The Journal of Experimental Biology 207, 3233-3242
Published by The Company of Biologists 2004
doi:10.1242/jeb.01049

Review

Erythropoietin and the hypoxic brain

Hugo H. Marti

Institute of Physiology and Pathophysiology, University of Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany

e-mail: hugo.marti@pio1.uni-heidelberg.de

Accepted 22 April 2004

Summary

Normal tissue function in mammals depends on adequate supply of oxygen through blood vessels. A discrepancy between oxygen supply and consumption (hypoxia) induces a variety of specific adaptation mechanisms at the cellular, local and systemic level. These mechanisms are in part governed by the activation of hypoxia-inducible transcription factors (HIF-1, HIF-2), which in turn modulate expression of hypoxically regulated genes such as those encoding vascular endothelial growth factor (*VEGF*) and erythropoietin (*EPO*).

EPO is a glycoprotein that is produced mainly by interstitial fibroblasts in the kidneys of the adult and in hepatocytes in the foetus. Released into the circulation, EPO makes its way to the bone marrow, where it regulates red cell production by preventing apoptosis of erythroid progenitor cells. Recently, EPO has emerged as a multifunctional growth factor that plays a significant role in the nervous system. Both EPO and its receptor are expressed throughout the brain in glial cells, neurones and endothelial cells. Hypoxia and ischaemia have been recognised as important driving forces of EPO expression in the brain. EPO has potent neuroprotective properties *in vivo* and *in vitro* and appears to act in a dual way by directly protecting neurones from ischaemic damage and by stimulating endothelial cells and thus supporting the angiogenic effect of VEGF in the nervous system. Thus, hypoxia-induced gene products such as VEGF and EPO might be part of a self-regulated physiological protection mechanism to prevent neuronal injury, especially under conditions of chronically reduced blood flow (chronic ischaemia).

In this review, I will briefly summarize the recent findings on the molecular mechanisms of hypoxia-regulated EPO expression in general and give an overview of its expression in the central nervous system, its action as a growth factor with non-haematopoietic functions and its potential clinical relevance in various brain pathologies.

Key words: hypoxia, ischaemia, neuroprotection, angiogenesis, VEGF, preconditioning, tolerance.

Introduction

It has been almost a century since Carnot and Deflandre postulated that a humoral factor regulates red blood cell production (Carnot and Deflandre, 1906b). The history of the discovery and characterisation of this factor – later termed erythropoietin (EPO) – has recently been reviewed by Fisher (2003). To fulfil its principal function, i.e. the regulation of red cell production, EPO binds to a surface receptor (EPO receptor) on erythroid progenitor cells. After activation of the receptor and subsequent intracellular signalling cascades, survival of these cells is promoted by anti-apoptotic mechanisms. A closed-loop feedback mechanism controls production of EPO, as a reduced number or saturation of oxygen carriers, i.e. erythrocytes, results in tissue hypoxia and subsequent activation of *EPO* gene expression. The resulting increase in blood EPO concentration leads to stimulation of erythropoiesis in the bone marrow, finally resulting in improvement of oxygen supply and repression of the activated *EPO* gene expression. *EPO* is a key example of a gene that is regulated in an oxygen-dependent manner. In fact, *EPO* was the first target gene for hypoxia-inducible factor-1 (HIF-1) to be identified (Semenza and Wang, 1992) and is still one of the best-characterized genes activated by reduced oxygen levels (Wenger, 2002).

The human *EPO* gene, a single-copy gene located on chromosome 7, consists of five exons and four introns. The resulting protein is a 165-amino-acid peptide with a molecular mass of ~30 kDa. EPO has four glycosylation sites, the glycosylation of which governs the biological half-life in the blood. The hormone is produced mainly in the adult kidney and the foetal liver. Peritubular fibroblasts in the kidney and hepatocytes in the liver have been identified as primary EPO-producing cells (Fisher, 2003). However, EPO expression is

not confined to liver and kidney, as EPO mRNA has also been detected at comparable levels in lung, testis and brain but not in muscle, intestine or bone marrow of rodents (Tan et al., 1992). In fact, Carnot and Deflandre had already suggested in their early publication on 'hemopoietin' that the brain contained a haematopoietic activity (Carnot and Deflandre, 1906a). More than 60 years later, a Romanian group presented evidence, in a series of experiments in dogs and rats, that EPO might be produced in the brain itself (Baciu et al., 2000). The physiological role of EPO in the central nervous system (CNS) remained enigmatic though, since the contribution of brain-derived EPO for erythropoiesis in the bone marrow appeared insignificant due to an impeded passage through the blood–brain barrier (BBB). To unravel whether EPO expression in the CNS serves a local physiological function, we and others began to study expression of EPO and its receptor, as well as their regulation in the CNS, in various species.

EPO and EPO receptor expression in the brain

EPO mRNA is constitutively expressed at comparable levels in the brain of mice (Digicaylioglu et al., 1995), monkeys and humans (Marti et al., 1996). *EPO* gene expression in the human brain is found in the temporal cortex, hippocampus and amygdala. In the monkey brain, EPO expression is detected in all investigated areas, which include temporal, frontal and occipital cortex, cerebellum, hypothalamus, hippocampus and caudate nucleus. The detection of EPO in the human brain immediately attracted a broader interest. At the protein level, immunoreactive EPO was found in the cortex and hippocampus of normal human and mouse brain (Bernaudin et al., 1999; Siren et al., 2001b). Furthermore, EPO was detected in the cerebrospinal fluid (CSF) of human adults (Marti et al., 1997) and neonates (Juul et al., 1997). At the cellular level, astrocytes (Marti et al., 1996; Masuda et al., 1994, 1997) and neurones (Bernaudin et al., 1999, 2000; Siren et al., 2001b) are a source of brain-derived EPO, as shown *in vitro* and *in vivo*. While no EPO expression was found in purified cultures of human microglial cells or oligodendrocytes (Nagai et al., 2001), expression was recently reported in oligodendrocytes isolated from embryonic rat brains (Sugawa et al., 2002). It remains to be established whether other cell types in the CNS, such as endothelial cells, have the ability to produce EPO.

EPO mediates its effects through binding to its cognate receptor. Thus, EPO receptor must be expressed at the site of action in the CNS to enable EPO to elicit biological functions. Indeed, expression of EPO receptor mRNA and protein was demonstrated in the brain of mouse, rat, monkey and humans (Digicaylioglu et al., 1995; Liu et al., 1997; Marti et al., 1996). RT-PCR and immunohistochemical analysis revealed that neurones and astrocytes carry the EPO receptor (Bernaudin et al., 1999, 2000; Siren et al., 2001b). In particular, the astrocytic processes surrounding the capillaries seem to strongly express the receptor. Moreover, EPO receptor immunoreactivity was also localised within endothelial cells (Brines et al., 2000). *In vitro* analysis in cell culture models confirmed expression on various endothelial cells including brain-derived endothelial cells (Anagnostou et al., 1994; Yamaji et al., 1996) as well as on neurones and astrocytes (Bernaudin et al., 1999, 2000; Morishita et al., 1997; Nagai et al., 2001). Finally, EPO receptor was shown in cultures of human microglial cells (Nagai et al., 2001) and in rat oligodendrocytes (Sugawa et al., 2002).

