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Signalling of hematopoietic growth factors in mammalian neural cells

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CONTENTS

1. Introduction to the scientific project

The present cumulative thesis includes two original publications investigating the signalling pathways activated by hematopoietic growth factors in neural cells and the biological effects of this activation. More than 20 proteins belong to hematopoietic growth factors including erythropoietin (EPO), thrombopoietin (TPO), and several colony stimulating factors such as granulocyte colony stimulating factor (GCSF) and interleukin-3 (Smithgall 1998). The present work focuses on activities of EPO, GCSF and TPO.

EPO, GCSF and TPO are glycoproteins which were originally described to regulate the survival, proliferation and differentiation of diverse blood cells (Kaushansky 2006). The main site of EPO production in adulthood is kidney (Kaushansky 2006). EPO acts on erythroid progenitor cells and is critical for survival (Kaushansky 2006). Mice deficient for EPO or EPO receptor (EPOR) genes die at embryonic day 13 (E13) because of severe anemia caused by deficiency in erythropoiesis. GCSF is released by macrophages and monocytes (Kaushansky 2006). It regulates myeloid development resulting in the generation of neutrophil lineage (Kaushansky 2006). Mice deficient for GCSF protein or receptor have severe deficits in granulopoiesis. The ability of deficient animals to control infections is impaired, nevertheless they are viable. TPO is mostly produced in the liver (Geddis *et al.* 2002). TPO controls the development of megakaryocytic cell lineage (Geddis *et al.* 2002). TPO and TPO receptor (TPOR or c-Mpl) null mice demonstrate severe thrombocytopenia though no signs of spontaneous bleeding are detected and the animals have normal lifespan.

Originally hematopoietic growth factors were believed to act exclusively in the hematopoietic system. However, in recent years it became clear that their effects are not restricted to providing instruction for hematopoiesis in the bone marrow, but appear to act more generally as growth and survival factors for multiple tissues expressing their corresponding receptors. In particular, expression of EPO receptor was detected in brain, as well as in neurons and glia cells (astrocytes/ oligodendrocytes/ microglia) *in vitro* (Hasselblatt *et al.* 2006). GCSF and TPO receptor expression is detected in the brain and in cultured neurons and astrocytes (Ehrenreich *et al.* 2005; Solaroglu *et al.* 2007). It is possible that hematopoietic growth factors produced outside the nervous

system could affect brain cells since it was demonstrated that EPO and GCSF cross blood-brain barrier (Ehrenreich *et al.* 2002; Ehrenreich *et al.* 2004; Brines and Cerami 2005; Schneider *et al.* 2005). The mechanism of this action remains still unknown. On the other hand, EPO, GCSF and TPO are also produced by neural cells (Brines and Cerami 2005; Ehrenreich *et al.* 2005; Hasselblatt *et al.* 2006; Solaroglu *et al.* 2007) suggesting that these factors can function in the brain in a paracrine and/or autocrine manner. Regulation of hematopoietic growth factor expression is stress-responsive: After such challenges as hypoxia-ischemia EPO and GCSF receptor and ligand expression is upregulated while TPO and TPOR expression is downregulated in the brain (Brines and Cerami 2005; Ehrenreich *et al.* 2005; Schneider *et al.* 2005).

Hematopoietic growth factors may play an essential role in brain development and neuronal survival. The best investigated factor in regard to brain development and neuronal survival is EPO (Brines and Cerami 2005; Chen *et al.* 2006). EPO is neuroprotective in a variety of *in vitro* models (Sirén and Ehrenreich 2001). There is strong evidence that EPO provides neuroprotective effects in neurodegenerative diseases and in the damaged brain during ischemic events. Treatment with EPO reduces neuronal damage and improves functional recovery in the animal models of acute and chronic brain diseases such as stroke and neurodegeneration, as well as after brain and spinal cord trauma, excitotoxic lesions and neuroinflammation (Sirén and Ehrenreich 2001; Brines and Cerami 2005; Hasselblatt *et al.* 2006). Reported neurotrophic effects of EPO include the ability to stimulate axonal regrowth, neurite formation and modulate neurotransmitter synthesis and release (Sirén and Ehrenreich 2001; Brines and Cerami 2005). The $EPOR^{-/-}$ fetuses exhibit increased apoptosis in the brain and a reduction in the number of neural progenitor cells, as well as increased sensitivity to hypoxia even prior to beginning of definitive erythropoiesis (Chen *et al.* 2006). However, EPOR expression in the brain is not required for survival, since transgene-rescued knockouts expressing EPOR exclusively in the cells of hematopoietic lineage are viable and fertile (Chen *et al.* 2006).

