (Pierce) reagent. The lower detection limit of our RIA was 4 U/l, the intrassay/interassay variances were <2% and <6%, respectively. All EPO

measurements were performed in duplicate. Mean values were calculated for both measurements.

2.2.7. Immunohistochemical detection of EPO receptor

Additional animals (*n*=3 each for wild-type and tg21 mice) were used for EPO receptor immunohistochemistry. Eyes were removed, frozen on dry-ice, and cut on a cryostate into 20 µm sections. Sections were fixed in 4% paraformaldehyde in 0.1 M PBS, washed, and immersed for 1 h in 0.1 M PBS containing 0.3% Triton (PBS-Tin 10% normal goat serum. Sections were incubated overnight at 4°C with polyclonal rabbit anti-EPO receptor (H-194, sc-5624; Santa Cruz), diluted 1:100 in PBS-T. After washing, sections were incubated in secondary FITClabeled anti-rabbit antibody, stained with 4', 6-diamidino-2-phenylindole (DAPI), and placed on coverslips.

2.2.8. Data analysis and statistics

Data were analyzed by two-tailed *t* tests (comparisons between 2 groups) and one-way ANOVA followed by LSD tests (comparisons between ≥3 groups) using SPSS for Windows 10.1. Results are presented as means ± SD values, with *n* values indicating the number of different samples analyzed. $P<0.05$ was considered significant.

2.3. Project 3: Post-injury administration of erythropoietin promotes neuronal survival and motor recovery after mild focal cerebral ischemia in mice (Kilic U, Kilic E, Vig R, Guo Z, Andres A-C, Gassmann M and Hermann DM. In preparation)

2.3.1. Animals

Experiments were performed in accordance to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and approved by local government authorities (Kantonales Veterinäramt; ZH169/2005). Adult male C57Bl6/j mice weighing 23-25 g were randomly assigned to one of three groups that were submitted to transient focal cerebral ischemia and were treated either with vehicle, 1 or 10 U daily EPO starting 3 days after ischemia. The EPO-treatment was continued for the subsequent 4 weeks. Erythropoietin was dissolved in saline. Vehicle or EPO were administrated intracerebroventricularly by using a mini-osmotic pump (Alzet, 2004/ 0.25 µl/ hr for 4 weeks connected with brain infusion kit 3).

2.3.2. Animal surgery

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. During the experiments, cerebral blood flow was measured by laser Doppler flow (LDF) measurements using a flexible 0.5 mm fiberoptic probe (Perimed, Stockholm, Sweden), which was placed above the core of the middle cerebral artery (MCA) territory (Kilic et al., 2006). LDF measurements were monitored up to 30 min after the onset of reperfusion. Focal cerebral ischemia was induced as described (Kilic et al., 2006) using an intraluminal filament technique. A midline neck incision was made, and the left common and external carotid arteries were isolated and ligated. A microvascular clip (FE691; Aesculap, Tuttlingen, Germany) was

temporarily placed on the internal carotid artery. A 8–0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicon resin (Xantopren; Bayer Dental, Osaka, Japan; diameter of the coated thread: 180–200 µm) was introduced through a small incision in the common carotid artery and advanced 9-mm distal to the carotid bifurcation for MCA occlusion. Thirty minutes after induction of ischemia, reperfusion was initiated by withdrawal of the monofilament. Anesthesia was discontinued and animals were placed back into their cages (Kilic et al., 2006).

2.3.3. Analysis of surviving neurons and capillary density

52 days after transient MCA occlusion, animals were deeply anesthetized (1 % halothane) and decapitated. Brains were removed and frozen on dry ice. Subsequently, brains were cut on a cryostat into 18 µm coronal sections, which were used for NeuN, CD31 and Iba1 immunohistochemistry (Kilic et al., 2008a, b).

2.3.4. Immunohistochemistry for NeuN and CD31

Brain sections were fixed in 4% paraformaldehyde / 0.1 *M* PBS, washed and immersed for 1 h in 0.1 *M* PBS containing 0.3% Triton X-100 (PBS-T) / 10% normal goat serum. Sections were incubated overnight at 4°C with monoclonal mouse anti-NeuN (MAB377; Chemicon, Lucerne, Switzerland; 1:100 in PBS) which stands for neuron-specific nuclear protein. NeuN is expressed in nucleus and cell body of most neuronal cell types in rodents, chicks and humans (Mullen et al., 1992). Sections were incubated overnight at 4°C with monoclonal rat anti-mouse CD31(catalogue number 557355; BD Biosciences, diluted 1 : 100) that is constitutively expressed on vascular endothelial cells, bone morrow stem cells, platelets and most circulating leucocytes. These were detected with anti-mouse Cy3 antibody. Sections were finally

incubated with DAPI and coverslipped. Surviving neurons or CD31-positive endothelial cells were microscopically evaluated by blinded researcher who counted the density of NeuN positive neurons in the same random region of interest (ROI) as above, both ipsilateral and contralateral to the stroke. Sections were evaluated by counting the number of NeuN or CD31 in rectangular fields, measuring 500 000 mm² and 25 000 mm², respectively. Mean values were calculated for all areas. These data allowed determination of the percentage of surviving neurons for each structure (Kilic et al., 2008a).

2.3.5. Analysis of microglial activation

Microglial activation was evaluated by immunohistochemistry for ionized calcium binding adaptor protein-1 (Iba1) which was found to highly and specifically recognize ramified microglia in normal rat brain. Brain sections were fixed in 4% Paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS), washed in water and boiled in 0.01 M citrate buffer for 15 min for epitope retrieval. Sections were incubated in 0.3% hydrogen peroxide peroxidase quenching solution for 10 min, followed by serum blocking solution for 30 min. Sections were then incubated with anti-Iba1 (Wako Chemicals GmbH, Neuss, Germany) antibodies, diluted 1:100 in 5% normal goat serum in 0.3% Triton X-100/0.1 M PBS for 60 min, followed by incubation in a biotinylated secondary antibody for 10 min. Following signal detection with diaminobenzidine, sections were counterstained with hematoxyline. Iba1-positive microglia was microscopically identified based on morphological criteria. Sections were analyzed by counting Iba1-positive cells in six random region of interest each in the ischemic striatum (measuring 62,500 μm²), for which mean values were calculated (Kilic et al., 2008b).

2.3.6. Evaluation of motor recovery and behavioral deficits

For assessment of functional neurologic deficits, grip strength, RotaRod, and Elevated 0 Maze tests were used as described below.

Grip strength test

The grip strength test consists of a spring force gauge calibrated in Newton, capacity 3N (Medio-Line Spring Scale, metric, 300 g; Pesola AG, Baar, 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., 2008b). Grip strength was evaluated at right paretic forepaw of mice submitted to left sided MCA occlusion, the left nonparetic forepaw being wrapped with adhesive tape. Grip strength was evaluated five times on occasion of each test, which took place on the day prior to stroke and on days 3, 14 and 42 after ischemia. For all five measurements, mean values were calculated.

RotaRod test

The RotaRod consists of a rotating drum with a speed accelerating from 4 to 40 rpm (Ugo Basile, model 47600, Comerio, Italy; ref. 32). Maximum speed is reached after 245 s, and the time at which the animal drops off the drum is evaluated (maximum testing time: 300 s). Measurements were repeated five times on occasion of each examination, which were scheduled on the same days as grip strength tests. For all measurements, mean values were determined (Kilic et al., 2008b).

Elevated Zero Maze

The zero maze consisted of a gray plastic annular runway (width 5.5 cm, outer diameter 46 cm, 40 cm above ground level). Two opposing 90° sectors were protected by inner and outer walls of gray polyvinyl-chloride (10 cm high). Animals were placed in one of the protected sectors and observed for 10 min. Time spent in unprotected sector was analyzed only when the animal moved into this section with all four paws. This test is a standard test to analyze anxiety-related behaviour by measuring the avoidance of the unsheltered open sectors (Zörner et al., 2003).

2.3.7. Data analysis and statistics

All data are given as mean ± S.D. Differences between groups were compared by one-way ANOVA (comparisons between ≥3 groups), and repeated measurements ANOVA (comparisons with ≥2 time points). P-values <0.05 were considered to be statistically significant.

3. RESULTS

3.1. Project 1: Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways (Kilic E, Kilic U, Soliz U, Gassmann M, Bassetti CL, Hermann DM. (2005). FASEB J 19:2026-2028)

3.1.1. Human EPO is expressed in the cerebral cortex and striatum of tg21 mice

To determine whether our tg21 mouse indeed expressed human EPO in the brain, we performed RIA measurements with tissue samples obtained from the cerebral cortex and underlying striatum, which are the main structures forming the MCA territory. Compared with wild-type animals, EPO levels were elevated ~2.1-fold in tg21 mice (Fig. 3).

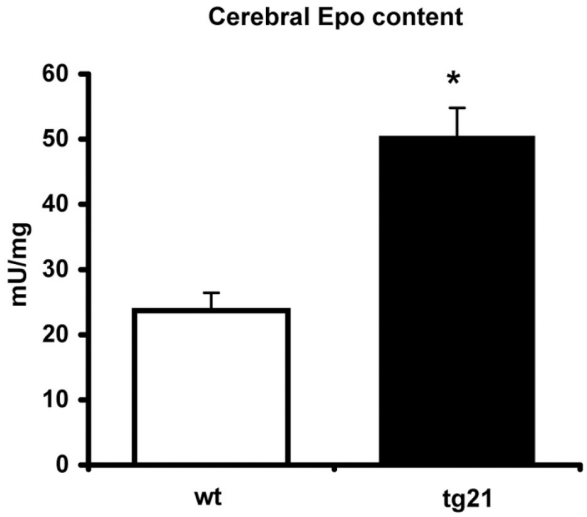

Fig.3. Human EPO is expressed in the brains of tg21 mice. Radioimmunoassays (RIA) for EPO are presented for wildtype and tg21 animals. Tissue samples examined involved both the cerebral cortex and striatum, that is, the core structures forming the middle cerebral artery (MCA) territory in mice. Note that EPO levels are increased by ~2.1-fold in tg21 compared with wild-type (wt) mice. Data are means ± SD values (n=3 animals/mouse line). *P < 0.05 compared with wildtype mice.

3.1.2. Brain neurons express the EPO receptor

To determine whether brain neurons of wild-type and tg21 mice similarly express the EPO receptor, immunohistochemical stainings were prepared. Microscopic analysis revealed a robust expression of EPO receptor on NeuN+ neurons both in the cerebral cortex and striatum (Fig. 4*A*, *B*). Expression levels of EPO receptor did not differ between wild-type and tg21 mice (Fig. 4*A*, *B*).

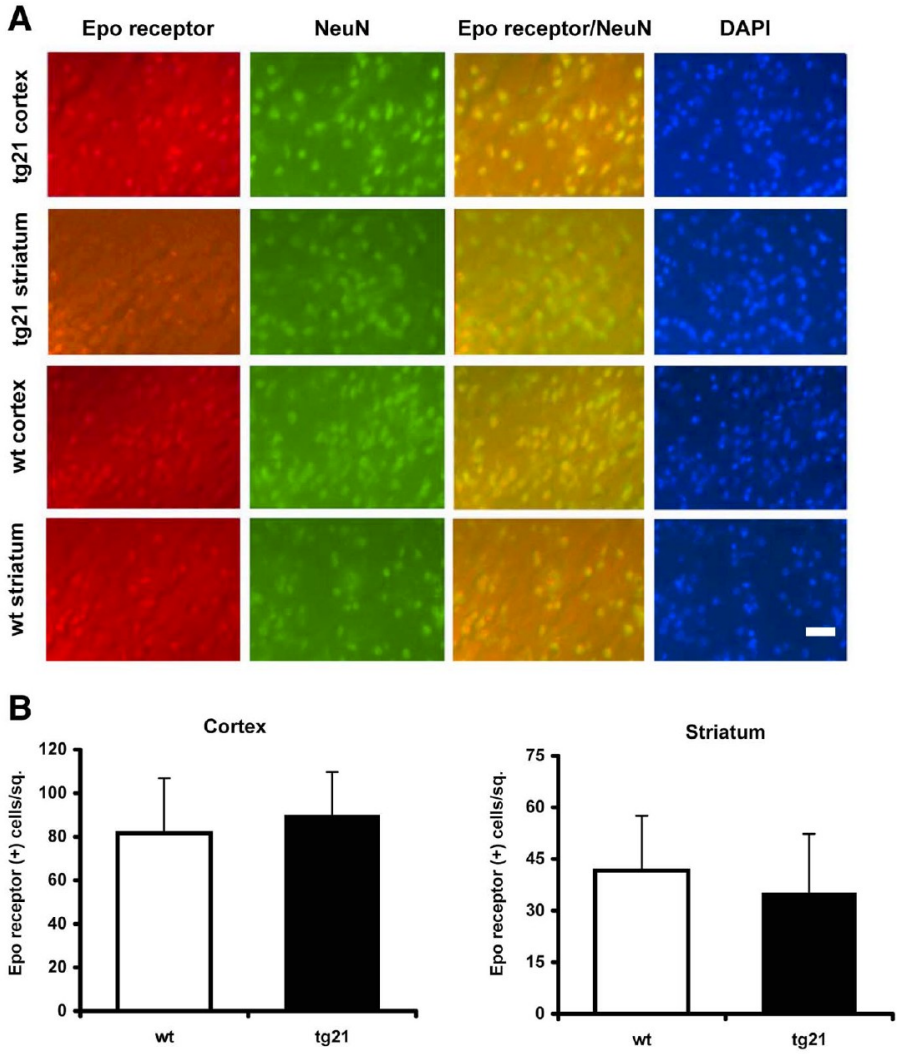

Fig.4. EPO receptor is expressed on brain neurons both in the cerebral cortex and striatum. ***A*)** Immunofluorescent double stainings for EPO receptor (red Cy-3 fluorescence) and the neuronal marker protein NeuN (green FITC fluorescence; superimposed: yellow fluorescence) are shown. Note the presence of EPO receptor on NeuN-positive neurons in both structures of wild-type (wt) and tg21 mice. ***B*)** No differences in EPO receptor staining are detectable between both mouse lines. Data are means ± SD values (*n*=4 animals/ group). Scale Bar: 200 *µ*m.