In summary, both EPO mRNA and protein are found in the brain of a variety of mammals including humans. EPO receptor is widely expressed in most cerebral cell types, including neurones, endothelial cells, microglial cells and astrocytes. Table 1 gives an overview of the cellular sites of EPO and EPO receptor expression in the CNS. All these data encouraged the investigation of mechanisms regulating expression of EPO and

Table 1. *Sites of erythropoietin (EPO) and EPO receptor expression in the central nervous system*

	EPO				EPO receptor				
	Rodents		Humans		Rodents		Humans		
Cell types	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	References
Neurones	+	+	–	+	+	+	+	+	Bernaudin et al. (1999, 2000); Siren et al. (2001b); Morishita et al. (1997); Nagai et al. (2001)
Astrocytes	+	+	+	+	+	+	+	+	Masuda et al. (1994, 1997); Marti et al. (1996); Bernaudin et al. (1999, 2000); Siren et al. (2001b)
Microglial cells	NA	NA	–	NA	NA	NA	+	NA	Nagai et al. (2001)
Oligodendrocytes	+	NA	–	NA	+	NA	–	NA	Nagai et al. (2001); Sugawa et al. (2002)
Endothelial cells	–	?	NA	?	+	+	+	+	Anagnostou et al. (1994); Yamaji et al. (1996); Brines et al. (2000)

+, expression detected; –, no expression; NA, not analysed; ?, not proven.

its receptor and the search for a physiological function of EPO in the brain.

Oxygen-dependent regulation of *EPO* gene expression

Many of the hypoxic adaptation processes in the body are based on transcriptional regulation by the hypoxia-inducible factors HIF-1 and HIF-2. HIF targets include genes involved in angiogenesis, vasomotor control, energy metabolism and apoptosis, as well as erythropoiesis. Indeed, oxygen-regulated *EPO* expression is controlled by HIF-1 (Wenger, 2000). As a consequence of these various functions, HIF-1 is also implicated in the pathophysiology of many human diseases (Semenza, 2000). HIF-1 is activated at physiologically relevant oxygen levels (Jiang et al., 1996), ensuring fast and adequate response to hypoxic stress. HIF-1 is a heterodimer composed of an α- and a β-subunit. HIF-1β is a constitutive nuclear protein identical to aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1α is an oxygen-labile protein that is very rapidly stabilized under hypoxic conditions. Upon stabilization, the HIF-1 heterodimer binds to specific DNA sequences located in hypoxia-response elements associated with oxygen-regulated genes such as *EPO* and vascular endothelial growth factor (*VEGF*) (Wenger, 2002). VEGF is the most important specific regulator of endothelial cell growth and differentiation and is also a survival factor for endothelial cells (Risau, 1997).

Oxygen availability influences multiple steps in HIF activation, and recent studies have suggested that at least two steps in this process are governed by a novel mode of signal transduction involving enzymatic hydroxylation of specific amino acid residues in the α-subunit by a group of oxygenases. In normoxia, oxygen availability enables a specific prolyl hydroxylation within the oxygen-dependent degradation domain of HIF-α. This prolyl hydroxylation allows binding of the von Hippel-Lindau protein (pVHL), leading to ubiquitylation and proteasomal degradation of HIF-α subunits (Ivan et al., 2001; Jaakkola et al., 2001). Three isoforms of the HIF prolyl hydroxylases were identified in mammalian cells and termed prolyl hydroxylase domain enzymes (PHD1–3; Bruick and McKnight, 2001; Epstein et al., 2001). All three enzymes are widely expressed but show a distinct maximal

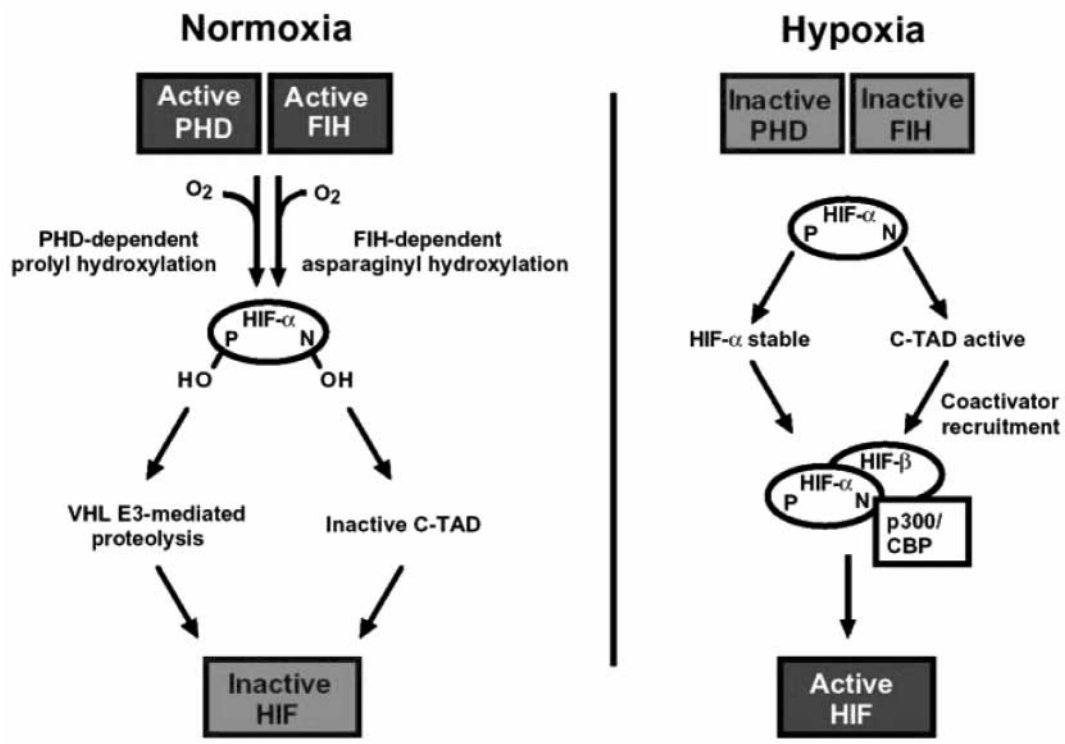

Fig. 1. Regulation of hypoxia-inducible factor (HIF) activity in response to cellular oxygen level. Two independent hydroxylation pathways regulate HIF activity in response to cellular oxygen level. In normoxia, oxygen availability enables hydroxylation of proline (P) residues of the HIF-α oxygen-dependent degradation domain *via* prolyl hydroxylase domain (PHD) enzymes. This prolyl hydroxylation allows binding of the von Hippel-Lindau (VHL) E3 ligase, leading to ubiquitylation and proteasomal degradation of HIF-α subunits. Oxygen availability also enables hydroxylation of asparagine (N) residues of the C-terminal transactivation domain (C-TAD), blocking interaction with the transcriptional co-activator p300/CBP (CREB binding protein). This event is governed by an asparaginyl hydroxylase termed factor-inhibiting HIF (FIH). As a consequence, in the presence of oxygen, active PHDs and FIH result in inactivation of HIF, and thus HIF-mediated gene transcription is blocked. In hypoxia, the PHD and FIH enzymes are inactive and the lack of hydroxylation results in stable HIF-α and an active C-TAD, which is able to form a DNA-binding heterodimer with the constitutive present HIF-β subunit and recruit the co-activator p300/CBP. Lack of oxygen and thus inactive PHD and FIH enzymes result in active HIF, which enables hypoxia-dependent gene expression of, for example, erythropoietin (EPO) and vascular endothelial growth factor (*VEGF*). Reproduced with permission from Masson and Ratcliffe (2003).

expression pattern (Masson and Ratcliffe, 2003). Oxygen availability also enables asparaginyl hydroxylation of the C-terminal transactivation domain of HIF-α, blocking interaction with transcriptional coactivators (Lando et al., 2002b). This event is governed by a specific asparaginyl hydroxylase, termed factor-inhibiting HIF-1 (FIH-1; Hewitson et al., 2002; Lando et al., 2002a). All these enzymes use dioxygen in the hydroxylation reaction. Thus, they are active during normoxia but get inactivated during hypoxia (Fig. 1). The lack of hydroxylation results in stable HIF-α, which is able to form a DNA-binding heterodimer with HIF-β/ARNT. The formed heterodimer then recruits the transcriptional coactivators at the transactivation domain, enabling transcriptional activity. Thus, under normoxic conditions, HIF-α subunits are modified by oxygen and iron-dependent prolyl and asparaginyl hydroxylation, leading to their instability and functional inactivation. In hypoxia, however, the PHD and FIH enzymes are inactive, resulting in stable and active HIF-α subunits. Therefore, HIF hydroxylases, providing a direct link between the availability of molecular oxygen and regulation of HIF, act as direct oxygen sensors (Lando et al., 2003; Pugh and Ratcliffe, 2003). For more details, see a recent review by Masson and Ratcliffe (2003).