Recently, GCSF was demonstrated to have neuroprotective properties similar to those of EPO (Solaroglu *et al.* 2007). GCSF was shown to improve behavioural outcome in several ischemia models *in vivo* and to protect neural cells against a variety of apoptotic inducers *in vitro* (Schabitz *et al.* 2003; Schneider *et al.* 2005). GCSF induces

neuronal differentiation of cultured adult neural stem cells and stimulates migration of neuronal progenitor cells to ischemic area in *vivo* (Schneider *et al.* 2005).

There are very few data concerning TPO role in the brain. Although EPO and TPO exhibit significant homology in their receptor-binding domains (Kaushansky 2006), TPO effect on neuronal survival is opposite to that of GCSF and EPO. It was first shown by our group that TPO had cell death promoting effect in rat hippocampal neurons *in vitro* (Ehrenreich *et al.* 2005). Furthermore, it augmented neuronal cell death after hypoxic-ischemic brain injury *in vivo* (Ehrenreich *et al.* 2005).

Hematopoietic growth factors act by binding to their specific transmembrane receptors. Receptors for EPO, GCSF and TPO have similar structure and belong to the same receptor family: Single-chain cytokine type I receptor family. This family includes also receptors for growth hormone (GH) and prolactin (Smithgall 1998). These receptors do not posses intrinsic tyrosine kinase activity; instead the signal is mediated by the Janus family tyrosine kinase 2 (JAK2) constitutively bound to the receptor cytoplasmic domain (Smithgall 1998). Currently it is not clear whether the receptors mediating effects of hematopoietic growth factors in brain are the same as those mediating actions in hematopoietic tissues (Brines and Cerami 2005). For example, heterodimeric complex comprised of the traditional EPOR subunit and the common β receptor subunit of the cytokine type I receptor (shared by the members of IL-3 receptor family) has been suggested to mediate the neuronal effects of EPO (Brines and Cerami 2005).

Signalling cascades induced by stimulation of cytokine type I receptors are well investigated in bone marrow precursor cells (Kaushansky 2006). Signalling in the brain is less known. First studies have characterized EPO and GCSF signalling in neurons (Brines and Cerami 2005; Solaroglu *et al.* 2007). Upon ligand binding the receptor dimerizes which leads to transactivation of JAK2 molecules. Once activated, JAK2 phosphorylates distal parts of receptors which subsequently serve as docking sites for downstream signalling molecules. Several signal transduction pathways are shown to be activated by hematopoietic growth factors including signal transducers and activators of transcription (Stat), phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/extracellular signal regulated kinase (ERK1/2) (Kaushansky 2006).