3.1.3. The EPO receptor-associated protein JAK-2 is activated by human EPO

To find out whether human EPO activates JAK-2, which binds with EPO receptor in vitro and mediates EPO receptor signaling into the cytosol (reviewed by Sirén et al. 2001), we performed Western blots for the phosphorylated, that is, activated JAK-2 protein. Increased phosphorylation levels of JAK-2 in the brains of tg21 mice (Fig. 5) indicated that JAK-2 activation took place in the presence of human EPO, thereby confirming that the EPO receptor is functional in tg21 mice.

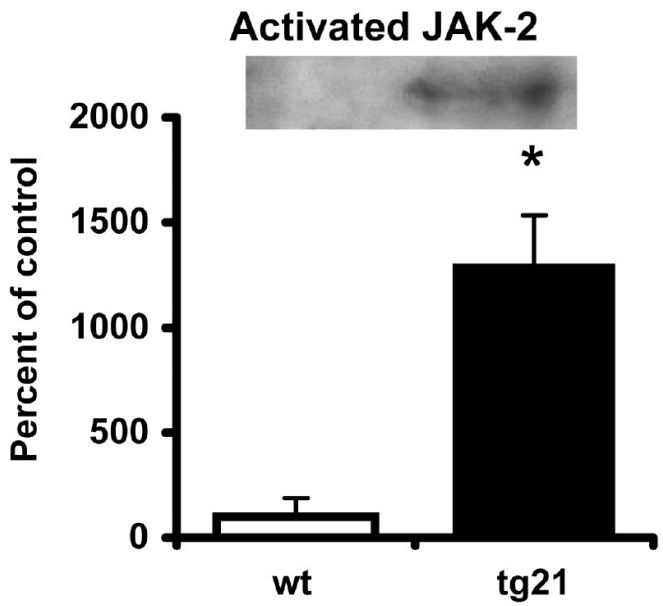

Fig.5. JAK-2 is activated by human EPO. Western blot revealing phosphorylated, that is, activated JAK-2 determined in brain tissue homogenates from the striatum of wild-type (wt) and tg21 mice after 30 min MCA occlusion. As from in vitro studies, JAK-2 is known to bind to EPO receptor, mediating EPO receptor signaling into the cytosol, our data indicate that the EPO receptor is functional in tg21 mice. Data are means ± SD values (n=3 different samples/ mouse line), normalized with corresponding blots for β-actin. *P < 0.05 compared with wild-type mice.

3.1.4. Cerebral blood flow is not influenced by human EPO

To ensure the reproducibility of ischemic treatment and to rule out hemodynamic differences between wild-type and tg21 mice, LDF was recorded above the MCA territory during ischemia and up to 30 min after reperfusion. LDF analysis did not exhibit any differences between wild-type and tg21 mice, either following 90 min (Fig. 6*A*) or 30 min (Fig. 7) of MCA occlusion. Insertion of the monofilament reproducibly resulted in a decline of blood flow to ~15–20% of preischemic levels (Fig. 6*A*, 7). Monofilament retraction was rapidly followed by a restoration of blood flow to basal values in animals submitted to 90 min MCA occlusion (Fig. 6*A*). In animals undergoing 30 min of ischemia, a short-lasting hyperperfusion response was found shortly after reperfusion onset (to ~120–140% of preischemic) (Fig. 7).

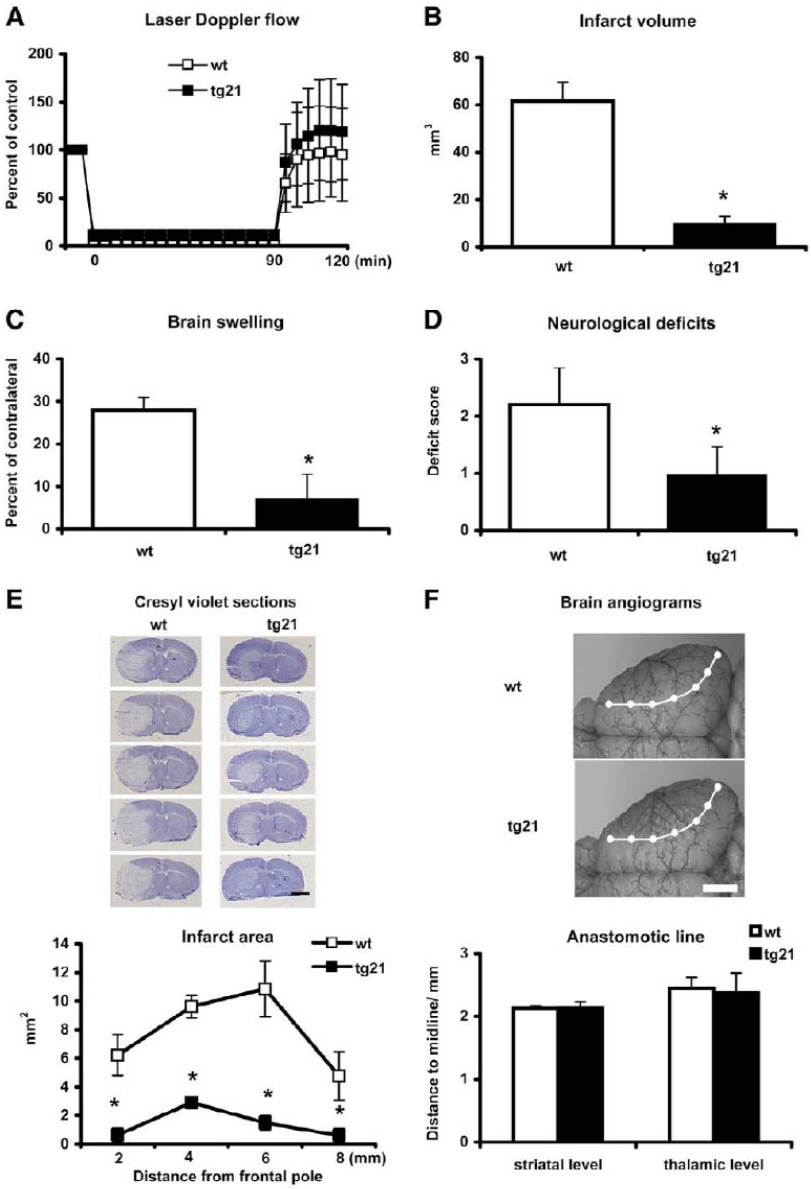

Fig.6. Human EPO protects against brain infarction following 90 min MCA occlusion. Laser Doppler flow (LDF) measurements (***A***), infarct volumes (***B***), brain swelling (***C***), and neurological deficits (***D***) are shown for wild-type (wt) and tg21 mice. Cresyl violet sections at the level of the striatum are presented for all animals (***E***), and results from vascular angiograms are also depicted (***F***). Note the remarkable reduction in infarct size by almost 85% induced by human EPO (***B***, ***E***), which is associated with reduced brain edema (***C***) and improved neurological deficits (***D***). Furthermore, note that the reduction of infarct size is neither attributed to differences in cerebral blood flow (***A***) nor to differences in the vascular anatomy of the MCA territory, reflected by the localization of the anastomotic line between the MCA and anterior cerebral artery territory, which was analyzed at the level of the mouse striatum and thalamus (***F***). Data are means ± SD values (*n*=5 animals/group (***A***–***E***) *n*=3 animals/group (***F***)). **P* < 0.05 compared with wild-type mice. Scale Bar: 1 mm.

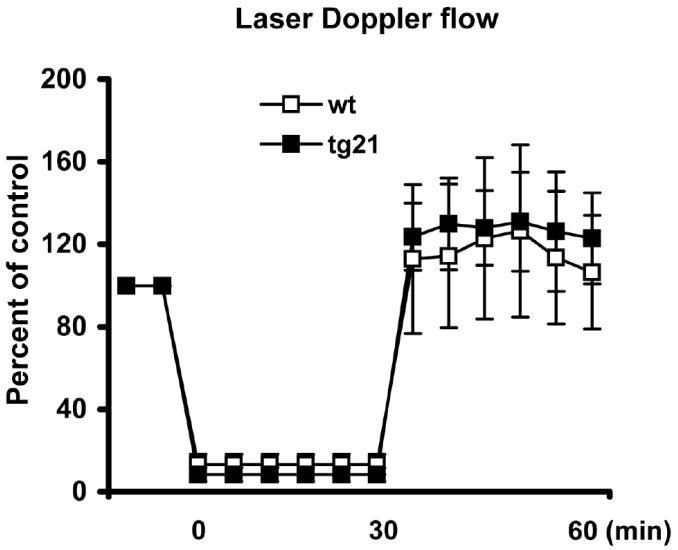

Fig.7. Human EPO does not influence cerebral blood flow after 30 min MCA occlusion. LDF recordings during ischemia and up to 30 min after reperfusion onset are shown for wild-type (wt) and tg21 mice. Data are means ± SD values (*n*=6 animals/ mouse line).

3.1.5. Neuronal expression of EPO reduces ischemic infarct volume and brain swelling, and improves neurological deficits

Brain infarcts were morphologically evaluated by cresyl violet staining at 24 h after 90 min MCA occlusion. In wild-type animals, reproducible brain infarcts were noted under these conditions (Fig. 6*B*, *E*). Infarcts always involved both cortical and striatal areas and were consistently associated with brain swelling (Fig. 6*C*) and neurological deficits (Fig. 6*D*). Human EPO reduced infarct volume by ~84% (Fig. 6*B*), drastically diminished brain edema (Fig. 6*C*), and improved the behavioral changes (Fig. 6*D*). Note that tg21 animals exhibited small striatal infarcts that almost completely spared the cerebral cortex (Fig. 6*E*). To exclude that differences in infarct size in tg21 mice were influenced by macroscopic differences of the brain vasculature, a phenomenon that exists when different mouse strains are compared (Maeda et al., 1999), latex angiograms were prepared. These angiograms allow us to outline the size of the MCA territory by tracing the anastomotic line between the MCA and anterior cerebral

artery territories (Wang et al., 2005). In our studies, the anatomical localization of the anastomotic line did not differ between wild-type and tg21 mice, indicating that MCA territories were identical in size (Fig. 6*F*). Our data confirm that the reduction of infarct size seen was indeed evoked by EPO.

3.1.6. EPO prevents disseminated neuronal injury and reduces neuronal NOS-1 and -2 expression

Whereas brain infarcts in animals undergoing 90 min MCA occlusion involved both the striatum and overlying cortex, 30 min of ischemia resulted in disseminated neuronal injury only in the striatum, but not the cortex, as analyzed by TUNEL staining (Fig. 8*A*, *B*) and activated caspase-3 immunohistochemistry (Fig. 8*A*, *C*) 3 days after reperfusion. Transgenic EPO significantly decreased the density of injured cells in the striatum (Fig. 8*A*–*C*). Neurological deficits after 30 min MCA occlusion were always mild (deficit score ≤1, irrespective of the mouse genotype) and were therefore not further evaluated. To characterize mechanisms mediating EPO's neuroprotective function, immunohistochemical stainings for NOS-1 and NOS-2 were analyzed. Microscopic analysis revealed that tissue protection by EPO was associated with decreased NOS-1 (Fig. 8*A*, *D*) and NOS-2 (Fig. 8*A*, *E*) levels in animals subjected to 30 min of ischemia. Double stainings for the neuronal marker NeuN revealed that NO synthase immunoreactivity was localized in neurons (Fig. 8*A*, *E*).