In the brain, the α-subunit of HIF-1 is strongly induced during hypoxia (Chavez et al., 2000), and sustained elevated levels of HIF-1 (Stroka et al., 2001) seem to be responsible for the continuous EPO upregulation seen in this organ during hypoxic exposure while hypoxic EPO expression in the kidney is attenuated (Chikuma et al., 2000). Depending on the severity of hypoxia, EPO mRNA levels increase between 3- and 20-fold in the brain compared with up to 200-fold induction in the kidney (Marti et al., 1996). Thus, hypoxic *EPO* gene activation in the brain appears to occur in a very similar way to in the kidney, albeit induction levels are lower.

On a cellular level, primary mouse astrocytes cultured *in vitro* upregulate EPO expression 100-fold in response to hypoxic exposure (Marti et al., 1996), and *EPO* gene expression is also stimulated by hypoxia in neurones (Bernaudin et al., 2000). By contrast, *EPO receptor* gene transcription is not directly influenced by exposure to systemic hypoxia *in vivo* (Digicaylioglu et al., 1995), but upregulation of its mRNA upon hypoxic exposure has been demonstrated in hippocampal neurones cultured *in vitro* (Lewczuk et al., 2000). Furthermore, anaemic stress and ischaemic conditions both enhanced EPO receptor expression *in vivo* (Bernaudin et al., 1999), ensuring an increased sensitivity of neuronal cells to EPO during these stress situations (Chin et al., 2000; Sadamoto et al., 1998).

Physiological role of EPO expression in the CNS

EPO has been suggested to play a role in brain development as this hormone and its receptor are abundantly expressed in the embryonic brain (Dame et al., 2001). Inspection of brain development in EPO-receptor-deficient mice indeed revealed an increased apoptosis rate in the developing brain that was accompanied by hypoplasia in the region of the fourth ventricle (Yu et al., 2001). Further evidence for a physiological effect of EPO in the brain comes from studies on neurones *in vitro* and *in vivo*. The first studies evaluating the function of brain-derived EPO suggested that it possesses trophic activity. EPO augmented choline acetyltransferase activity in primary cultured mouse septal neurones and promoted regeneration of septal cholinergic neurones in adult rats that had undergone fimbria–fornix transections (Konishi et al., 1993). Recently, it was also demonstrated that addition of EPO enhanced survival and dopaminergic differentiation of CNS precursor cells *in vitro* (Studer et al., 2000), reminiscent of its survival function during erythropoiesis in the bone marrow. Furthermore, hypoxia-induced EPO appears to act directly on forebrain neural stem cells, promoting the production of neuronal progenitors and suggesting that EPO is involved in neurogenesis after hypoxia (Shingo et al., 2001). These results suggest that EPO can act on neurones as a neurotrophic factor.

In 1998, Sasaki and colleagues provided good evidence that endogenous brain-derived EPO is also crucial for neuronal survival *in vivo*. Infusion of a soluble EPO receptor into the brain of gerbils submitted to a mild ischaemia that did not produce neuronal damage by itself resulted in neuronal cell death in the hippocampus (Sakanaka et al., 1998). These results indicate that brain-derived EPO may be an endogenous protective agent for neurones against mild forms of tissue hypoxia and ischaemia. Indeed, a crucial role for the endogenous EPO/EPO receptor system was also recently demonstrated in a model of transient global retina ischaemia. Neutralisation of endogenous EPO exacerbated ischaemic injury, supporting the role of brain-derived EPO for survival and recovery of neurones after hypoxic and ischaemic episodes (Junk et al., 2002). Along the same lines, it was also shown that HIF-1 induced *EPO* expression in the hypoxic retina protected against light-induced retinal degeneration (Grimm et al., 2002).

At a cellular level, EPO has been implicated in the regulation of calcium flux in neuronal cells *in vitro*. Externally applied EPO to the human neuroblastoma cell line SK-N-MC led to an increase in calcium influx *via* plasma membrane T-type voltage-dependent calcium channels (Assandri et al., 1999) and increased calcium uptake in the pheochromocytoma cell line PC12 (Koshimura et al., 1999; Masuda et al., 1993). EPO also elevated intracellular concentrations of monoamines (Masuda et al., 1993) and increased dopamine release and tyrosine hydroxylase activity in PC12 cells (Koshimura et al., 1999). Finally, EPO stimulated dopamine release and enhanced potassium-induced acetylcholine release from rat striatal slices (Yamamoto et al., 2002). Recently, it was demonstrated that EPO improved synaptic transmission during oxygen and glucose deprivation in rat hippocampal slices (Weber et al., 2002). Thus, EPO might stimulate neuronal function and viability *via* activation of calcium channels and release of neurotransmitters.

A second target for physiological EPO action in the brain is the vasculature. Originally, it was demonstrated that EPO has

a mitogenic and chemotactic effect on endothelial cells derived from the human umbilical vein and bovine adrenal capillaries (Anagnostou et al., 1990). It has also been shown that vessel outgrowth of rat aortic rings is stimulated by EPO (Carlini et al., 1995), suggesting that EPO has angiogenic properties. Indeed, neovascularisation *in vivo* was stimulated in the endometrium after EPO injection into the mouse uterine cavity (Yasuda et al., 1998) and in the chick embryo chorioallantoic membrane after EPO administration (Ribatti et al., 1999). The fact that brain capillary endothelial cells express two forms of EPO receptor mRNA (Yamaji et al., 1996) implicates EPO in brain angiogenesis. Indeed, EPO showed a dose-dependent mitogenic activity on brain capillary endothelial cells (Yamaji et al., 1996).

Finally, glial cells are additional targets for EPO action in the brain. For example, it was demonstrated that EPO promoted the maturation and differentiation of oligodendrocytes and the proliferation of astrocytes *in vitro* (Sugawa et al., 2002). Furthermore, EPO was shown to exert anti-apoptotic effects on rat microglial cells *in vitro*. Thus, the EPO/EPO receptor system might serve as an endogenous system to protect brain cells from damage caused by intermittent episodes of hypoxia. Along this line, EPO has been implicated in the mechanisms of ischaemic tolerance or preconditioning. Preconditioning means that practically any stimulus capable of causing injury to a tissue can, when applied below the threshold level of damage, activate endogenous protective mechanisms and thus potentially lessen the impact of subsequent, more severe insults (reviewed in Dirnagl et al., 2003). It has been shown in models of ischaemic preconditioning both *in vitro* and *in vivo* that hypoxia-induced EPO release from astrocytes can inhibit hypoxia-induced apoptosis in neurones (Ruscher et al., 2002) and thus provide stroke tolerance (Prass et al., 2003).