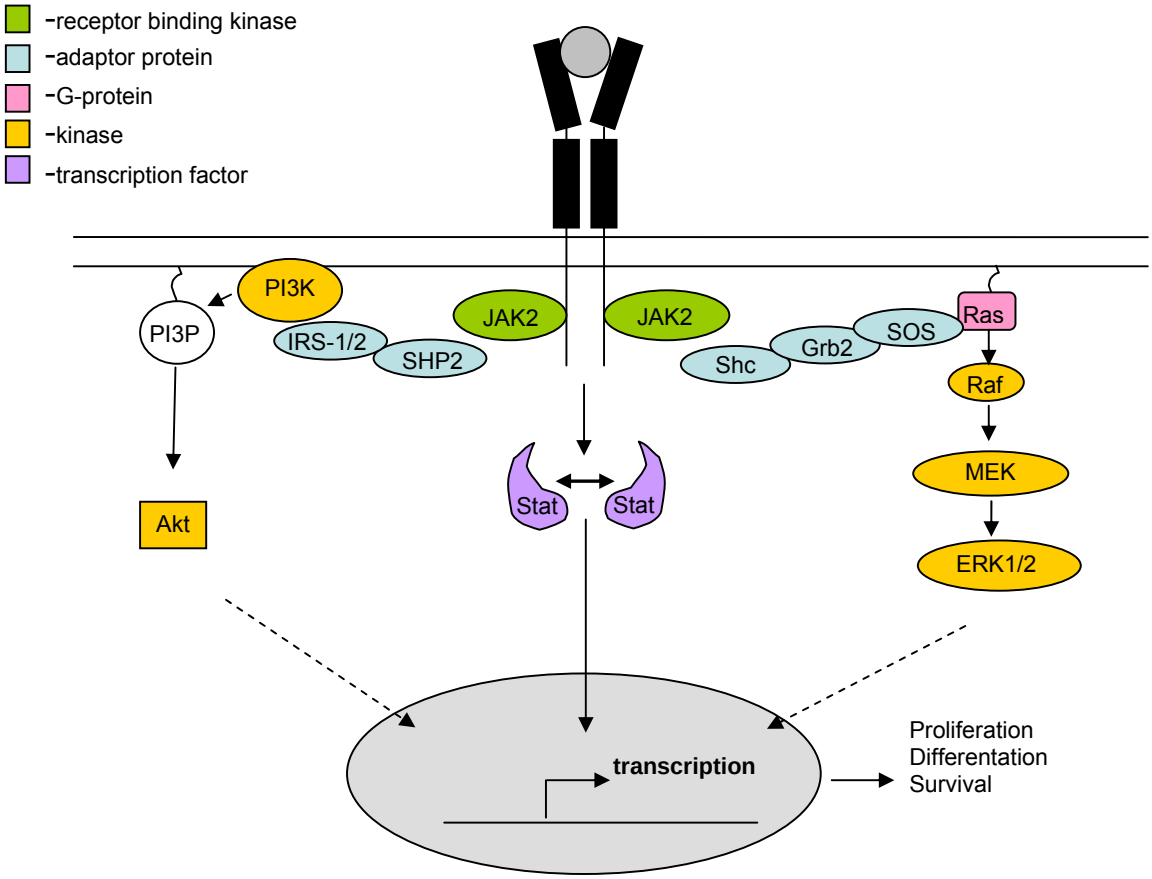
Stat represent a family of conserved proteins, seven of which have been identified in mammals (Stat 1, 2, 3, 4, 5a, 5b, 6). They are transcription factors known to

play a significant role in signal transduction of a wide range of cytokines. After recruitment to the activated receptor Stat becomes phosphorylated by JAK2, dissociates from the receptor, homodimerizes and translocates to the nucleus, where it induces transcription of response genes. For example, Stat3 and Stat5 activate proproliferative and antiapoptotic genes such as cyclin D1 and Bcl-X (Bowman *et al.* 2000). Stat3 has been proposed to regulate neuronal survival, brain maturation and to stimulate recovery after injury; nevertheless the precise role of any of the Stat transcriptional factors in the neural cells is not well known (Cattaneo *et al.* 1999; Schweizer *et al.* 2002).

Activation of PI3K through its recruitment to the activated receptor results in conversion of the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3). PI(3,4,5)P3 anchors the serine/threonine kinase Akt to the plasma membrane and induces a conformational change, which consequently allows phosphorylation of Akt by two currently undefined kinases. Once activated Akt phosphorylates and inactivates members of the Forkhead transcription factor family. Subsequently, this leads to reduced expression of several apoptotic proteins that are normally activated by the Forkhead transcription factors. In the brain the PI3K/Akt pathway is largely associated with neuronal survival (Rodgers and Theibert 2002).

Ligand binding to the cytokine type I receptor also activates monomeric GTPase Ras (Kaushansky 2006). Ras recruits to the cell membrane Raf kinases with their subsequent phosphorylation. Activated Raf is the point of entry into a cascade of the consequent kinases in which Raf phosphorylates and activates MAP/ERK kinase (MEK), and MEK phosphorylates and activates ERK1/2. ERK1/2 targets several cytoskeletal and membrane proteins as well as downstream kinases and a number of transcription factors. Ras/ERK signalling pathway was shown to regulate proliferation, differentiation and cell survival of neural cells (Huang and Reichardt 2003). Activation of ERK in neurons could lead to either cell death or cell survival depending on stimuli (Subramaniam and Unsicker 2006). The classical example of PC12 cells shows that terminal differentiation into neuronal-like cells correlates with sustained activation of ERK, whereas proliferative signals cause its transient activation (Huang and Reichardt 2003).