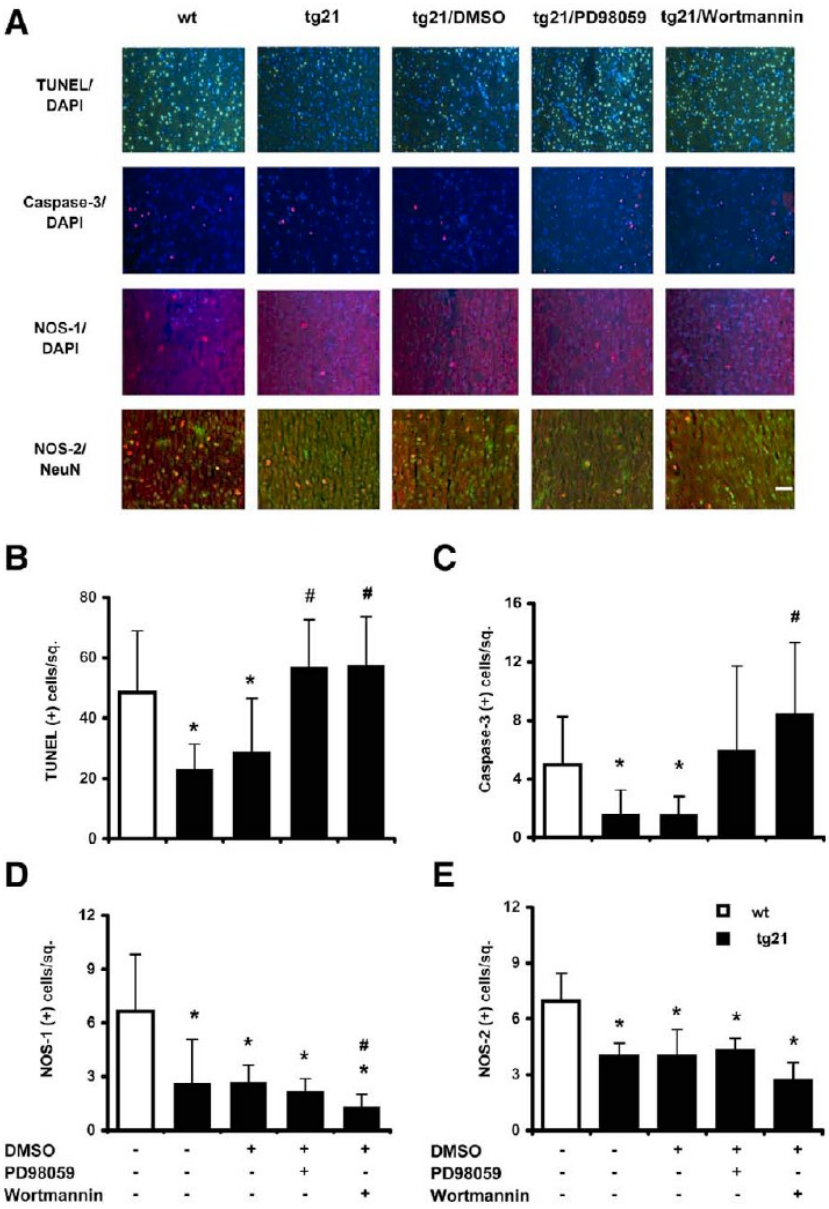

Fig.8. Human EPO reduces disseminated neuronal injury after 30 min MCA occlusion by dual activation of ERK-1/-2 and Akt. TUNEL stainings, as well as immunohistochemistries for activated caspase-3, NOS-1, and NOS-2 were evaluated in the ischemic mouse striatum of wild-type (wt) and tg21 mice (representative sections shown in ***A***). Densities of DNA fragmented (***B***), activated caspase-3+ (***C***), NOS-1+ (***D***) and NOS-2+ (***E***) cells were quantified. Animals were either untreated or received intracerebroventricular injections of the solvent DMSO, which was applied in the presence or absence of the ERK-1/-2 inhibitor PD98059 and PI3K/Akt inhibitor wortmannin. Note that human EPO reduces delayed neuronal death in the striatum (***A***, ***B***), at the same time decreasing activated caspase-3 levels (***A***, ***C***) and reducing NOS-1 (***A***, ***D***) and NOS-2 (***A***, ***E***) expression in ischemic neurons. Interestingly, antagonization of either ERK-1/-2 and Akt pathways completely abolishes tissue protection induced by EPO (***A–C***). On the other hand, inhibition of ERK-1/-2 and Akt does not reverse the inhibition of NOS-1 and -2 in tg21 mice (***A***, ***D***, ***E***), indicating that NOS-1 and -2 inhibition by EPO occurs in an ERK-1/-2 and Akt-independent manner. Fluorescence colors in ***A***: TUNEL, NeuN: green (FITC); caspase-3, NOS-1/-2: red (Cy-3); DAPI: purple; superimposed: TUNEL/DAPI: bright green, caspase-3/DAPI // NOS-1/DAPI: pink; NOS-2/NeuN: yellow. Data are means ± SD values (*n*=6 animals/group). **P* < 0.05 compared with wild-type mice; #*P* < 0.05 compared with DMSO-treated mice. Scale Bar: 200 *μ*m.

3.1.7. EPO activates ERK-1/-2, Akt, JNK and Bcl-XL signaling

To further analyze cell signaling pathways responsible for EPO's neuroprotective properties, we performed Western blots with tissue samples obtained from the mouse striatum. Blots of nonischemic tissue showed that total levels of ERK-1/-2, Akt, and JNK-1/-2 (assessed with antibodies recognizing both the phosphorylated and unphosphorylated proteins) did not differ between tg21 and wild-type animals (Fig. 9). Importantly, however, phosphorylated, i.e., activated ERK-1/-2, Akt, and JNK-1/-2 levels were higher, while Bcl-XL expression was slightly lower in tg21 than wild-type mice (Fig. 9). Upon ischemia, phosphorylated but not total levels of ERK-1/-2 and JNK-1/-2 further increased in tg21 animals but remained unchanged or decreased in wild-type mice (Fig. 9). Akt phosphorylation remained high in tg21 animals (Fig. 9). Bcl-XL expression increased above levels of wild-type mice (Fig. 9). Our data suggested a role of ERK-1/-2, Akt, and Bcl-XL pathways in EPO's neuroprotective function.

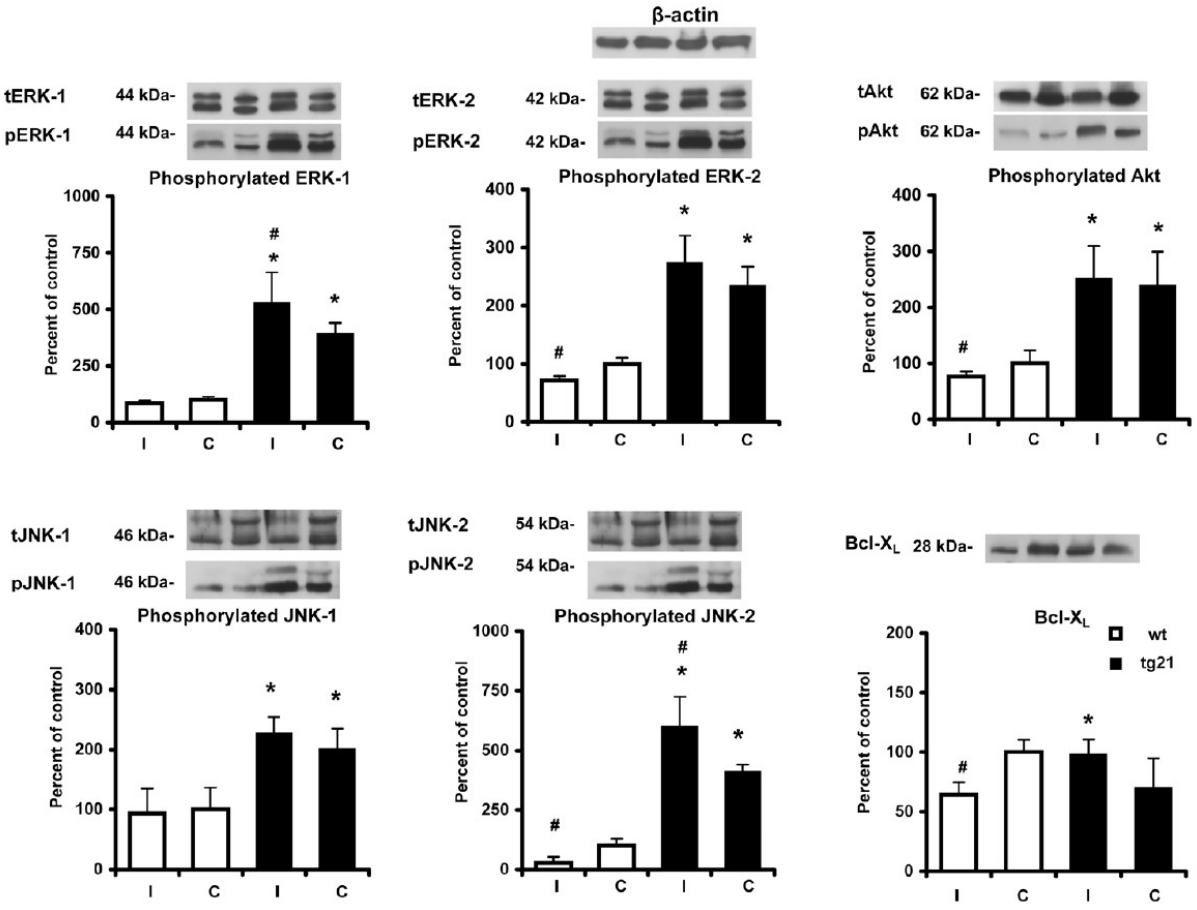

Fig.9. Human EPO elevates ERK-1/-2, Akt and JNK-1/-2 phosphorylation and stimulates Bcl-XL expression after focal cerebral ischemia, induced by 30 min MCA occlusion. Western blots with brain tissue homogenates of wild-type (wt) and tg21 mice obtained from the ischemic striatum are presented. Note that phosphorylated, but not total levels of ERK-1/-2, Akt, and JNK-1/-2, as well as Bcl-XL expression, are increased by human EPO. Our data indicate that ERK-1/-2, Akt, and Bcl-XL are involved in EPO's neuroprotective function. Data are means ± SD values (*n*=3 different samples/ group), normalized with corresponding blots for *β*-actin, which are also shown. I: ischemic; C: contralateral nonischemic. **P* < 0.05 compared with wild-type mice; #*P* < 0.05 compared with nonischemic tissue.

3.1.8. EPO's neuroprotective function in vivo requires dual activation of ERK-1/-2 and Akt

To define as whether ERK-1/-2 or Akt signaling pathways were responsible for EPO's neuroprotective function, we applied the signal transduction inhibitors PD98059 (ERK-1/-2) or wortmannin (Akt) into the intracerebroventricular space. Interestingly, both inhibitors completely abolished the tissue neuroprotection induced by EPO (Fig.

8*A*–*C*). Our data indicate that EPO's neuroprotective activity crucially depends on the dual activation of both pathways.

3.1.9. Inhibition of NO synthases by human EPO does not depend on ERK-1/-2 and Akt

To determine whether ERK-1/-2 or Akt mediates the inhibition of NOS-1 and -2 by human EPO, we also prepared immunohistochemical stainings of animals treated with PD98059 and wortmannin. In the brains examined, neither ERK-1/-2 (PD98059) nor Akt (wortmannin) inhibition restored NOS-1 and -2 levels in ischemic neurons (Fig. 8*A*, *D*, *E*). This observation indicates that the NOS-1 and -2 inhibition by EPO occurs in an ERK-1/-2- and Akt-independent manner.

3.2. Project 2: Erythropoietin protects from axotomy-induced degeneration of retinal ganglion cells by activating ERK-1/-2 (Kilic U, Kilic E, Soliz J, Gassmann M, Bassetti CL, Hermann DM. (2005). FASEB J 19: 249-251)

3.2.1. The retinae of tg21 mice express human EPO

To determine whether our tg21 mouse indeed expresses human EPO in the retina, we did RIA analysis. These experiments revealed significantly elevated EPO levels in the retinae of tg21 mice, as compared with nontransgenic wild-type animals (94.4±6.6 vs. 59.5±2.1 mU EPO/mg protein). Our data confirm that in tg21 mice, increased expression of EPO occurs not only in the brain, as previously shown (Wiessner et al., 2001), but also in the eyes.

3.2.2. EPO receptor is expressed on RGCs

Immunohistochemical analysis revealed a robust expression of EPO receptor on RGCs (Fig. 10). In contrast to EPO, expression levels of EPO receptor did not differ between wild-type and tg21 mice (Fig. 10).