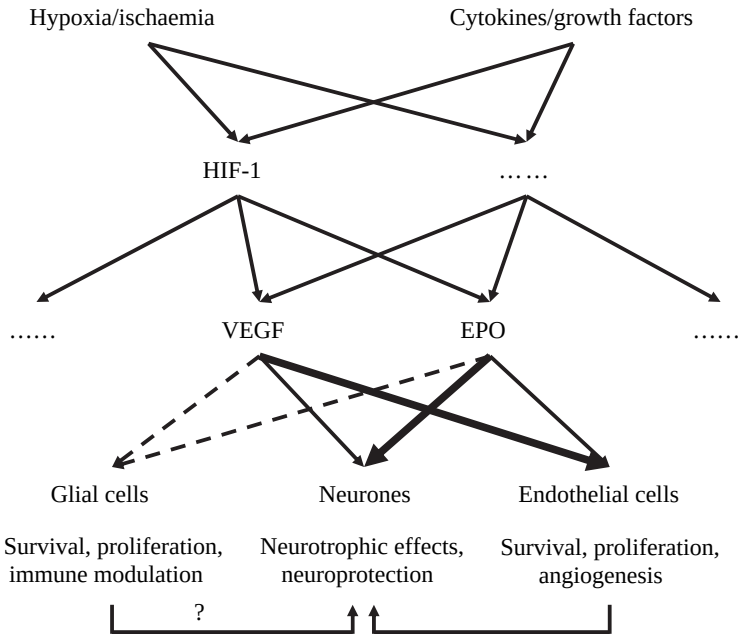

Fig. 2. Hypoxia-induced neuronal protection mechanisms in the central nervous system. Tissue hypoxia and cerebral ischaemia activate hypoxia-inducible factor-1 (HIF-1), which in turn activates gene transcription of a variety of oxygen-regulated factors, among them erythropoietin (EPO) and vascular endothelial growth factor (VEGF). These factors, as well as HIF-1 itself, might also be activated by hypoxia-independent stimuli such as growth factors or cytokines. EPO and VEGF then confer cellular protection. The main target for EPO (indicated by a thicker arrow) is neurones, while VEGF mainly prevents apoptosis and stimulates proliferation of endothelial cells, resulting in new vessel growth (angiogenesis) and ultimately better oxygenation of hypoxic tissues. However, to a lesser extent, EPO also contributes to endothelial cell proliferation, and VEGF is also a direct neuroprotective factor (indicated by thinner arrows). In addition, both EPO and VEGF also have neurotrophic properties. Finally, as receptors for both EPO and VEGF are expressed on microglial cells and astrocytes, glial cells might be a target for both factors, although the effects on these cells are less clear (indicated by broken arrows) and the contribution to neuronal survival remains to be established.

From the data available so far, one might conclude that EPO acts at least in a dual way, firstly by acting as a direct neurotrophic or neuroprotective factor and, secondly, by inducing angiogenesis (Marti et al., 2000) (Fig. 2). Indirect neuronal protection by EPO would be achieved by affecting endothelial cell growth and survival. Hypoxia- or ischaemia-induced EPO might stimulate new vessel growth, enabling the transport of more red blood cells and thereby increasing the amount of oxygen delivered to the hypoxic tissue, which in turn counteracts the detrimental effects of hypoxia on neurones (Marti and Risau, 1999). Finally, EPO could influence neuronal survival by modulation of glial cell activation. Thus, EPO seems to be part of an endogenous defensive system enabling the brain to counteract detrimental effects of hypoxia and ischaemia. A model of such a protective system in the brain is depicted in Fig. 2. It includes operation of various growth factors such as EPO, VEGF and others promoted by a number of stimuli *via* activation of several transcription factors. Activation of HIF-1 by tissue hypoxia is an obvious pathway, but many others might be involved as well. Indeed, it has been shown that hypoxia-independent activation of HIF-1 occurs, e.g. by cytokines, as well as activation of other transcription factors such as AP-1 and nuclear factor κB (NF-κB) by oxygen depletion.

The molecular mechanisms of EPO action in the CNS

The molecular mechanisms by which EPO mediates its effects in the CNS are not well understood. By analogy to the erythroid precursor cells in the bone marrow, where EPO promotes cell viability by repressing apoptosis (Koury and Bondurant, 1990), one might speculate that similar mechanisms operate in neuronal and endothelial cells. Indeed, it has been shown that EPO protects hippocampal neurones from delayed cell death in a global cerebral ischaemia model

in gerbils (Sakanaka et al., 1998) and inhibits programmed cell death in the ischaemic penumbra after middle cerebral artery occlusion in rats (Siren et al., 2001a). In primary hippocampal neurones, hypoxia-induced cell death was reversed by addition of EPO (Lewczuk et al., 2000). Similarly, treatment with EPO reduced death of primary cortical neurones in an *in vitro* model of cerebral ischaemia, consisting of combined oxygen and glucose deprivation. Interestingly, this effect was cell-specific and remained restricted to neurones, whereas hypoxic astrocytes were not protected (Sinor and Greenberg, 2000). EPO could interfere at any step within the well-described cascade of neuronal cell death (Lee et al., 2000; Lipton, 1999). For example, it was shown that EPO prevented excitotoxic neuronal death induced by various glutamate receptor agonists. Addition of EPO to neuronal cells was protective against kainate- (Siren et al., 2001a) as well as *N*-methyl-D-aspartate (NMDA)- (Bernaudin et al., 1999; Morishita et al., 1997; Sakanaka et al., 1998) and α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA)-induced toxicity (Sinor and Greenberg, 2000). In addition, EPO blocked the calcium-induced exocytotic glutamate release on cerebellar granule neurones submitted to chemical ischaemia *in vitro* (Kawakami et al., 2001). Thus, EPO can suppress ischaemic cell death by inhibiting exocytosis of glutamate, which in turn prevents glutamate action at the corresponding receptor (for a recent review, see Buemi et al., 2003). Overactivation of glutamate receptors on neurones can lead to excessive production of NO, which, after reaction with superoxide and formation of peroxynitrite, induces neuronal cell death (Nicotera and Lipton, 1999). EPO might also act on the level of NO toxicity. It has been proposed that EPO decreases NO-mediated injury, although it could not affect NO levels directly (Digicaylioglu and Lipton, 2001).

EPO may also repress cell death in neurones by activation of anti-apoptotic genes or by suppression of caspases, as is the case in erythroid precursor cells, where EPO maintains expression of anti-apoptotic genes such as *bcl-2* and *bcl-xL* (Silva et al., 1996). Indeed, increased gene expression of *XIAP* and *c-IAP2*, members of apoptosis-inhibitor genes, was demonstrated in cerebrocortical cells following pre-incubation with EPO (Digicaylioglu and Lipton, 2001). Furthermore, infusion of EPO caused more intense expression of *bcl-xL* in the hippocampal CA1 region of ischaemic gerbils than did vehicle infusion (Wen et al., 2002). Taken together, these results demonstrate that EPO has multiple protective effects in the CNS that are at least partially mediated through upregulation of anti-apoptotic molecules (Chong et al., 2003).

An important issue with regard to potential therapeutic interference is the identification of signalling pathways that mediate the protective signal of EPO within the cell. Cell survival in haematopoietic precursor cells is mediated by EPO receptor activation, leading to activation of Janus-tyrosine kinase-2 (JAK2), which in turn phosphorylates molecules in several downstream signalling pathways, including MAPK (Ras-mitogen-activated protein kinase), phosphatidylinositol 3-kinase (PI3K)-Akt and Stat-5 (signal transducer and activator of transcription) (Ihle, 1995). The same pathways seem to be involved in EPO-mediated neuroprotection (for a recent review, see Chong et al., 2003). EPO induced phosphorylation of Stat-5, Akt and the MAPK ERK1 in rat hippocampal neurones. Furthermore, inhibition of MAPK and PI3K pathways largely abolished the EPO-induced protection against hypoxia-mediated cell death in these cells (Siren et al., 2001a). Recently, it was demonstrated that EPO-mediated neuroprotection may also involve cross-talk between JAK2 and NF-κB, resulting in activation of NF-κB signalling pathways (Digicaylioglu and Lipton, 2001). However, the mechanism of NF-κB activation in EPO-mediated signalling awaits further clarification, as it was also demonstrated that the activity of JAK2 was dispensable for induction of NF-κB by the EPO receptor (Bittorf et al., 2001). Taken together, it appears that, in neurones, EPO binding to its receptor results in phosphorylation of JAK2 and Stat-5 as well as in activation of NF-κB. Stat-5 and NF-κB then translocate to the nucleus and bind to DNA, promoting expression of anti-apoptotic genes (Juul, 2002).