In general, the contribution of each signalling pathway downstream of the receptor to the activities of hematopoietic growth factors in neural cells is not well understood and may differ in different brain cell types.



Signalling pathways downstream of cytokine type I receptor investigated in the present thesis. Binding of EPO, GCSF or TPO to receptor induces receptor dimerization and activation of receptor-associated Janus tyrosine kinase 2 (JAK2). Tyrosine phosphorylation of the receptor by JAK2 creates high-affinity binding sites for effector molecules with SH2 domains. Effector molecules are recruited to the receptor and phosphorylated by JAK2 kinase. They include adaptor proteins such as SHP2 or Shc and the transcriptional factors such as signal transducer and activator of transcription (Stat).

Once activated two Stat molecules dimerize and in dimer form are translocated to the nucleus, where they act as transcriptional factors.

Recruitment and consequent activation of two adaptor proteins SHP2 and IRS-1/2 leads to activation of a membrane bound phosphatidylinositol 3-kinase (PI3K). Activated PI3K phosphorylates inositol phospholipids to generate phosphatidylinositol-3,4,5-triphosphate (PI3P) providing docking site for serine/threonine kinase Akt. Once activated Akt phosphorylates and regulates a variety of proteins including cytosolic and nuclear targets.

Phosphorylation of Shc promotes association with the Grb2/SOS guanine nucleotide exchange complex activating small GTPase, Ras. Ras activates serine/threonine kinase Raf. Activated Raf leads to the activation of extracellular signal regulated kinase 1/2 (ERK1/2) via the intermediate dual-specificity kinase MEK. Activated ERK1/2 can phosphorylate several cytosolic targets or translocate to the nucleus and phosphorylate several transcriptional factors.

Depending upon the cellular context, Stat, PI3K/Akt and Ras/ERK1/2 signalling pathways have been shown to influence proliferation, differentiation and survival.

2. Focus of the present work

In the two original publications of the present thesis the effects of hematopoietic growth factors in neural cells and the molecular mechanisms executing these effects are investigated.

2.1 Aims of project I

The focus of the first original publication was to investigate functional and signalling interactions between TPO and GCSF in two different brain cell types –neurons and astrocytes. Primary cultures of rat hippocampal neurons and cortical astrocytes were used as models.

Our first aim was to investigate and to compare the molecular mechanisms of the newly discovered proapoptotic activity of TPO with the neuroprotective activity of GCSF in neurons. To study neuroprotection by GCSF, TPO and exposure to hypoxia were used as cell death challenges. We tested whether TPO and GCSF activate signalling molecules such as Stat3, ERK1/2 and Akt. The impact of the activated pathways in GCSF-induced neuroprotection was elucidated using pharmacological inhibitors.

The second aim was to test TPO and GCSF activities and intracellular signalling in astrocytes. We studied the effects of TPO and GCSF on cell viability of astrocytes both under normal and stressed conditions (hypoxia and oxygen-glucose deprivation). Next we elucidated the potential of these factors to influence astrocytic proliferation. Activation of Stat3, ERK1/2 and Akt was determined.

2.2 Aims of project II

The second original publication focuses on the role of Stat5 signalling pathway in neuroprotective and neurotrophic effects of EPO. We elucidated the impact of Stat5 by comparing the neuronal effects of EPO in wild type ($Stat5^{+/+}$) and Stat5 deficient ($Stat5^{-/-}$) neurons. Primary embryonic day 18 (E18) hippocampal neuronal cultures from $Stat5^{+/+}$ and $Stat5^{-/-}$ mice were used.

The first aim was to study the involvement of Stat5 in neuroprotective activity of EPO. Neuroprotective effect was tested after glutamate challenge. Since we found that EPO protected both $Stat5^{+}/^{+}$ and $Stat5^{-}/^{-}$ neurons against glutamate exitotoxicity, we further elucidated the role of the antiapoptotic pathway PI3K/Akt in EPO-mediated effect in Stat5 deficient cells.