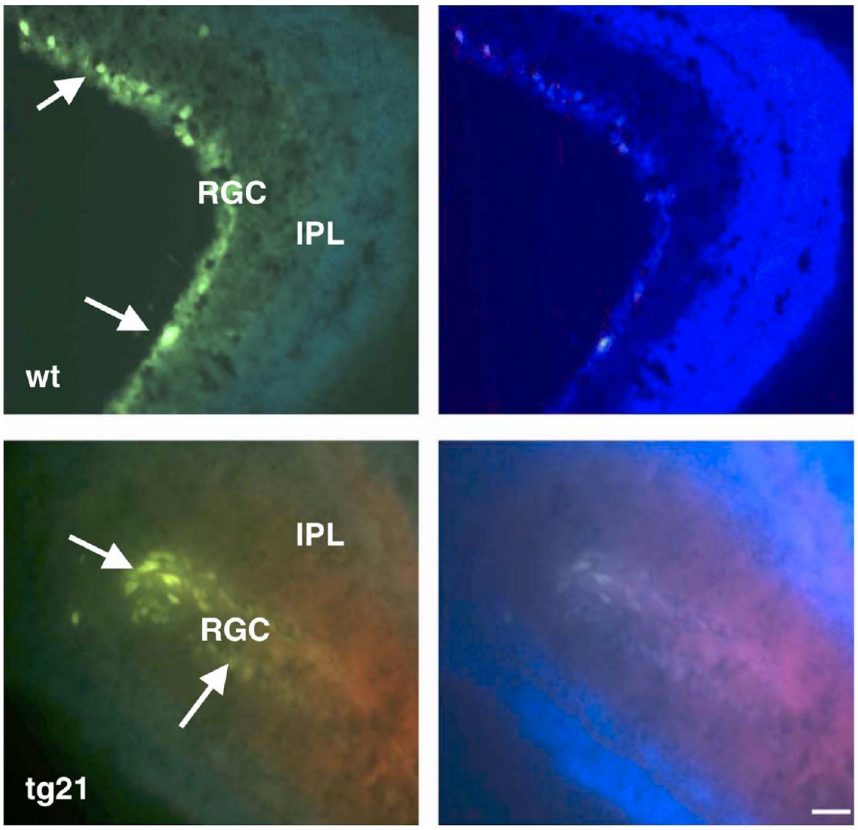

Fig.10. EPO receptor is expressed on mouse RGCs. Representative immunohistochemistries for EPO receptor of wildtype and tg21 animals (left) are contrasted with DAPI stainings (right). Note that the expression level of the EPO receptor does not differ between tg21 and wild-type mice. IPL, inner plexiform layer; RGC, retinal ganglion cell layer. Bar, 50 μm.

3.2.3. EPO protects against RGC degeneration

Stereological analysis of fluorogold-prelabeled RGCs in axotomized mice revealed that EPO markedly increased the density of surviving RGCs (see Fig. 11*A*–*C*). While only 21.3 ± 7.6% of ganglion cells were viable at 14 days after lesioning in nontransgenic wild-type mice, as many as 61.4 ± 20.6% of cells were preserved in transgenic animals ($P<0.01$). Neuroprotection was similarly seen in all retinal eccentricities, both close to the posterior pole of the eye and distant to it (Fig. 11*B*). Ganglion cell densities in nonlesioned retinae did not differ between wild-type and tg21 mice (Fig. 11*C*, 11*D*).

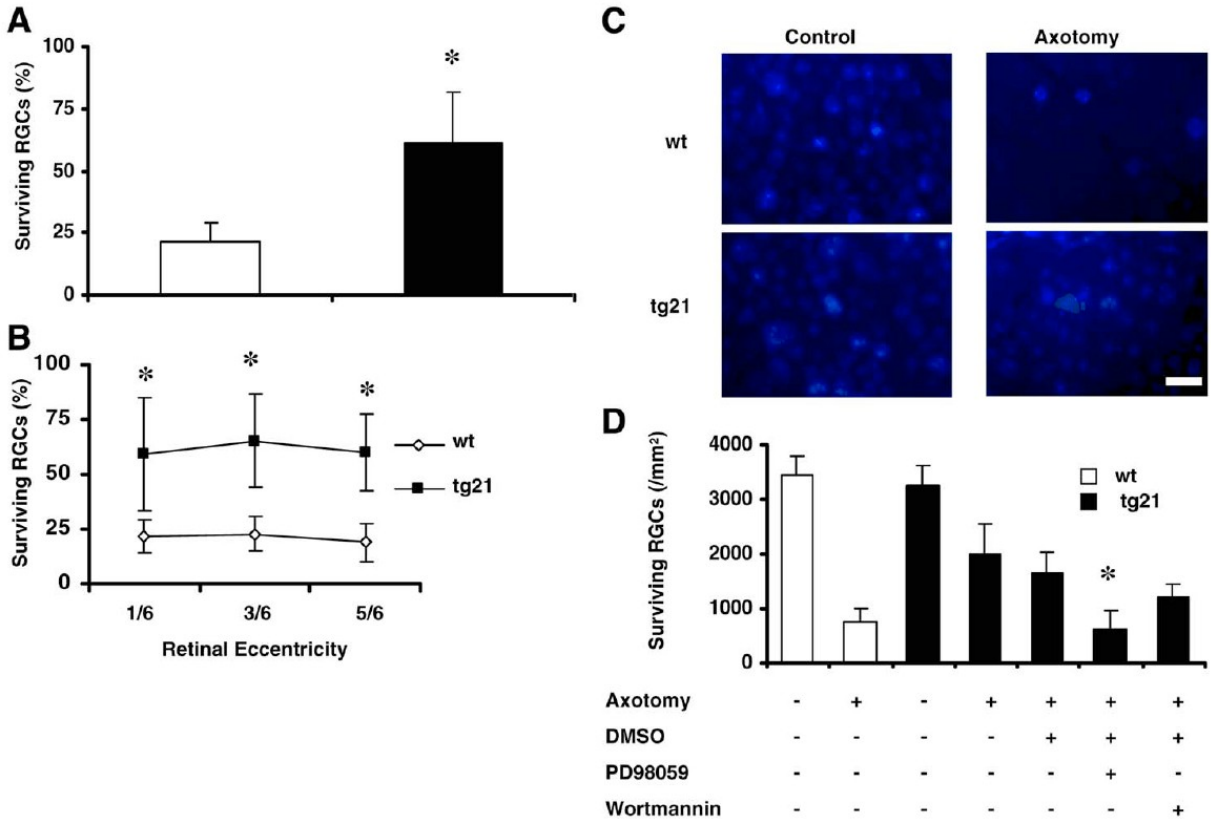

Fig.11. EPO protects against the delayed degeneration of axotomized RGCs via ERK-1/-2 signaling. Mean percentage values of surviving RGCs (as compared with the contralateral nonlesioned eye) averaged over the whole retina (*A*), mean values determined in various retinal eccentricities (1/6th, 3/6th, 5/6th) (*B*), and microphotographs of representative wild-type and tg21 mice (*C*) are shown. Absolute numbers of surviving RGCs in nonlesioned and lesioned retinae treated with the solvent DMSO and the signal transduction inhibitors PD98059 (ERK-1/-2) and Wortmannin (Akt) (*D*) are also depicted. While transgenic EPO markedly protected against RGC degeneration (*A*–*C*), only ERK-1/-2 inhibition, but not Akt inhibition, reversed EPO's neuroprotective activity (*D*). Data are mean ± SD. **P* < 0.05 compared with axotomized wild-type mice (*A*, *B*); **P* < 0.05 compared with DMSO-treated axotomized tg21 animals (*D*). Bar, 100 µm.

3.2.4. Constitutive ERK-1/-2, Akt, and STAT-5 activities are elevated in nonlesioned retinae of tg21 mice

To elucidate signaling mechanisms mediating EPO's neuroprotective activity, we performed Western blots using retinal homogenates. Blots of nonlesioned control retinae showed that activities of ERK-1/-2, Akt, and STAT-5 were higher in tg21 than in wild-type mice (Fig. 12). Constitutive JAK-2, JNK, Bcl-XL, and caspase-3 levels, on the other hand, did not differ between both mouse lines (Fig. 12).

3.2.5. ERK-1/-2 and Akt signaling are increased, while JNK and caspase-3 signaling are decreased after axotomy in tg21 mice

In tg21 mice, ERK-1/-2 phosphorylation markedly increased after RGC axotomy (Fig. 12). Akt phosphorylation, on the other hand, remained high after lesioning, while JNK activity mildly decreased (Fig. 12). In wild-type mice, optic nerve transection did not induce any signalling changes, of phosphorylated Akt or of MAP kinases (Fig. 12). Our data suggested a role of both ERK-1/-2 and Akt signaling in EPO's neuroprotective activity. Caspase-3 activity was markedly inhibited in axotomized retinae of EPO expressing, but not in those of control mice (Fig. 12), indicating that EPO signaling converged in the inhibition of the executioner caspase-3.

3.2.6. JAK-2, STAT-5, and Bcl-XL signaling are attenuated after optic nerve transection in tg21 mice

Optic nerve transection markedly reduced retinal JAK-2 and Bcl-XL levels and also mildly decreased STAT-5 phosphorylation levels in tg21 mice (Fig. 12). In nontransgenic animals, on the other hand, JAK-2 and Bcl-XL levels remained unchanged after lesioning, while STAT-5 activity markedly increased, even above levels of transgenic mice (Fig. 12). The reduced STAT-5 and Bcl-XL signaling argues against an involvement of these pathways in EPO's neuroprotective activity, at least in the experimental paradigm and mouse line used.

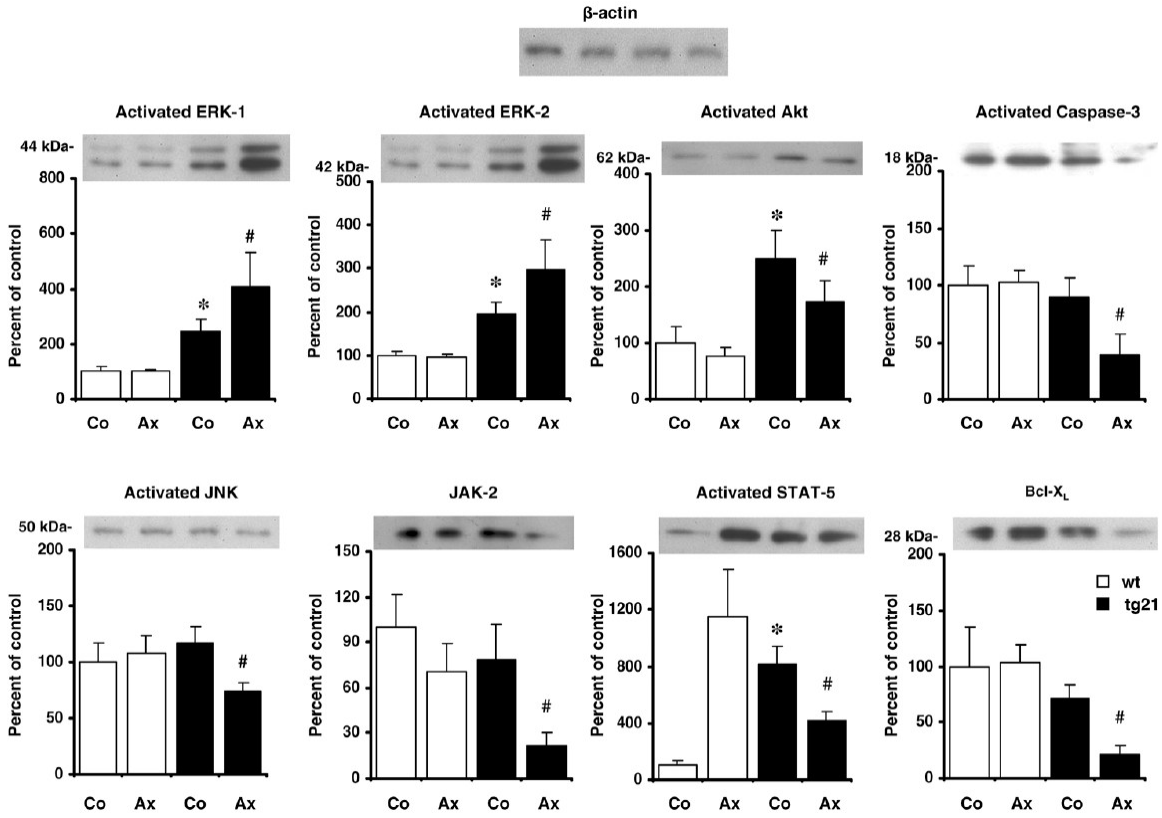

Fig.12. Effects of RGC axotomy on the expression and activation state of various signaling proteins, as determined in Western blots using homogenates of nonlesioned and lesioned retinae of wild-type and tg21 mice. Note that optic nerve transection stimulates both ERK-1/-2 and Akt phosphorylation and inhibits caspase-3 activity and JNK phosphorylation in lesioned retinae of tg21 mice, suggesting a role of these signaling factors in EPO-induced neuroprotection. Furthermore, decreased expression levels of JAK-2 and Bcl-XL and reduced phosphorylation levels of STAT-5 in RGCs of axotomized tg21 animals indicate that the corresponding pathways are not crucial for EPO's neuroprotective activity. Co, nonlesioned control eye; As, axotomized eye. Data are mean ± SD (*n*=3 different samples/group), normalized with corresponding blots for β-actin, for which a representative blot is also shown. **P* < 0.05 compared with nonlesioned wild-type mice; #*P* < 0.05 compared with axotomized tg21 mice.