With regard to endothelial functions of EPO, it is noteworthy that endothelial cells and haematopoietic cells are believed to be derived from the same mesenchymal precursor, the so-called haemangioblast (Risau, 1997). This may explain why endothelial cells carry the EPO receptor and can be stimulated by EPO (Ribatti et al., 1999; Yasuda et al., 1998). Very recently, it was demonstrated that EPO is a potent physiological stimulus for endothelial progenitor cell mobilisation and stimulates postnatal neovascularisation (Heeschen et al., 2003). As in the case for erythroid precursor cells and neurones, EPO also seems to be a survival factor for endothelial cells by preventing cell injury and DNA fragmentation through activation of Akt1 and inhibition of cytochrome *c* release and caspase activity (Chong et al., 2002). EPO might influence endothelial cells indirectly through activation of the VEGF/VEGF receptor (VEGFR) system. It was demonstrated that EPO-induced proliferation of bovine aortic and glomerular endothelial cells was prevented by a specific anti-VEGF antibody (Nitta et al., 1999; Victoria et al., 1998). Furthermore, mRNA expression for both the VEGFR-1 and VEGFR-2 was upregulated in the aortic cells after EPO pre-treatment (Victoria et al., 1998). Finally, incubation of glomerular endothelial cells with EPO resulted in a dose-dependent release of VEGF, which was abolished by incubation with an anti-EPO antibody (Nitta et al., 1999). The physiological significance of these results remains, however, unclear, since it appears that endothelial cells, at least *in vivo*, don't produce significant amounts of VEGF (Marti and Risau, 1998). Furthermore, Plate and colleagues found no effect of EPO treatment on VEGFR-2 expression in cerebral slice cultures while stimulation by hypoxia or VEGF resulted in a clear upregulation of VEGFR-2 mRNA and protein levels (Kremer et al., 1997). Thus, more work is definitely needed to delineate the putative involvement of the VEGF/VEGFR system in EPO-mediated angiogenesis. Nevertheless, it appears likely that increased expression of EPO and its

receptor in blood vessels during cerebral ischaemia in mice (Bernaudin et al., 1999) as well as in humans (Siren et al., 2001b) contributes to new vessel growth in the tissue area suffering from hypoxia.

Pathophysiology of EPO in the CNS

EPO has been shown to be neuroprotective in the brain after exposure to a variety of insults, including cerebral ischaemia, head injury, seizures and experimental autoimmune encephalomyelitis.

Upregulation of EPO and its receptor during cerebral ischaemia implicates the EPO/EPO receptor system in the pathophysiology of ischaemic diseases (Bernaudin et al., 1999; Siren et al., 2001b). Indeed, in a model of global ischaemia, infusion of EPO into the lateral ventricles of gerbils prevented ischaemia-induced learning disability and rescued hippocampal CA1 neurones from lethal ischaemic damage. In line with these results, electron microscopy showed enhanced numbers of synapses within the hippocampal region CA1 in EPO-treated ischaemic gerbils compared with in vehicle-treated controls (Sakanaka et al., 1998). Furthermore, intracerebroventricular injection of EPO offered significant protection of neuronal tissue in focal cerebral ischaemia models in mice and rats with permanent occlusion of the middle cerebral artery (Bernaudin et al., 1999; Sadamoto et al., 1998). The protective effect of EPO in brain ischaemia was later confirmed in several animal studies *in vivo* (Brines et al., 2000; Calapai et al., 2000). These findings clearly demonstrated that ischaemic neuronal injury was reduced by direct or systemic administration of EPO. The fact that a differential temporal and cellular modulation of the EPO/EPO receptor system by ischaemia was also detected in human brain tissue (Siren et al., 2001b) indicated that EPO might have a beneficial effect for the treatment of stroke patients (see below).

The neuroprotective effect of EPO during ischaemia and the fact that EPO affects survival of cholinergic neurones and dopamine release led to the hypothesis that EPO may have beneficial effects in Parkinson's disease. In a mouse model of experimental Parkinsonism, mice received intraperitoneal injections of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), leading to selective dopaminergic neuronal cell death. Simultaneously, mice were treated by direct bilateral injection of EPO or saline into the brain parenchyma. The EPO-treated mice showed a significant improvement of locomotor activity compared with control animals. Furthermore, MPTP-induced loss of dopaminergic neurones was prevented by EPO (Genc et al., 2001). These results indicate that EPO can protect dopaminergic neurones against MPTP-induced toxicity. It remains to be established whether these results have any implications for patients suffering from Parkinson's disease.

The potential beneficial effect of EPO treatment was subsequently tested in other pathologies of the brain. In a model of epilepsy, seizures were induced in mice by administration of the glutamate analogue kainic acid. Pre-treatment with EPO for 24 h before administration of kainate significantly delayed the onset of status epilepticus and reduced the mortality rate when compared with controls. However, no protection from seizures was achieved by administration of EPO 30 min before kainate exposure (Brines et al., 2000). These results suggest that EPO can modulate neuronal excitability rather by activation of gene expression than due to an acute activity on glutamate channels. The same group also demonstrated a reduction in the clinical severity of experimental autoimmune encephalomyelitis (EAE) after systemic EPO administration (Brines et al., 2000). EPO was shown to exert an anti-inflammatory effect on the CNS in EAE by delaying the increase of the pro-inflammatory cytokines tumour necrosis factor (TNF) and interleukin 6 (IL-6) (Agnello et al., 2002). As EAE is considered to be an appropriate animal model for multiple sclerosis, EPO might act as a protective factor in this inflammatory pathology of the CNS. Thus, EPO may also play an important immunomodulatory role. Furthermore, treatment with EPO proved to be effective also for brain injury. The systemic EPO administration, started either as a pre-treatment 24 h before or up to 6 h after impact and continued for 4 days, revealed a significant protection associated with a marked reduction of inflammatory infiltrate in a model of blunt brain trauma (Brines et al., 2000).

Clinical impact and outlook of EPO in the CNS

Based on all these studies, EPO treatment could be of interest in a large variety of brain injuries. As only intravenous or subcutaneous drug application is feasible in human patients, systemically applied EPO must cross the BBB to be useful for therapy of CNS diseases. Results from various studies indicated, though, that endogenous kidney-derived EPO only gets access into the brain after breakdown of the BBB (Buemi et al., 2000; Marti et al., 1997). However, immunohistochemical studies have demonstrated that EPO receptors are present at high density around brain capillaries, especially within astrocytic endfeet and on the luminal side of capillary endothelial cells. Therefore, it was suggested that circulating EPO could bind to these receptors and might be transported through the BBB by transcytosis. Indeed, when large doses of EPO were administered systemically, EPO appeared within the CSF; however, the transfer was less than 1% of the administered dose (Brines et al., 2000; Juul et al., 2004). Nevertheless, these findings demonstrate that systemically applied EPO can reach the CNS and accumulate in the CSF in a time- and dose-dependent fashion and open the possibility for an easy intravenous administration of EPO to patients with brain disorders. This approach might be even more promising as many brain disorders such as cerebral ischaemia are associated with an opening of the BBB (Gloor et al., 2001; Rosenberg, 1999) and, thus, intravenous applied agents get better access to the CNS. However, to circumvent the limited access of intravenously applied EPO through the BBB into the brain, and thus the need for high EPO dosages

to obtain protective EPO levels in the CNS, alternative therapeutic strategies should be considered. Minimising the danger of elevated haematocrit levels that could result from high levels and repeated application of EPO should be a primary goal, because elevated blood viscosity resulting from a high haematocrit can lead to perfusion deficits in the brain. Indeed, transgenic mice with systemic overexpression of EPO resulting in high haematocrit levels of over 80% showed significantly larger cerebral infarcts compared with their nontransgenic littermates (Wiessner et al., 2001). A promising approach might be a recently developed nonerythropoietic variant of EPO. Asialoerythropoietin was generated by total enzymatic desialylation and possesses a very short plasma half-life, resulting in no erythropoietic activity. Asialoerythropoietin, however, can cross the BBB and exert a full neuroprotective effect in the CNS (Erbayraktar et al., 2003).