Our second aim was to elucidate the importance of functional Stat5 for the neurotrophic properties of EPO. For this purpose, the ability of EPO to stimulate neurite outgrowth was measured in hippocampal cultures from early embryonic stage (E15).

Since $Stat5^{-}/^{-}$ mice are known to have deficits not only in EPO but also in GH signalling, the last aim of the study was to compare EPO and GH effects and intracellular signalling in neurons. GH was also of particular interest for us since its receptor belongs to cytokine type I receptor family which include receptors of all hematopoietic growth factors investigated in the frame of the project.

3. Signalling of TPO and GCSF in neurons and astrocytes

3.1 Overview of project I

Hematopoietic growth factors, first discovered as the regulators of proliferation, differentiation and survival of bone marrow precursor cells, are now shown to have pleiotropic functions in various tissues. The ability of these factors to influence distinct biological processes depends on the expression of appropriate receptors and activation of the specific intracellular signalling pathways. The specific pathway activated may differ in various cell types and mediate distinct biological functions. In the present publication we investigated the interaction between TPO and GCSF in two functionally distinct neural cell types - neurons and astrocytes - both under baseline and hypoxic conditions (see also Focus of the present work, paragraph 2.1). We were specifically interested in the molecular mechanism underlying growth factor effects on neural viability and proliferation.

The novel proapoptotic effect of TPO on cultured hippocampal neurons had been just discovered (Ehrenreich *et al.* 2005) while GCSF was known to protect neurons against different apoptotic inducers both *in vitro* and *in vitro* (Schneider *et al.* 2005; Solaroglu *et al.* 2007). GCSF had been shown to protect cortical astrocytes against ischemic damage following middle cerebral artery occlusion *in vivo* (Solaroglu *et al.* 2006). To the best of our knowledge TPO effects on glial survival as well as the effects of TPO and GCSF on astrocytic proliferation have not been studied before.

In the present study we demonstrated that TPO and GCSF utilized two distinct transduction pathways (Ras/ERK and PI3K/Akt respectively) for their opposite effects on neuronal survival. Next we have shown that in cortical astrocytes TPO and GCSF induced antiproliferative response and had no effect on cellular viability. In astrocytes TPO and GCSF modulate different signalling pathways (Ras/ERK1/2, Stat3) from those activated by these factors in hippocampal neurons. These observations underline the fact that despite high structural similarity between hematopoietic growth factor receptors both effects and signalling pathways stimulated by their activation are different depending on cell type.

The exact role of such diversity of hematopoietic growth factor functions in a physiological context is not fully understood. It is likely that regulated survival and proliferation of neuronal and glial precursors play a role during brain development, especially since the levels of these factors and their receptors are known to be developmentally regulated in the brain (Ehrenreich *et al.* 2005). Since TPO and GCSF promote opposite effects on viability of adult neurons, the balance between these two factors could play a role in brain plasticity. Also taking into account that TPO/GCSF balance is shifted after hypoxic-ischemia damage (TPO level is downregulated, while GCSF level is upregulated (Ehrenreich *et al.* 2005; Schneider *et al.* 2005)) it can be also implicated in repair processes in the injured CNS.

Regulation of cell proliferation and migration during brain development, modulation of neural excitability and survival, and neuron-glia interactions are the research fields in which more substantial evidence for a physiological role of these factors is emerging.

3.2 Original publication:

Byts N, Samoylenko A, Woldt H, Ehrenreich H & Sirén A-L (2006) Cell type specific signaling by hematopoietic growth factors in neural cells, Neurochem Res 31(10): 1219-1230

Personal contribution:

I performed alone all cell culture work on astrocytes and worked on the hippocampal neuronal cultures. I performed alone all experiments involving Western blotting and immunocytochemical staining. I performed data analysis and wrote the first draft of the paper.