3.2.7. EPO-induced neuroprotection in vivo is mediated by ERK-1/-2 signaling

To define whether ERK-1/-2 or Akt signaling pathways are responsible for EPO-induced neuroprotection, we locally applied the solvent DMSO with or without signal transduction inhibitors into the eye's vitreous space. While the PI3K/Akt inhibitor Wortmannin did not influence RGC survival to a major extent, inhibition of ERK-1/-2 using the MEK-1 inhibitor PD98059 completely abolished the protective effects of EPO (see Fig. 11*D*). Our data imply that EPO-induced neuroprotection critically depends on the activation of the ERK-1/-2, but not of the Akt pathway.

3.3. Project 3: Post-injury administration of erythropoietin promotes neuronal survival and motor recovery after mild focal cerebral ischemia in mice (Kilic U, Kilic E, Vig R, Guo Z, Andres A-C, Gassmann M and Hermann DM. In preparation)

To ensure the reproducibility of ischemiac lesions, laser dopler flow (LDF) recordings were performed above the core of the MCA territory. Transient MCA occlusion resulted in an expected decrease of LDF values to approximately 15% of the pre-ischemic control values (Fig. 13). After reperfusion, LDF values rapidly normalized (Fig. 13). No differences were found between vehicle-treated and EPO-treated animals (Fig. 13).

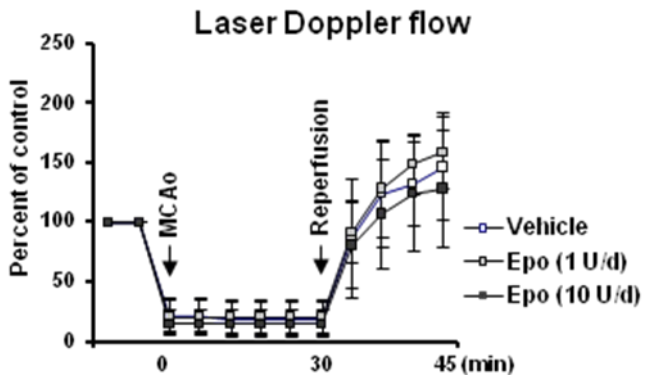

Fig.13. Erythropoietin delivered 72 h after setting a 30-min MCA occlusion. LDF during and immediately after ischemia in vehicle-treated and mice treated with EPO (1 or 10 U/day Note that LDF does not differ between animal groups. Data are mean ± S.D. (n=8 animals per group).

To investigate EPO`s effects on neuronal survival, we stained brain slides with NeuN, that identifies neuron-specific nuclear protein in most neuronal cell types. Thirty minutes of MCA occlusion resulted in disseminated neuronal injury in the striatum, but not in the overlying cortex (Fig. 14A). Interestingly, the number of surviving neurons in the striatum, as assessed by NeuN, was significantly higher in animals treated with high EPO dose than in low dose EPO or vehicle-treated animals (not shown), indicating that high doses of EPO exerted structural rescue effects even when applied 72h after injury.

To evaluate capillary density in the ischemic brain, CD31 expression was also examined by immunohistochemistry. CD31 stainings revealed that EPO stimulated angiogenesis in the ischemic striatum (Fig. 14B). No significant angiogenesis was observed in cortex and corpus callosum (data not shown).

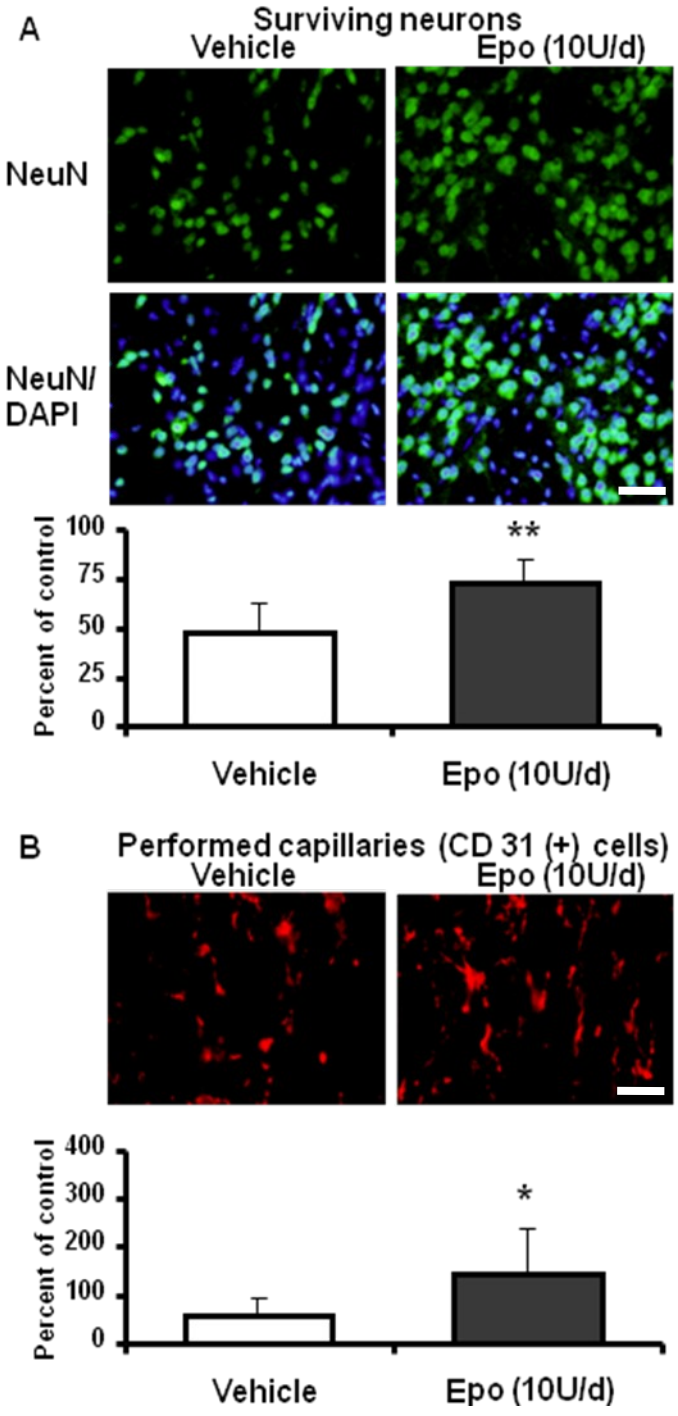

Fig.14. Erythropoietin (10 U/d), delivered starting at 72 h following 30-min MCA occlusion promotes neuronal survival and stimulates angiogenesis. Percentage of surviving neurons in the ischemic striatum, assessed by NeuN (A) and angiogenesis assessed by CD31 (B) immunohistochemistry in vehicle-treated and EPO-treated mice. Data are mean ± S.D. (n=8 animals per group). *P< 0.05; **P< 0.01 compared with vehicle treated control animals. Scale Bar: 50 µm.

To determine the influence of EPO on brain activation of microglia, immunohistochemistry for the microglia-specific Iba1 marker was performed. Iba1 is a novel calcium-binding protein and specifically expressed in microglia in the brain. Iba1 plays an important role in regulation of microglial function, especially in the activated microglia (Ito et al., 1998). It has been suggested that microglia may be rapidly and time dependently activated after ischemia, and microglial activation may reflect the extend of severity of ischemic injury (Kato et al., 1996; Rupalla et al., 1998). Significant microglial reactivity was noted in ischemic brain areas in animals subjected to 30 min of ischemia (Fig. 15). EPO treatment increased the density of Iba1-positive ramified microglia in the ischemic tissue (Fig. 15).

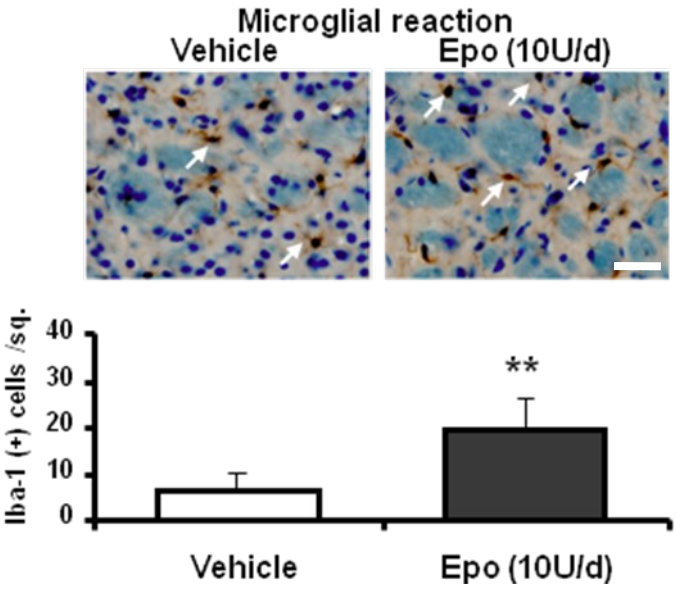

Fig.15. EPO treatment activates microglia in the stroke tissue. Density of Iba1 -positive microglia in EPOtreated animals submitted to 30 min MCA occlusion, followed by 52 days reperfusion. Representative Iba1-positive microglias in the striatum are also depicted; (counterstained with hematoxyline; labeled cells: see flashes). Data are means ± SD (n=8 animals/group). *P< 0.05 compared with vehicle treated control animals. (two-tailed t-tests). Scale Bar: 50 µm.

To elucidate how EPO influences motor recovery, grip strength, and RotaRod tests were performed. Grip strength tests performed at 7 or 14 days after stroke revealed a mild paresis of the right forelimb that was associated with coordination deficits in RotaRod tests (Fig. 16). High doses of EPO significantly enhanced motor performance as compared with low dose or non-treated animals 42 days after reperfusion (Fig. 16).

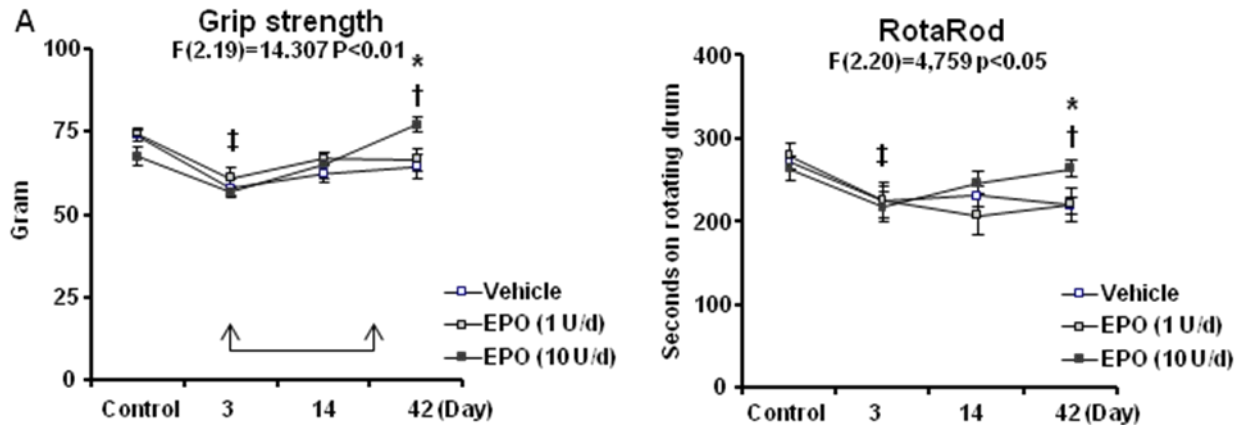

Fig.16. EPO (10 U/d) enhances motor recovery in animals submitted to 30-min MCA occlusion. Grip strength and RotaRod tests in mice prior to ischemia and at 3-, 14- and 42 day poststroke. Note the mild paresis of the right forelimb, as a consequence of left MCA occlusion revealed by grip strength tests, as well as the more pronounced coordination deficit in RotaRod tests that is almost completely reversed by high EPO dose. Data are mean ± S.D. (n=8 animals per group). Data were analyzed by repeated measurement ANOVA, for which condition x time interaction effects are shown, followed by one-way ANOVA LSD tests. ‡ P< 0.05 compared with baseline; *†P< 0.05 compared with vehicle treated control or low dose EPO treated animals, respectively.