An alternative strategy aims to mimic the hypoxic response of the brain by increasing HIF-1 activity in the CNS. This approach has the advantage of activating the whole endogenous hypoxic response of the organism. Induction of HIF-1 could occur either specifically, with targeted inducers, through gene therapy or through the action of hypoxia mimetics (for a recent review, see Giaccia et al., 2003). An example of the latter is the iron chelator and known HIF-1-inducer desferrioxamine. Intraperitoneal administration of desferrioxamine to mice resulted in increased EPO mRNA levels in the brain cortex (Bernaudin et al., 2000) and induced tolerance against focal cerebral ischaemia in rodents that coincided with activation of HIF-1 DNA binding and *EPO* gene transcription (Prass et al., 2002).

In summary, the primary goal, not only for stroke patients but also for other diseases of the CNS, is to protect neural function. Imitation of brain endogenous protective mechanisms may be the key to future successful approaches to neuroprotection, as activation and mimicry of endogenous mechanisms can be expected to be efficient and well tolerated (Ehrenreich and Siren, 2001). In this respect, EPO might be a showpiece. Originally identified as a haematopoietic factor, EPO is expressed in the CNS, including the human brain. Brain-derived EPO is upregulated by hypoxia, and expression of both EPO and EPO receptor is specifically modulated during cerebral ischaemia. Furthermore, EPO has a neuroprotective potential both *in vitro* and *in vivo* in various animal models of CNS diseases by inhibition of apoptosis in neurones and inducing angiogenesis. EPO eventually also modulates inflammatory responses. Thus, hypoxically upregulated EPO is a naturally self-regulated physiological protective mechanism in the mammalian brain, especially during ischaemia. As EPO is also a clinically extremely well studied and tolerated compound, its use in stroke patients is tempting. Results from a first clinical Phase I/Phase II study are promising. Intravenous high-dose EPO in a total of 53 stroke patients was well tolerated and associated with an improvement in clinical outcome at one month without any signs of elevated haematocrit levels (Ehrenreich et al., 2002). Currently, a larger multi-centre study is under way. Very recently, it was also demonstrated in animal models that EPO can protect the myocardium from ischaemia-reperfusion injury (Cai et al., 2003; Calvillo et al., 2003), suggestive of a general protective role of EPO against hypoxic damage in various tissues.

Taken together, all these results support the idea that EPO acts in the CNS primarily as a direct protective factor in neurones *via* activation of anti-apoptotic pathways. The protective effect on neurones might be supported by the action of EPO and other growth factors such as VEGF on endothelial cells, resulting in cell survival and stimulation of new vessel growth (angiogenesis), as well as on glial cells leading to modulation of inflammatory responses (Fig. 2).

I am indebted to Edwige Petit, Myriam Bernaudin and Christian Bauer for helpful discussions and to Robert Feldmann for critically reading the manuscript. This work was supported by a grant from the Swiss National Science Foundation (3100-067256.01).

References

Agnello, D., Bigini, P., Villa, P., Mennini, T., Cerami, A., Brines, M. and Ghezzi, P. (2002). Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Res.* **952**, 128-134.

Anagnostou, A., Lee, E. S., Kessimian, N., Levinson, R. and Steiner, M. (1990). Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc. Natl. Acad. Sci. USA* **87**, 5978-5982.

Anagnostou, A., Liu, Z.-Y., Steiner, M., Chin, K., Lee, E. S., Kessimian, N. and Noguchi, C. T. (1994). Erythropoietin-receptor mRNA expression in human endothelial cells. *Proc. Natl. Acad. Sci. USA* **91**, 3974-3978.

Assandri, R., Egger, M., Gassmann, M., Niggli, E., Bauer, C., Forster, I. and Görlach, A. (1999). Erythropoietin modulates intracellular calcium in a human neuroblastoma cell line. *J. Physiol. Lond.* **516**, 343-352.

Baciu, I., Oprisiu, C., Derevenco, P., Vasile, V., Muresan, A., Hriscu, M. and Chis, I. (2000). The brain and other sites of erythropoietin production. *Rom. J. Physiol.* **37**, 3-14.

Bernaudin, M., Marti, H. H., Roussel, S., Divoux, D., Nouvelot, A., MacKenzie, E. T. and Petit, E. (1999). A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* **19**, 643-651.

Bernaudin, M., Bellail, A., Marti, H. H., Yvon, A., Vivien, D., Duchatelle, I., MacKenzie, E. T. and Petit, E. (2000). Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. *Glia* **30**, 271-278.

Bittorf, T., Büchse, T., Sasse, T., Jaster, R. and Brock, J. (2001). Activation of the transcription factor NF-κB by the erythropoietin receptor: structural requirements and biological significance. *Cell. Signalling* **13**, 673-681.

Brines, M. L., Ghezzi, P., Keenan, S., Agnello, D., de Lanerolle, N. C., Cerami, C., Itri, L. M. and Cerami, A. (2000). Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. *Proc. Natl. Acad. Sci. USA* **97**, 10526-10531.

Bruick, R. K. and McKnight, S. L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* **294**, 1337-1340.

Buemi, M., Allegra, A., Corica, F., Floccari, F., D'Avella, D., Aloisi, C., Calapai, G., Iacopino, G. and Frisina, N. (2000). Intravenous recombinant erythropoietin does not lead to an increase in cerebrospinal fluid erythropoietin concentration. *Nephrol. Dial. Transplant.* **15**, 422-423.

Buemi, M., Cavallaro, E., Floccari, F., Sturiale, A., Aloisi, C., Trimarchi, M., Corica, F. and Frisina, N. (2003). The pleiotropic effects of erythropoietin in the central nervous system. *J. Neuropathol. Exp. Neurol.* **62**, 228-236.

Cai, Z., Manalo, D. J., Wei, G., Rodriguez, E. R., Fox-Talbot, K., Lu, H., Zweier, J. L. and Semenza, G. L. (2003). Hearts from rodents exposed to

intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation* **108**, 79-85.

Calapai, G., Marciano, M. C., Corica, F., Allegra, A., Parisi, A., Frisina, N., Caputi, A. P. and Buemi, M. (2000). Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. *Eur. J. Pharmacol.* **401**, 349-356.

Calvillo, L., Latini, R., Kajstura, J., Leri, A., Anversa, P., Ghezzi, P., Salio, M., Cerami, A. and Brines, M. (2003). Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc. Natl. Acad. Sci. USA* **100**, 4802-4806.

Carlini, R. G., Reyes, A. A. and Rothstein, M. (1995). Recombinant human erythropoietin stimulates angiogenesis in vitro. *Kidney Int.* **47**, 740-745.

Carnot, P. and Deflandre, C. (1906a). Sur l'activité hémopoiétique des différents organes au cours de la régénération du sang. *C. R. Acad. Sci. Paris* **143**, 432-435.

Carnot, P. and Deflandre, C. (1906b). Sur l'activité hémopoiétique du sérum au cours de la régénération du sang. *C. R. Acad. Sci. Paris* **143**, 384-386.

Chavez, J. C., Agani, F., Pichiule, P. and LaManna, J. C. (2000). Expression of hypoxia-inducible factor-1α in the brain of rats during chronic hypoxia. *J. Appl. Physiol.* **89**, 1937-1942.

Chikuma, M., Masuda, S., Kobayashi, T., Nagao, M. and Sasaki, R. (2000). Tissue-specific regulation of erythropoietin production in the murine kidney, brain, and uterus. *Am. J. Physiol.* **279**, E1242-E1248.

Chin, K., Yu, X., Beleslin-Cokic, B., Liu, C., Shen, K., Mohrenweiser, H. W. and Noguchi, C. T. (2000). Production and processing of erythropoietin receptor transcripts in brain. *Brain Res. Mol. Brain Res.* **81**, 29-42.

Chong, Z. Z., Kang, J. Q. and Maiese, K. (2002). Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. *Circulation* **106**, 2973-2979.

Chong, Z. Z., Kang, J. Q. and Maiese, K. (2003). Erythropoietin: cytoprotection in vascular and neuronal cells. *Curr. Drug Targets Cardiovasc. Haematol. Disord.* **3**, 141-154.