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ORIGINAL PAPER

Cell Type Specific Signalling by Hematopoietic Growth Factors in Neural Cells

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Abstract Correct timing and spatial location of growth factor expression is critical for undisturbed brain development and functioning. In terminally differentiated cells distinct biological responses to growth factors may depend on cell type specific activation of signalling cascades. We show that the hematopoietic growth factors thrombopoietin (TPO) and granulocyte colony-stimulating factor (GCSF) exert cell type specific effects on survival, proliferation and the degree of phosphorylation of Akt1, ERK1/2 and STAT3 in rat hippocampal neurons and cortical astrocytes. In neurons, TPO induced cell death and selectively activated ERK1/2. GCSF protected neurons from TPO- and hypoxia-induced cell death via selective activation of Akt1. In astrocytes, neither TPO nor GCSF had any effect on cell viability but inhibited proliferation. This effect was accompanied by activation of ERK1/2 and inhibition of STAT3 activity. A balance between growth factors, their receptors and signalling proteins may play an important role in regulation of neural cell survival.

Keywords Thrombopoietin · Granulocyte colony-stimulating factor · Neurons · Astrocytes · Rat · Phosphorylation

Abbreviations

ECL	enhanced chemoluminescence
EPO	erythropoietin
ERK	extracellular signal-regulated kinase
FCS	foetal calf serum
GCSF	granulocyte colony-stimulating factor
GFAP	glial fibrillary acidic protein
GH	growth hormone
JAK	Janus kinase
MAP	microtubule-associated protein
OGD	oxygen/glucose deprivation
NF-200	neurofilament 200
PI3K	phosphatidylinositol-3′ kinase
STAT	signal transducer and activator of transcription
TPO	thrombopoietin

Introduction

Regeneration and cell survival in the central nervous system is regulated by growth factors [1]. Cell fate and differentiation during brain development depends on correct timing and spatial location of expression of several growth factors. Regenerative capacity of growth factors in terminally differentiated cells may rely on a cell type specific pattern of intracellular signalling that has been programmed during brain

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maturation. In recent years the list of possible candidate substances has expanded from purely brain derived trophic factors to peripheral factors such as bone marrow stimulating growth factors [2–13]. For example, mRNA and protein expression of the hematopoietic growth factors erythropoietin (EPO), thrombopoietin (TPO) and granulocyte colony-stimulating factor (GCSF) and their receptors is found in neural cells [2, 3, 7, 8, 11–14]. Apart from the intensive research efforts concentrating on brain effects of EPO, the role of hematopoietic factors in the brain is still obscure. It is not clear, why hematopoietic growth factors are expressed in the brain, how they interact with each other in neural cells, and why, despite high structural similarity, some factors elicit opposite effects on neuronal function [12].

In the bone marrow precursor cells hematopoietic growth factors act by binding to their specific transmembrane receptors which belong to the cytokine single chain receptor family [14, 15]. Ligand binding leads to receptor dimerization and transphosphorylation of the constitutively receptor bound Janus kinase 2 (JAK2) and to signal transduction through downstream second messenger pathways which in the bone marrow precursor cells include the signal transducers and activators of transcription (STAT), phosphatidyl-inositol-3′ kinase (PI3K)/Akt1 and the Ras/extracellular signal-regulated kinase-1/2 (ERK1/2) [14, 16, 17]. Functional Akt1 and ERK1/2 pathways that are activated quickly upon neurotrophin treatment have been earlier characterized in neurons [18–22]. In neurons, Akt1 and STAT5 have been identified as key cytoprotective pathways upon EPO signalling [12, 23–25] while STAT3, Akt1 and ERK1/2 are activated by GCSF in rat cortical neurons [13].

The aim of the present study was to shed light on the puzzling interaction of brain-expressed hematopoietic growth factors with different brain cells by studying their effects on cell viability both at rest and under stressed (hypoxic) conditions, and by monitoring the phosphorylation status of intracellular signalling intermediates of growth factor receptors in neurons and astrocytes. Specifically, we wanted to elucidate on the mechanism of our unexpected recent finding that TPO is a potent proapoptotic factor for newly generated neurons [12]. Thus we (1) investigated the distinct biological responses to TPO in terminally differentiated neurons and astrocytes under normoxic and hypoxic conditions, (2) studied in detail the cell type specific intracellular signalling mechanisms associated with TPO receptor in these cells, (3) compared the effects of TPO to those elicited by a recently identified neuroprotective growth factor, GCSF.