To investigate how EPO affects anxiety, elevated 0 maze tests were evaluated. Time spent in the non-protected area was significantly lower in all groups of animals assessed on day 14 and 42 after reperfusion, indicating that EPO had no effect on anxious behaviour of the animals (Fig. 17).

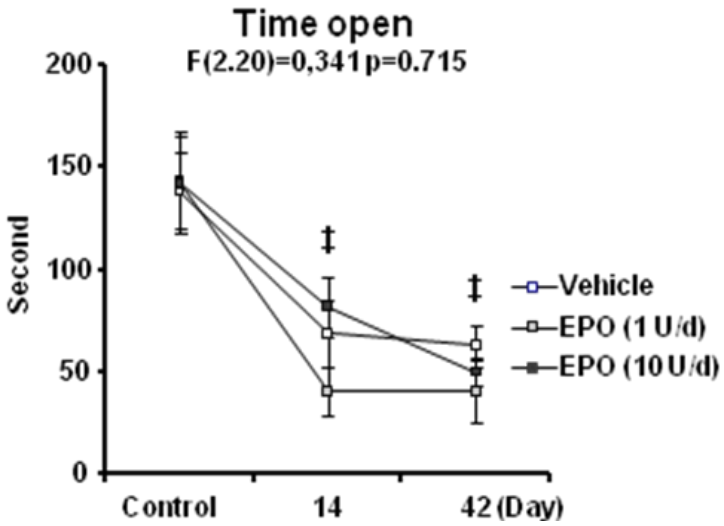

Fig. 17. EPO does not improve anxiety. Anxiety tests in mice prior to ischemia, and at 14- and 42 day poststroke. EPO- treatments showed no significant improvement in anxious behavior. Data are mean ± S.D. (n=8 animals per group). Data were analyzed by repeated measurement ANOVA, for which condition x time interaction effects are shown, followed by one-way ANOVA LSD tests.

4. DISCUSSION

4.1. Project 1: Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways (Kilic E, Kilic U, Soliz U, Gassmann M, Bassetti CL, Hermann DM. (2005). FASEB J 19:2026-2028)

Using the transgenic mouse line tg21 that expresses human EPO in the CNS, we demonstrate that the hematopoietic growth factor EPO protects against focal cerebral ischemia in vivo via ERK-1/-2 and Akt signaling. Already by the mid 1990s, it was realized that EPO, apart from its expression in the liver and kidney, is expressed in the CNS of rodents, monkeys, and humans (Digicaylioglu et al., 1995; Marti et al., 1996). After observations that induction of EPO in the brain takes place in an oxygen dependent manner via activation of hypoxia-inducible factor-1 (HIF-1) (Grimm et al., 2005; Höpfl et al., 2004), it was subsequently shown that HIF-1-induced EPO expression is involved not only in high altitude adaptation, but also mediates resistance against ischemic stroke (Brines et al., 2000; Siren et al., 2001). Indeed, recent clinical studies have proven that EPO protects stroke patients against ischemic damage (Ehrenreich et al., 2002). These data evoked great excitement in the field, since they provided evidence that neuroprotection is feasible in human patients. Until that point, there had been no clinically applicable compound with proven neuroprotection efficacy.

Tg21 mice as model for neuroprotection studies

Despite being in clinical trials, little was known about the signaling mechanisms mediating EPO's neuroprotective activity in vivo. This may be due to the fact that conventional systemic delivery of EPO invariably causes fluctuations of tissue EPO

levels, which in turn lead to variable signaling responses. The tg21 mouse appeared particularly suitable for deciphering EPO's protective functions, since it shows a very stable expression of human EPO in the brain, without inducing excessive erythrocytosis. In fact, blood hematocrit in these animals is normal (~42%). Effects of transgenic EPO have previously been assessed in another mouse line, tg6, in which EPO levels are elevated not only in the brain but also in blood plasma (Wiessner et al., 2001). As a consequence, blood hematocrit in these animals is markedly increased (up to 80%). When submitted to focal ischemia, tg6 mice revealed enlarged brain infarcts, an effect that can be attributed to hemodynamic disturbances brought about by the elevated blood viscosity.

EPO's neuroprotective activity in vivo involves ERK-1/-2 and PI3K/ Akt signalling

In contrast to mice with excessive erythrocytosis, we show in the present study that overexpressed human EPO markedly protected tg21 mice from ischemic injury. Thus, neuroprotection was observed both after long-lasting ischemia (90 min MCA occlusion), where infarct volume was reduced by ~84%, as well as after mild ischemias (30 min) leading to disseminated neuronal injury. Our data confirm that EPO is a highly potent neuroprotectant. Our pharmacological studies revealed that EPO's neuroprotective action was mediated via the dual activation of both ERK-1/-2 and PI3K/ Akt pathways. Antagonization of either signaling pathway with the ERK-1/-2 inhibitor PD98059 or PI3K/ Akt inhibitor wortmannin completely reversed EPO's neuroprotection in vivo, which indicates that both pathways are required for sustained tissue protection. In brain ischemia, the delivery of signal transduction inhibitors is a well-established approach. When delivered via intracerebroventricular application,

PD98059 (Alessandrini et al., 1999) and wortmannin (Han and Holtzman, 2000) induce a potent inhibition of ERK-1/-2 and Akt pathways in the brain. In nontransgenic animals, PD98059 and wortmannin do not aggravate ischemic injury, as previously shown (Alessandrini et al., 1999; Han and Holtzman, 2000) PD98059 given at identical concentration even mediated neuroprotection after 30 min MCA occlusion (Alessandrini et al., 1999), while wortmannin had no influence on neuronal viability after brain ischemia, even at much higher doses (Han and Holtzman, 2000). The absence of an injury-aggravating effect in nontransgenic animals shows that the infarct changes induced by PD98059 and wortmannin in tg21 mice indeed reflect specific actions on cell signaling, rather than unspecific side effects of the two compounds.

Role of mitochondrial signaling pathways in EPO's neuroprotective function

Although in vitro studies already suggested that EPO modulates ERK-1/-2 (Sirén et al., 2001) and PI3K/ Akt (Chong et al., 2003a), our earlier findings following retinal ganglion cell axotomy, a model of subacute CNS neuronal degeneration, indicated that EPO's protective function in these neurons is predominantly driven by ERK-1/-2 (Kilic et al., 2005). On the basis of our present data, EPO's neuroprotective action in brain ischemia differs from models of more delayed neurodegeneration in the sense that not only ERK-1/-2 but also Akt is required for neuronal survival. In ischemic neurons, Akt signaling exhibits a strong stabilizing influence on mitochondrial function that plays a crucial role in ischemic injury. Via phosphorylation of Bad, activated Akt releases Bcl-XL inside the mitochondria (Ruscher et al., 2002), which, in turn, prevents formation of the mitochondrial permeability transition pore. Bcl-XL, on the other hand, once released from Bad, inhibits the secretion of cytochrome *c* from

injured mitochondria, thereby impeding activation of executioner caspases-9 and -3 (Kilic et al., 2002). Our observation of elevated Bcl-XL levels in ischemic tg21 mice, which we again had not seen after retinal ganglion cell axotomy (Kilic et al., 2005), underlines the importance of mitochondrial signaling after stroke and confirms earlier in vitro findings suggesting a role of Bcl-XL in EPO's neuroprotective activity (Chong et al., 2003a; Chong et al., 2003b).

Transgenic EPO activates JNK-1/-2 signaling

In line with in vitro studies demonstrating that EPO activates JNK-1/-2 signaling (Nagata et al., 1998; Jacobs-Helber et al., 2000), transgenic EPO also stimulated JNK-1/-2 phosphorylation after focal cerebral ischemia. Similar to ERK-1/-2, JNK-1/-2 also belong to the MAP kinase family, mediating stress responses to ischemia (Irving and Bamford, 2002). Pharmacological inhibition of JNK-1/-2 prevented mitochondrial Bax translocation (Okuno et al., 2004) and reduced brain injury after stroke (Hirt et al., 2004). On the basis of its potential death promoting activity, an involvement of JNK-1/-2 in EPO's neuroprotective function seemed unlikely. We therefore did not modulate JNK-1/-2 activity pharmacologically. We interpret the JNK-1/-2 response as a stress reaction of the rescued tissue. It is noteworthy that we did not see this JNK-1/-2 activation after optic nerve transection. Our data suggest that the role of JNK-1/-2 depends on the type of tissue and experimental model used, which is in line with observations in the literature that JNK-1/-2 activation differs strongly between model systems (Irving and Bamford, 2002).

Inhibition of NO synthases by EPO does not depend on ERK-1/-2 and Akt signaling

Apart from the activation of ERK-1/-2, Akt, and JNK-1/-2, an inhibition of NOS-1 and -2 by human EPO was noted in our studies. Immunocytochemical double stainings confirmed that NOS expression was localized in neurons. Interestingly, the NOS-1/-2 inhibition was not reversed either by the ERK-1/-2 inhibitor PD98059 or the PI3K/ Akt inhibitor wortmannin, suggesting that NOS-1/-2 inhibition by EPO occurs in an ERK-1/-2- and Akt-independent manner. Inhibition of NOS-1 and -2 by EPO has not yet been shown after brain ischemia. Inhibition of NOS-2 has previously been described following splanchnic artery occlusion, in which it was interpreted as part of a mechanism protecting against endotoxin-induced shock (Squadrito et al., 1999). The inhibition of NOS-1 and -2 by human EPO may be a hitherto unknown player also in the context of EPO's neuroprotective function. Indeed, both NO synthase isoforms are known to exacerbate ischemic damage after stroke, as they contribute to free radical stress in the brain parenchyma, via formation of nitrite and nitrate (Dalkara and Moskowitz, 1994). On the basis of our data, EPO may protect the brain tissue by reducing levels of these highly toxic compounds.

4.2. Project 2: Erythropoietin protects from axotomy-induced degeneration of retinal ganglion cells by activating ERK-1/-2 (Kilic U, Kilic E, Soliz J, Gassmann M, Bassetti CL, Hermann DM. (2005). FASEB J 19: 249-251)

EPO protects against RGC degeneration in vivo

Using the transgenic mouse line tg21, which expresses human EPO predominantly in the CNS, we were able to demonstrate in the RGC axotomy model that the hematopoietic growth factor EPO protects CNS neurons in vivo against delayed neuronal degeneration via ERK-1/-2 signaling. Already in the mid-1990s, it was realized that EPO, apart from its expression in the liver and kidney, is expressed in the CNS of rodents, monkeys, and humans (Digicaylioglu et al., 1995; Marti et al., 19996). Induction of EPO transcription takes place in an oxygen-dependent manner, via activation of hypoxia inducible factor-1 (HIF-1) (for review, see refs. (Gassmann et al., 2003; Höpfl et al., 2004). HIF-1-induced EPO expression is involved not only in high-altitude adaptation (Gassmann et al., 2003), but may also mediate resistance against brain ischemia (Brines et al., 2000; Siren et al., 2001) as well as against light-induced photoreceptor degeneration (Grimm et al., 2002). We now expand these observations showing that EPO expression in vivo is protective not only against rather rapidly progressing acute, but also against rather slowly developing subacute, neuronal injury. We provide evidence that human EPO is indeed expressed in the retinae of tg21 mice. In our RIAs, we observed an ~60% increase of total retinal EPO content, as compared with wild-type animals. Considering that EPO is secreted by neurons, we assume that this ~60% increase represents an underestimation of local EPO concentrations surrounding ganglion cells. Moreover, in view that retinal EPO acts most likely in a paracrine fashion, we consider this increase as physiologically relevant. A great advantage of the retinotectal projection in the study of neuronal

degeneration and protection is the highly coordinated signaling response induced in axotomized RGCs, resulting in the activation of executive cell death programs and leading to the loss of ~80% of RGCs within 14 days after lesioning (Garcia-Valenzuela et al., 1994; Isenmann et al.,1997). Because of the highly standardized injury pattern, the RGC model has already previously been used to assess actions of various growth factors in rats (Klöcker et al., 2000; Kermer et al., 2000) and mice (Kilic et al., 2002; Kilic et al., 2004). Thus, it is noteworthy that the neuroprotection efficacy of EPO in the present experiments was higher than that previously seen for classical neurotrophins, such as brain-derived neurotrophic factor (BDNF) (Klöcker et al., 2000), insulin-like growth factor-I (IGF-1) (Kermer et al., 2000), or glial cell line-derived neurotrophic factor (GDNF) (Kilic et al., 2004), all of which achieved RGC survival rates of <50%. This raises the question about the mechanisms underlying EPO-induced neuroprotection.