Dame, C., Juul, S. E. and Christensen, R. D. (2001). The biology of erythropoietin in the central nervous system and its neurotrophic and neuroprotective potential. *Biol. Neonate* **79**, 228-235.

Digicaylioglu, M. and Lipton, S. A. (2001). Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-κB signalling cascades. *Nature* **412**, 641-647.

Digicaylioglu, M., Bichet, S., Marti, H. H., Wenger, R. H., Rivas, L. A., Bauer, C. and Gassmann, M. (1995). Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc. Natl. Acad. Sci. USA* **92**, 3717-3720.

Dirnagl, U., Simon, R. P. and Hallenbeck, J. M. (2003). Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci.* **26**, 248-254.

Ehrenreich, H. and Siren, A. L. (2001). Neuroprotection – what does it mean? – what means do we have? *Eur. Arch. Psyc. Clin. Neurosci.* **251**, 149-151.

Ehrenreich, H., Hasselblatt, M., Dembowski, C., Cepek, L., Lewczuk, P., Stiefel, M., Rustenbeck, H. H., Breiter, N., Jacob, S., Knerlich, F. et al. (2002). Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol. Med.* **8**, 495-505.

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A. et al. (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54.

Erbayraktar, S., Grasso, G., Sfacteria, A., Xie, Q. W., Coleman, T., Kreilgaard, M., Torup, L., Sager, T., Erbayraktar, Z., Gokmen, N. et al. (2003). Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity *in vivo*. *Proc. Natl. Acad. Sci. USA* **100**, 6741-6746.

Fisher, J. W. (2003). Erythropoietin: physiology and pharmacology update. *Exp. Biol. Med.* **228**, 1-14.

Genc, S., Kuralay, F., Genc, K., Akhisaroglu, M., Fadiloglu, S., Yorukoglu, K., Fadiloglu, M. and Gure, A. (2001). Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. *Neurosci. Lett.* **298**, 139-141.

Giaccia, A., Siim, B. G. and Johnson, R. S. (2003). HIF-1 as a target for drug development. *Nat. Rev. Drug Discov.* **2**, 803-811.

Gloor, S. M., Wachtel, M., Bolliger, M. F., Ishihara, H., Landmann, R. and Frei, K. (2001). Molecular and cellular permeability control at the blood–brain barrier. *Brain Res. Rev.* **36**, 258-264.

Grimm, C., Wenzel, A., Groszer, M., Mayser, H., Seeliger, M., Samardzija, M., Bauer, C., Gassmann, M. and Reme, C. E. (2002). HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. *Nat. Med.* **8**, 718-724.

Heeschen, C., Aicher, A., Lehmann, R., Fichtlscherer, S., Vasa, M., Urbich, C., Mildner-Rihm, C., Martin, H., Zeiher, A. M. and Dimmeler, S. (2003). Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* **102**, 1340-1346.

Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M. et al. (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J. Biol. Chem.* **277**, 26351-26355.

Ihle, J. N. (1995). Cytokine receptor signalling. *Nature* **377**, 591-594.

Ivan, M., Kondo, K., Yang, H. F., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: Implications for O_2 sensing. *Science* **292**, 464-468.

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., von Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001). Targeting of HIFα to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* **292**, 468-472.

Jiang, B. H., Semenza, G. L., Bauer, C. and Marti, H. H. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am. J. Physiol.* **271**, C1172-C1180.

Junk, A. K., Mammis, A., Savitz, S. I., Singh, M., Roth, S., Malhotra, S., Rosenbaum, P. S., Cerami, A., Brines, M. and Rosenbaum, D. M. (2002). Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. USA* **99**, 10659-10664.

Juul, S. (2002). Erythropoietin in the central nervous system, and its use to prevent hypoxic-ischemic brain damage. *Acta Paediatr.* **Suppl. 91**, 36-42.

Juul, S. E., Harcum, J., Li, Y. and Christensen, R. D. (1997). Erythropoietin is present in the cerebrospinal fluid of neonates. *J. Pediatr.* **130**, 428-430.

Juul, S. E., McPherson, R. J., Farrell, F. X., Jolliffe, L., Ness, D. J. and Gleason, C. A. (2004). Erythropoietin concentrations in cerebrospinal fluid of nonhuman primates and fetal sheep following high-dose recombinant erythropoietin. *Biol. Neonate* **85**, 138-144.

Kawakami, M., Sekiguchi, M., Sato, K., Kozaki, S. and Takahashi, M. (2001). Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. *J. Biol. Chem.* **276**, 39469-39475.

Konishi, Y., Chui, D.-H., Hirose, H., Kunishita, T. and Tabira, T. (1993). Trophic effect of erythropoietin and other hematopoietic factors on central cholinergic neurons in vitro and in vivo. *Brain Res.* **609**, 29-35.

Koshimura, K., Murakami, Y., Sohmiya, M., Tanaka, J. and Kato, Y. (1999). Effects of erythropoietin on neuronal activity. *J. Neurochem.* **72**, 2565-2572.

Koury, M. J. and Bondurant, M. C. (1990). Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* **248**, 378-381.

Kremer, C., Breier, G., Risau, W. and Plate, K. H. (1997). Up-regulation of flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system. *Cancer Res.* **57**, 3852-3859.

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L. and Bruick, R. K. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* **16**, 1466-1471.

Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J. and Whitelaw, M. L. (2002b). Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. *Science* **295**, 858-861.

Lando, D., Gorman, J. J., Whitelaw, M. L. and Peet, D. J. (2003). Oxygen-dependent regulation of hypoxia-inducible factors by prolyl and asparaginyl hydroxylation. *Eur. J. Biochem.* **270**, 781-790.

Lee, J. M., Grabb, M. C., Zipfel, G. J. and Choi, D. W. (2000). Brain tissue responses to ischemia. *J. Clin. Invest.* **106**, 723-731.

Lewczuk, P., Hasselblatt, M., Kamrowski-Kruck, H., Heyer, A., Unzicker, C., Siren, A. L. and Ehrenreich, H. (2000). Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin. *Neuroreport* **11**, 3485-3488.

Lipton, P. (1999). Ischemic cell death in brain neurons. *Physiol. Rev.* **79**, 1431-1568.

Liu, C., Shen, K., Liu, Z. Y. and Noguchi, C. T. (1997). Regulated human erythropoietin receptor expression in mouse brain. *J. Biol. Chem.* **272**, 32395-32400.

Marti, H. H. and Risau, W. (1998). Systemic hypoxia changes the organ-

specific distribution of vascular endothelial growth factor and its receptors. *Proc. Natl. Acad. Sci. USA* **95**, 15809-15814.

Marti, H. H. and Risau, W. (1999). Angiogenesis in ischemic disease. *Thromb. Haemost.* **82 (Suppl.)**, 44-52.

Marti, H. H., Wenger, R. H., Rivas, L. A., Straumann, U., Digicaylioglu, M., Henn, V., Yonekawa, Y., Bauer, C. and Gassmann, M. (1996). Erythropoietin gene expression in human, monkey and murine brain. *Eur. J. Neurosci.* **8**, 666-676.

Marti, H. H., Gassmann, M., Wenger, R. H., Kvietikova, I., Morganti-Kossmann, M. C., Kossmann, T., Trentz, O. and Bauer, C. (1997). Detection of erythropoietin in human liquor: intrinsic erythropoietin production in the brain. *Kidney Int.* **51**, 416-418.

Marti, H. H., Bernaudin, M., Petit, E. and Bauer, C. (2000). Neuroprotection and angiogenesis: a dual role of erythropoietin in brain ischemia. *News Physiol. Sci.* **15**, 225-229.

Masson, N. and Ratcliffe, P. J. (2003). HIF prolyl and asparaginyl hyroxylases in the biological response to intracellular O_2 levels. *J. Cell Sci.* **116**, 3041-3049.