EPO modulates ERK-1/-2, PI3K/Akt, and JNK pathways in vivo

Based on in vitro studies suggesting that EPO induces a large number of signaling changes in various cell types, including alterations of MAP kinases ERK-1/-2 (Siren et al., 2001), PI3K/Akt (Siren et al., 2001; Chong et al., 2003), JNK (Nagata et al., 1998; Jacobs-Helber et al., 2000), STAT-5 (Siren et al., 2001;Ihle, 1995), Bcl-XL (13), and caspase-3 (Chong et al., 2003), we hypothesized that the pronounced rescue effects of EPO may be due to its pleiotropic actions. Yet, it remained unclear whether signaling responses previously reported in vitro were similarly relevant in vivo. Interestingly, as in earlier in vitro studies using neuronal cell cultures (Siren et al., 2001; Chong et al., 2003), we were able to confirm in the present experiments that EPO expression indeed induced a pronounced activation of both ERK-1/-2 and

Akt signaling pathways after optic nerve transection, while JNK phosphorylation was mildly reduced in axotomized tg21 mice. An inhibition of JNK phosphorylation by EPO has not been shown in neuronal cells so far. In erythropoietic precursor cells, EPO delivery increased JNK phosphorylation (Nagata et al., 1998; Jacobs-Helber et al., 2000), an effect made responsible for the trophic actions of EPO. Yet, the role of JNK in the CNS differs from that in erythropoietic precursor cells, since JNK activates rather than inhibits cell death cascades in the CNS (for review, see ref. Chong et al., 2002). Accordingly, differential effects of EPO on JNK signaling in the CNS and blood most likely represent cell-type-specific actions.

ERK-1/-2 but not PI3K/Akt is required for EPO's neuroprotective activity

In neuronal cell cultures, both ERK-1/-2 and PI3K/Akt have similarly been shown previously to contribute to neuroprotection induced by EPO, as seen after delivery of signal transduction inhibitors (Siren et al., 2001). The question arose whether both signaling cascades were also involved in EPO induced neuroprotection in vivo. To clarify this issue, we made use of a procedure that has previously been applied successfully to unravel signal transduction mechanisms following optic nerve transection in vivo (Klöcker et al., 2000; Kermer et al., 2000) by injecting the MEK-1 inhibitor PD98059 and PI3K/Akt inhibitor Wortmannin directly into the eye's vitreous space. Interestingly, in our studies, only inhibition of ERK-1/-2, but not of PI3K/Akt, reversed EPO's neuroprotective activity. Thus, the mechanism of action of EPO differs from classical neurotrophins, such as BDNF, for which neuroprotection could not be abolished by MEK-1 inhibition following RGC axotomy (Klöcker et al., 2000).

STAT-5 and Bcl-XL signaling are not activated after RGC axotomy in tg21 mice

While it has previously been shown that EPO stimulates the JAK-2, STAT-5, and Bcl-XL pathways in vitro (Siren et al., 2001; Chong et al., 2002; Ihle, 1995; Chong et al., 2003), the latter signaling cascades were not activated after axotomy in tg21 mice. JAK-2 is a cytosolic protein, which binds to the Box-1 motif of EPO receptor and is thereby phosphorylated and activated, subsequently stimulating activation of STAT-5 (Siren et al., 2001; Ihle, 1995) and, in turn, expression of the mitochondrial anti-apoptotic protein Bcl-XL (Chong 2003). It has long been believed that inhibition of programmed cell death by EPO is critically dependent on the activation of EPO receptor and, consecutively, JAK-2 (Joneja and Wojchowski 1997). Indeed in cells carrying dominant JAK-2 deletions or a biologically incompetent JAK-2, EPO was no longer effective in inhibiting apoptosis (Zhuang et al., 1995; Digicaylioglu et al., 2001). On the other hand, a very recent report shows that EPO derivatives not binding to the classical homodimeric EPO receptor (such as carbamylated EPO) may still exhibit preserved neuroprotective activity, while the erythropoietic potency of such compounds is absent (Leist et al., 2004). Thus, we cannot exclude that EPO receptor/JAK-2 is not required for EPO's neuroprotective effects. Further studies are needed to prove this notion. The lack of STAT-5 and Bcl-XL activation in axotomized RGCs argues against an involvement of STAT-5 and Bcl-XL signalling in EPO-induced neuroprotection, at least in the mouse strain and model used. Our data also suggest that EPO's neuroprotective activity is not mediated via a mitochondrial loop, but directly via cytosolic pathways.

4.3. Project 3: Post-injury administration of erythropoietin promotes neuronal survival and motor recovery after mild focal cerebral ischemia in mice (Kilic U, Kilic E, Vig R, Guo Z, Andres A-C, Gassmann M and Hermann DM. In preparation)

The preparation of clinical trials testing neuroprotective compounds requires careful animal studies, in which clinically relevant structural as well as functional improvements have to persist in the postacute stroke phase. This requires histologic as well as behavioural neurologic studies, in which drug effects are assessed in both phases. We herein examined the effects of EPO- that was administrated 3 days after stroke- on functional recovery in a model of mild focal cerebral ischemia, i.e., 30-min MCA occlusion. By histological analysis of brain injury, angiogenesis and microglia reaction, as well as assessment of motor function, coordination and anxious behaviour, we show that EPO (i) exerts neuroprotective activity (ii) stimulates angiogenesis; (iii) promotes the recovery of motor and coordination deficits. Besides these effects EPO-treatment activated microglia. However, EPO had no effect on anxious behaviour.

In initial rodent studies, brain inflammation and microglia activation were found to be detrimental for the survival of newly born hippocampal neurons post partum (after birth). However, recent experimental evidence indicates that under certain circumstances microglia can be beneficial and support the different steps in neurogenesis, progenitor proliferation, survival, migration, and differentiation (Ekdahl et al., 2008). Additionally, inflammation is a defence reaction against diverse insults that serves to remove noxious agents and to limit their detrimental effects. There is increasing evidence that post-ischemic inflammation plays an important role in brain ischemia. However, whether inflammatory processes are deleterious or beneficial to recovery is presently a matter of debate (Kriz, 2006). Moreover, microglia play an

important role in the pathogenic cascade following cerebral ischemia, since these cells express various growth factors, chemokines and regulatory cytokines as well as free radicals and other toxic mediators (Yanagisawa et al., 2008). It has also been shown that besides neurons, EpoR are expressed on microglia (Nagai et al., 2001). Herein we have also observed activated microglia in ischemic tissues associated with functional recovery and neuroprotection.

In addition to its neuroprotective activity, EPO enhanced angiogenesis in the ischemic brain. Angiogenesis and blood supply to ischemic tissues is correlated with functional recovery (Wang et al., 2004; Li et al., 2007). We observed that EPO also increased angiogenesis in the ischemic striatum, while it did not increase angiogenesis in non-ischemic tissues including overlying cortex and corpus callosum. The 30-min MCA occlusion model we used exhibits very low mortality (in our hands <10%) and induces only mild motor deficits (Kilic et al., 2005, Winter et al., 2005). Of note, this “mild” effect was important regarding locomotion tests, as hemiparesis would invariably have resulted in pathologic findings and thus would have made data interpretation impossible. We previously showed that 30-min MCA occlusion induces disseminate neuronal injury in the mouse striatum that evolves over approximately 3 days (Hermann et al., 2001). Unlike in longer ischemias, overt brain infarcts are not observed under such conditions. We were surprised that EPO had a beneficial effect on neuronal survival, when delivered only 3 days after stroke. Our data support earlier conclusions that EPO is indeed a highly potent neuroprotectant and imperfectly, can be applied non-acutely, a fact that is novel.

The time window in which drugs are able to rescue brain tissue is a critical factor in drug development. As such, thrombolytic compounds are efficacious only when

applied within a 3 to 4.5 h window after stroke onset in human patients (NINDS 1995; ATLANTIS, ECASS and NINDS 2004). In a clinical setting, a short time window precludes broader application, limiting the number of patients that may receive treatment. In case of thrombolytic compounds, less than 10% of the patients are currently eligible for recanalization therapies, even in larger University hospitals. Thus, our observation that EPO prevented ischemic injury even when delivered 3 days after injury is very promising. It raises hope that morel patients will benefit from such treatment. It has been shown that Epo exerts neuroprotective activity when applied up to 6 hours after focal cerebral ischemia (Brines et al., 2000) and 24 h after permanent cerebral ischemia (Wang et al., 2004). The Göttingen EPO-stroke trial in which EPO was given intravenously within an 8-h time frame, was able to demonstrate that the EPO-treated patients showed significantly better clinical outcome as well as reduced infarct size compared to the placebo treated patients,also no sides effect were observed. The second and third application of EPO followed 24 and 48 h later (Ehrenreich et al., 2002). In our study, we applied EPO at first 3d after injury and show that EPO in a dose-dependent manner, promotes neuronal survival, stimulates angiogenesis, activates microglia in the stroke tissue and enhances motor recovery.

In behavioural tests we show for the first time that EPO promotes the recovery of motor coordination. However, it has no effect on anxiety of ischemic mice. In our studies, only a mild paresis of the right forelimb was noticed in ischemic mice after 30-min MCA occlusion, a result that is in line with findings from another group using SV129 mice (Winter et al., 2005). Despite the mild motor weakness, the ischemic animals had significant deficits in the RotaRod test, an observation that was almost completely reversed by EPO application.

5. CONCLUSION AND PERSPECTIVE

After the proof-of-concept trial by Ehrenreich and coworkers (Ehrenreich et al., 2002), which proved the safety and efficacy of recombinant Epo in ischemic stroke patients, chances appear favorable that it will soon be possible to establish a clinically applicable neuroprotection therapy in humans based on Epo. The more than 80% reduction of infarct volume seen in ischemic tg21 animals after focal cerebral ischemia confirms that human Epo is indeed a very potent neuroprotectant. Notably, Epo's actions in our present study were superior to other growth factors that we previously examined in our laboratory, such as glial cell line-derived neurotrophic factor (GDNF) (Kilic et al., 2003), vascular endothelial growth factor (VEGF) (Wang et al., 2005), and melatonin (Kilic et al., 2005).

The neuroprotective action of Epo that is presented here using optic nerve axotomy model might open new perspectives for the treatment of subacute and more chronic neurodegenerative diseases under clinical conditions. Neurodegenerative illnesses, such as Parkinson's and Alzheimer's disease, are extremely frequent disorders and are major causes of long-term disability and impose tremendous burdens to public health care. Unfortunately, there are no efficacious treatments available at present that allow prevention of neuronal degeneration in a causative manner. Establishing efficacious strategies in this context would create new perspectives for neurological therapy and would have favorable socioeconomically consequences.

The successful development of a treatment would create new perspectives for neurological therapy and have favorable consequences for health care systems, as clinical improvements would invariably result in reduced rehabilitation costs. The

delayed neuroprotective action of Epo on functional recovery starting 3 days after stroke onset, and continuing for 30 days provides evidence that EPO induces functionally relevant neurologic improvements in mice suffering from stroke. In view of the fact that EPO is a well known drug with little to no side effects future studies in patients to evaluate time windows and dose–response relationships of EPO are obvious.