Masuda, S., Masaya, N., Takahata, K., Konishi, Y., Gallyas, F., Tabira, T. and Sasaki, R. (1993). Functional erythropoietin-receptor of the cells with neural characteristics. *J. Biol. Chem.* **268**, 11208-11216.

Masuda, S., Okano, M., Yamagishi, K., Nagao, M., Ueda, M. and Sasaki, R. (1994). A novel site of erythropoietin production: oxygen-dependent production in cultured rat astrocytes. *J. Biol. Chem.* **269**, 19488-19493.

Masuda, S., Chikuma, M. and Sasaki, R. (1997). Insulin-like growth factors and insulin stimulate erythropoietin production in primary cultured astrocytes. *Brain Res.* **746**, 63-70.

Morishita, E., Masuda, S., Nagao, M., Yasuda, Y. and Sasaki, R. (1997). Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents *in vitro* glutamate-induced neuronal death. *Neuroscience* **76**, 105-116.

Nagai, A., Nakagawa, E., Choi, H. B., Hatori, K., Kobayashi, S. and Kim, S. U. (2001). Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. *J. Neuropathol. Exp. Neurol.* **60**, 386-392.

Nicotera, P. and Lipton, S. A. (1999). Excitotoxins in neuronal apoptosis and necrosis. *J. Cereb. Blood Flow Metab.* **19**, 583-591.

Nitta, K., Uchida, K., Kimata, N., Honda, K., Kobayashi, H., Kawashima, A., Yumura, W. and Nihei, H. (1999). Recombinant human erythropoietin stimulates vascular endothelial growth factor release by glomerular endothelial cells. *Eur. J. Pharmacol.* **373**, 121-124.

Prass, K., Ruscher, K., Karsch, M., Isaev, N., Megow, D., Priller, J., Scharff, A., Dirnagl, U. and Meisel, A. (2002). Desferrioxamine induces delayed tolerance against cerebral ischemia *in vivo* and *in vitro*. *J. Cereb. Blood Flow Metab.* **22**, 520-525.

Prass, K., Scharff, A., Ruscher, K., Löwl, D., Muselmann, C., Victorov, I., Kapinya, K., Dirnagl, U. and Meisel, A. (2003). Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. *Stroke* **34**, 1981-1986.

Pugh, C. W. and Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat. Med.* **9**, 677-684.

Ribatti, D., Presta, M., Vacca, A., Ria, R., Giuliani, R., Dell'Era, P., Nico, B., Roncali, L. and Dammacco, F. (1999). Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Blood* **93**, 2627-2636.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Rosenberg, G. A. (1999). Ischemic brain edema. *Prog. Cardiovasc. Dis.* **42**, 209-216.

Ruscher, K., Freyer, D., Karsch, M., Isaev, N., Megow, D., Sawitzki, B., Priller, J., Dirnagl, U. and Meisel, A. (2002). Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an *in vitro* model. *J. Neurosci.* **22**, 10291-10301.

Sadamoto, Y., Igase, K., Sakanaka, M., Sato, K., Otsuka, H., Sakaki, S., Masuda, S. and Sasaki, R. (1998). Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. *Biochem. Biophys. Res. Commun.* **253**, 26-32.

Sakanaka, M., Wen, T. C., Matsuda, S., Masuda, S., Morishita, E., Nagao, M. and Sasaki, R. (1998). *In vivo* evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl. Acad. Sci. USA* **95**, 4635-4640.

Semenza, G. L. (2000). HIF-1 and human disease: one highly involved factor. *Genes Dev.* **14**, 1983-1991.

Semenza, G. L. and Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol.* **12**, 5447-5454.

Shingo, T., Sorokan, S. T., Shimazaki, T. and Weiss, S. (2001). Erythropoietin regulates the *in vitro* and *in vivo* production of neuronal progenitors by mammalian forebrain neural stem cells. *J. Neurosci.* **21**, 9733-9743.

Silva, M., Grillot, D., Benito, A., Richard, C., Nunez, G. and Fernandez-Luna, J. L. (1996). Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through bcl-x_L and bcl-2. *Blood* **88**, 1576-1582.

Sinor, A. D. and Greenberg, D. A. (2000). Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity. *Neurosci. Lett.* **290**, 213-215.

Siren, A. L., Fratelli, M., Brines, M., Goemans, C., Casagrande, S., Lewczuk, P., Keenan, S., Gleiter, C., Pasquali, C., Capobianco, A. et al. (2001a). Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc. Natl. Acad. Sci. USA* **98**, 4044-4049.

Siren, A. L., Knerlich, F., Poser, W., Gleiter, C. H., Brück, W. and Ehrenreich, H. (2001b). Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. *Acta Neuropathol.* **101**, 271-276.

Stroka, D. M., Burkhardt, T., Desbaillets, I., Wenger, R. H., Bauer, C., Gassmann, M. and Candinas, D. (2001). HIF-1 is expressed in normoxic tissue and displays an organ specific regulation under systemic hypoxia. *FASEB J.* **15**, 2445-2453.

Studer, L., Csete, M., Lee, S. H., Kabbani, N., Walikonis, J., Wold, B. and McKay, R. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J. Neurosci.* **20**, 7377-7383.

Sugawa, M., Sakurai, Y., Ishikawa-Ieda, Y., Suzuki, H. and Asou, H. (2002). Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation. *Neurosci. Res.* **44**, 391-403.

Tan, C. C., Eckardt, K.-U., Firth, J. D. and Ratcliffe, P. J. (1992). Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. *Am. J. Physiol.* **263**, F474-F481.

Victoria, M., Arroyo, A., Castilla, M. A., Pacheco, F. R. G., Tan, D., Riesco, A., Casado, S. and Caramelo, C. (1998). Role of vascular endothelial growth factor on erythropoietin-related endothelial cell proliferation. *J. Am. Soc. Nephrol.* **9**, 1998-2004.

Weber, A., Maier, R. F., Hoffmann, U., Grips, M., Hoppenz, M., Aktas, A. G., Heinemann, U., Obladen, M. and Schuchmann, S. (2002). Erythropoietin improves synaptic transmission during and following ischemia in rat hippocampal slice cultures. *Brain Res.* **958**, 305-311.

Wen, T. C., Sadamoto, Y., Tanaka, J., Zhu, P. X., Nakata, K., Ma, Y. J., Hata, R. and Sakanaka, M. (2002). Erythropoietin protects neurons against chemical hypoxia and cerebral ischemic injury by up-regulating Bcl-x_L expression. *J. Neurosci. Res.* **67**, 795-803.

Wenger, R. H. (2000). Mammalian oxygen sensing, signalling and gene regulation. *J. Exp. Biol.* **203**, 1253-1263.

Wenger, R. H. (2002). Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. *FASEB J.* **16**, 1151-1162.

Wiessner, C., Allegrini, P. R., Ekatodramis, D., Jewell, U. R., Stallmach, T. and Gassmann, M. (2001). Increased cerebral infarct volumes in polyglobulic mice overexpressing erythropoietin. *J. Cereb. Blood Flow Metab.* **21**, 857-864.

Yamaji, R., Okada, T., Moriya, M., Naito, M., Tsuruo, T., Miyatake, K. and Nakano, Y. (1996). Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur. J. Biochem.* **239**, 494-500.

Yamamoto, M., Koshimura, K., Kawaguchi, M., Sohmiya, M., Murakami, Y. and Kato, Y. (2002). Stimulating effect of erythropoietin on the release of dopamine and acetylcholine from the rat brain slice. *Neurosci. Lett.* **292**, 131-133.

Yasuda, Y., Masuda, S., Chikuma, M., Inoue, K., Nagao, M. and Sasaki, R. (1998). Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. *J. Biol. Chem.* **273**, 25381-25387.

Yu, X., Lin, C.-S., Costantini, F. and Noguchi, C. T. (2001). The human erythropoietin receptor gene rescues erythropoiesis and developmental defects in the erythropoietin null mouse. *Blood* **98**, 475-477.