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1989- 1994	Faculty of Veterinary Medicine, University of Ankara, Ankara, Turkey
1994-1996	Research assistant at the Department of Food Hygiene and Technology Faculty of Veterinary Medicine, University of Ankara, Ankara, Turkey
1994-1996	Quality assurance authorized agent at food companies by order of Turkish veterinary society
2/1996-9/1996	Language course (German)
1996-2000	DVM at the Department of Hygiene and Technology of Food Animal Origin/ Microbiology and Molecular biology, Ludwig-Maximilians University of Munich (LMU), Munich, Germany. (Scholarship for Foreign study from the Turkish Federal Ministry of Education)

POST-GRADUATE/DOC WORKS

2000 Nov-2001 July	Postdoc scientist in Brain Research Laboratory, Department of Neurology, University of Tübingen, Germany
2001 Aug-2002 Dec	Postdoc scientist in Brain Research Laboratory, Department of Neurology, University of Göttingen, Germany
2003 Jan-2007 Oct	Postdoc scientist in Brain Research Laboratory, Department of Neurology, University of Zürich, Switzerland
2007 Oct-2008 July	Postdoc scientist in Cardiovascular Research, Institute of Physiology, University of Zürich, Switzerland
2008 Aug-2008 Dec	Instructor in Medical Biology, Medical School Yeditepe University, Istanbul, Turkey
Since 2009-	Assist. Prof. Department of Medical Biology, Medical School Yeditepe University, Istanbul, Turkey

Certificates

Experimental courses

1-LTK Modul-1: Introductory Course in Laboratory Animal Science (Mouse/ Rat/ Hamster/ Guinea Pig/ Rabbit corresponding to FELASA-Category B. Institute for Laboratory Animals, University of Zürich, 2003

2-Mouse Model for Human Diseases, Cell Biology, ETH Zürich, 2005

3-LTK Modul-8: Production of polyclonal and monoclonal antibodies, Institute for Laboratory Animals, University of Zürich, 2006

4-Teaching course “Basics in Apoptosis signalling” Institute of Pathology, University of Bern, 2006

5-LTK Modul6E: In vitro Cell Culture: Primary Cells, Embryos, ES Cells; LT. Institute for Laboratory Animals, University of Zürich, 2007

Biosafety (BSO)

1-Basic Regulatory and Technical Principles in Biosafety, BUWAL, Swiss Expert Committee for Biosafety, Bern 2004

2-BioInfoseminar 1-2005 AWEL, Specialist Department of Biological Safety in Eastern Switzerland, 2005

3- Biosafetyconcept of a diagnostic Laboratory with Biosafetylevel 2 (BSL2). Biosafetytraining b-safe GmbH, Bern, Switzerland, 2006

AfH-Didactica, ETH Zurich, Pro-wiss, University of Zurich

1- Project management in scientifical Projects, Pro-WISS, University of Zurich, Arbeitstelle für Hochschuldidaktik AfH, 2004

2- Self-management and time-management. Pro-WISS, University of Zurich, Arbeitstelle für Hochschuldidaktik AfH, 2004.

3- Tutorial-tips, University of Zurich, Arbeitstelle für Hochschuldidaktik AfH ETH Zürich, Didaktik Zentrum DiZ, 2004

4- Discussing with and Guiding students in the education. Gespräche leiten und moderieren in der Lehre, University of Zurich, Arbeitstelle für Hochschuldidaktik AfH, 2004

5-The teaching Portfolio, Universität Zürich, Arbeitstelle für Hochschuldidaktik AfH, ETH Zürich, Didaktik Zentrum DiZ, 2004

6- Teaching and learning by www: ETH Zürich, Didaktik Zentrum DiZ, 2004

Membership in Professional Societies

Schweizerische Geselschaft für Lebensmittel- und UmweltChemie	seit 2004-
Society for Neuroscience	seit 2006-
Turkish Neuroendocrinology Society	seit 2008-
Medical Biology and Genetics Society	seit 2009-

LIST OF PUBLICATIONS

ORIGINAL RESEARCH PUBLICATIONS:

1. Osto E, Matter CM, Kouroedov A, Malinski T, Bachschmid M, Camici GG, **Kilic U**, Stallmach T, Boren J, Iliceto S, Lüscher TF, Cosentino F .c-Jun N-Terminal Kinase 2 Deficiency Protects Against Hypercholesterolemia-Induced Endothelial Dysfunction and Oxidative Stress. **Circulation**. Nov 11;118 (20):2073-80. Oct 27. **2008**
2. Kilic E, Spudich A, **Kilic U**, Rentsch KM, Vig R, Matter CM, Wunderli-Allenspach H, Fritschy JM, Bassetti CL, Hermann DM. ABCC1: a gateway for pharmacological compounds to the ischaemic brain. **Brain**. Oct;131 (Pt 10):2679-89. **2008**
3. **Kilic U,** Kilic E, Bassetti CL, Hermann DM. TLR-4 deficiency protects against focal cerebral ischemia and axotomy-induced neurodegeneration. **Neurobiology Dis.** Jul; 31(1):33-40. **2008**
4. Hédou GF, Koshibu K, Farinelli M, Kilic E, Gee CE, **Kilic U**, Baumgartel K. Hermann DM, Mansuy IM. PP1-dependent bidirectional synaptic plasticity controls ischemic recovery in the adult brain, **Journal of Neuroscience** Jan 2; 28(1):154-62. **2008**
5. Kilic E, **Kilic U,** Bacigallupi M. Guo Z, Ben Abdallah N, Reiter RJ, Wolfer DP. Hermann DM, Bassetti CL. Delayed melatonin administration promotes neuronal survival, neurogenesis and motor recovery, and attenuates hyperactivity and anxiety after mild focal cerebral ischemia in mice. **J Pin Res** J Pineal Res. Sep;45(2):142-8.**2008**
6. Kilic E, **Kilic U**, Vogel P,Böttiger B, Hermann DM. Poxvirus-derived cytokine response modifier a (crma) does not protect against focal cerebral ischemia in mice. **Brain Res.** 1185: 293-300. **2007**

7. **Kilic U**, Kilic E, Järve A, Guo Z, Spudich A, Bieber K, Barzena U, Bassetti CL, Marti HH, Hermann DM. Human VEGF protects axotomized retinal ganglion cells *in vivo* by activating ERK-1/-2 and Akt pathways **Journal of Neuroscience** 26 (48):12439-46 2006

8. Kilic E, **Kilic U,** Wang Y, Bassetti CL, Marti HH, Hermann DM. The phosphatidylinositol-3 kinase/ Akt pathway mediates VEGF's neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. **FASEB J.** Jun 20: 81185-7 **2006**

9. Matter CM, Ma L, von Lukowicz T, Meier P, Lohmann C, Zhang D, **Kilic U,** Hofmann E, Ha SW, Hersberger M, Hermann DM, Lüscher TF. Increased balloon-induced inflammation, proliferation, and neointima formation in apolipoprotein E (ApoE) knockout mice. Stroke. Oct; 37(10):2625-32. **2006**

10. Spudich A, Kilic E, Xing H, **Kilic U**, Wunderli-Allenspach H, Bassetti CL, Hermann DM. Inhibition of multidrug resistance transporter-1 facilitates neuroprotective therapies after focal cerebral ischemia. **Nature Neuroscience** 9: 487-488 **2006**

11. Kilic, E**, Kilic U**, Bähr M and Hermann, D.M.´´NMDA Antagonist MK-801 Reverses detrimental Effects of *Tissue*-Plasminogen Activator Following Intraluminal Thread Occlusion in Mice. **Neurodegenerative diseases** 2: 49-55 **2005**

12. Kilic E, **Kilic U**, Soliz U, Gassmann M, Bassetti CL, Hermann DM. Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways. **FASEB J** 19:2026-2028. **2005**

13. **Kilic U**, Kilic E, Soliz J, Gassmann M, Bassetti CL, Hermann DM. Erythropoietin-induced neuroprotection following retinal ganglion cell axotomy is mediated by ERK-1/-2 signaling *in vivo* **FASEB J** 19: 249-251. **2005**

14. **Kilic U**, Kilic E, Reiter RJ, Bassetti CL, Hermann DM. Signal transduction pathways mediating melatonin-induced neuroprotection after focal cerebral ischemia in mice. **Journal of Pineal Research** Jan; 38(1):67-71. **2005**

15. Kilic E, **Kilic U**, Reiter RJ, Bassetti CL, Hermann DM. Tissue-plasminogen activator-induced ischemic brain injury is reversed by melatonin: role of iNOS and Akt. **Journal of Pineal Research** 39; 151-156. **2005**

16. Wang Y, Kilic E, **Kilic U**, Weber B, Bassetti CL, Marti HH and Hermann DM. Brain-selective VEGF overexpression induces post-ischemic neuroprotection, but also facilitates hemodynamic steal phenomena. **Brain.** Jan; 128(1) 52-63. **2005**

17. **Kilic U,** Kilic E, Bassetti CL and Hermann DM. Post-ischemic delivery of rosuvastatin induces neuroprotection after intraluminal thread occlusion in mice via Erk-1/-2, Bcl-X_L and caspases-9/ -3, but not endothelial NO synthetase (eNOS) pathways. **Neuroscience**. 134:901-906. **2005**

18. Kilic E, **Kilic U**, Matter C, Lüscher TF, Bassetti CL and Hermann DM. Inhibition of endothelial NO synthase by *tissue*-plasminogen activator after focal cerebral ischemia: Signaling mechanisms and reversal by statin. **Stroke** Feb;36(2):332-6. **2005**

19. Spudich A, Frigg R, Kilic E, **Kilic U**, Bassetti CL, Hermann DM. Aggravation of brain injury by prion protein deficiency, but lack of neuroprotection by enhanced prion protein expression following focal cerebral ischemia in mice: Role of STAT-1 and JNK-1/-2 **Neurobiology of Disease** Nov; 20(2): 442-9. **2005**

20. **Kilic U**, Kilic E, Dietz GPH and Bähr M. The TAT protein transduction domain enhances the neuroprotective effect of Glial Cell Line-Derived Neurotrophic Factor after Optic Nerve Transection in Mice. **Neurodegenerative Diseases** 1, 144-49. **2004**

21. **Kilic U**, Yulug B, Kilic E and Bähr M. Rifampicin attenuates brain damage in focal ischemia. (Equal contribution), **Brain Research** 996: 76-80. **2004**

22. **Kilic U**, Kilic E, Lingor P, Yulug B and Bähr M. Rifampicin inhibits neurodegeneration in the optic nerve transection model *in vivo* and after MPP^+-intoxication *in vitro* **Acta Neuropathologica** 108, 65-68. **2004**

23. Kilic E, **Kilic U**, Reiter RJ, Bassetti CL, Hermann DM. Prophylactic use of melatonin protects against focal cerebral ischemia in mice: role of endothelin converting enzyme-1. **Journal of Pineal Research**. Nov; 37(4):247-51. **2004**

24. Kilic E, Weishaupt JH, **Kilic U**, Rohde G, Yulug B, Peters K, Hermann DM and, Bahr M. The superoxid dismutase 1 (sod1) G93A mutation does not promote neuronal injury after focal brain ischemia and optic nerve transection in mice. **Neuroscience** 128 (2), 359-364. **2004**

25. Kilic E, **Kilic U**, Hermann DM, Yulug B and Reiter RJ Melatonin reduces disseminate neuronal death after mild focal ischemia in mice via inhibition of caspase-3 and is suitable as an add-on treatment to *tissue*-plasminogen activator **Journal of Pineal Research 36**, 171-176. **2004**

26. Kilic E, **Kilic U**, Bassetti CL, and Hermann DM. Intravenously administrated recombinant *tissue*-plasminogen activator attenuates neuronal injury after mild focal ischemia. **Neuroreport** 15:687-689. **2004**

27. **Kilic, U**, Kilic E, Dietz GPH and Bähr M. Intravenous TAT-GDNF is protective after focal cerebral ischemia in mice´´. **Stroke** 34, 1304-1310. **2003**

28. **Kilic U**, Schalch B, Stolle A. Ribotyping of *Clostridium perfringens* from industrially produced ground meat**. Letters of Applied Microbiology** 34(4) 238-243. **2002**

29. Kilic E, DM Hermann, S Kügler, H., **Kilic U**, Schmeer, C., Holzmüller and M. Bähr. `Adenovirus-mediated neuron-specific Bcl-X_L expression protects against disseminated neuronal injury and brain infarction following transient middle cerebral artery occlusion in mice. **Neurobiology of Disease** 11:275-284. **2002**

30. **Kilic U**, Schalch B, Solle A. Zur Feintypisierung von *Clostridium perfringens* aus rohem Fleisch. **Archiv für Lebensmittelhygiene** 52:13-15. **2001**

REVIEW ARTICLES:

1. Kilic E, **Kilic U**, and Hermann DM. TAT-GDNF in neurodegeneration and ischemic stroke. **CNS Drug Reviews** 11:353-362. **2005**

2. Kilic E, **Kilic U**, and Hermann DM TAT fusion proteins against ischemic stroke: Current status and future perspectives. **Frontiers in Bioscience** 11:1716-1721. **2006**

3. Grimm, C., Hermann, D.M., Bogdanova, A., Hotop, S., **Kilic, Ü**., Wenzel, A., Kilic, E., and Gassmann, M. Neuroprotection by hypoxic preconditioning: HIF-1 and erythropoietin protect from retinal degeneration. **Sem. Cell Dev. Biol.** 16: 531-538. **2005**

4. Reiter RJ, Tan DX, Leon J, **Kilic U**, Kilic E. When melatonin gets on your nerves: Its benefical actions in experimental models of stroke. **Experimental Biology and Medicine** Feb; 230(2):104-17. **2005**

5. Reiter RJ, Tan DX, Gitto E, Sainz RM, Mayo JC, Leon J, Manchester LC, Vijayalaxmi, Kilic E, **Kilic U**. Pharmacological utility of melatonin in reducing oxidative cellular and molecular damage. **Polish Journal of Pharmacology** Mar-Apr; 56 (2): 159-70. **2004**

Declaration of Originality

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

Istanbul, 18.06.2009

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