Experimental procedures

All experiments were approved by and conducted in accordance with the regulations of the local Animal Care and Use Committee

Cell culture

Primary hippocampal neuronal cultures

Hippocampal neuronal cultures were prepared from newborn Wistar–Imamichi rats using an established culture method [26] with slight modifications. We have previously shown by using this method that at day 6 in culture rat hippocampal neurons grow extended neuronal processes, express markers for mature neurons (MAP-2, NF-200), display high vulnerability to hypoxia and show less than 10% contamination with astrocytes [23, 27–29]. Briefly, hippocampi were removed, digested with solution containing papain and DNAse and subjected to density gradient centrifugation. Neuron-enriched fractions were resuspended in serum-free growth medium [Neurobasal A/B27 with 5 ng/ml basic fibroblast growth factor (Invitrogen, Karlsruhe, Germany), 0.5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin]. For survival assay and immuno-cytochemistry, neurons were plated on 12-mm cover slips coated with poly-D-lysine in 4-well-plates, at a density of 5,000 cells per cover slip. For Western blot analysis, cells were grown in poly-D-lysine-coated 6-well-plates at a density of about 100,000 cells per well. Neurons were cultured for 5 days without medium change in a humidified incubator at 37°C, 5% CO_2.

Primary cortical astrocytic cultures

Astrocyte cultures were prepared from newborn Wistar–Imamichi rats as described previously [30]. Briefly, cortices were isolated and homogenized, the homogenate was filtered through a nylon sieve and diluted with glutamine-free DMEM containing 1 g/l glucose, and supplemented with 20% foetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. For survival and proliferation assays and for immunocytochemistry, astrocytes were plated on 24-well-plates, at a density of about 20,000 cells per well. The cells for survival assays were harvested on day 10, for proliferation assays on semiconfluent cultures on day 8 and for immunofluorescence on subconfluent cultures on day 6. For Western blot analysis, 400,000 astrocytes were plated per 10 cm

culture dish and cultured for 10 days. The cells were placed in a humidified incubator at 37°C, 10% CO_2. The medium was changed after 3 days. FCS concentration was lowered after five days either to 10% (for Western blot analysis) or to 3% (for immunocytochemistry, cell survival and proliferation assays). About 98% of cells were positively stained for the astrocytic marker GFAP.

Chemicals

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers: TPO (R&D Systems, Minneapolis, MN, USA), GCSF (Neupogen, Amgen, Munich, Germany), PI3K inhibitor LY294002 and MEK-inhibitors PD98059 and U0126 (Biomol, Plymouth Meeting, PA, USA).

Hypoxia

Medium of *neuronal cultures* on day 5 was replaced with freshly prepared medium without or supplemented with 10^{-10} M TPO and/or 10^{-9} M GCSF and/or 10^{-4} M PI3K inhibitor LY294002. Hypoxic conditions were induced by purging an incubator with N_2 as previously described [27]. Cultures were exposed to hypoxia (<1% O_2, 5% CO_2) or normoxia (control) for 15 h.

Medium of *astrocytic cultures* on day 9 was changed to glutamine- and glucose-free DMEM supplemented with 0.75 mM (glucose deprived) or 5.5 mM (control) glucose with or without 10^{-9} M TPO or 10^{-9} M GCSF. Hypoxic conditions were induced by purging an incubator with N_2 as previously described [31]. Cultures were exposed to hypoxia (<1% O_2, 10% CO_2) or normoxia (control) for 15 h.

Cell survival and proliferation assays

Cell survival was measured by Trypan blue exclusion method. Neuronal survival on day 6 was estimated under the microscope by counting Trypan blue-stained cells on 12-mm cover slips in four distinct, non-overlapping fields of 2 different cover slips, i.e., 100–200 cells, for each condition. Astrocytic survival on day 10 was estimated after astrocytes were treated with trypsin for 5 min at 37°C. After reaction was stopped by FCS the cells were stained with Trypan blue and counted in Neubauer chamber. For each condition six different cover slips were used. For *proliferation assays* astrocytes on day 6 in culture were treated either with vehicle, TPO (10^{-9} M) or GCSF (10^{-9} M) in medium containing 1% FCS, treatments were replenished after 12 h and incubation continued for a total of 36 h. Trypsinized astrocytes were stained with Trypan blue and the viable cells were counted in Neubauer chamber. For each condition six different cover slips were used.

Western blot analysis

Western blot analysis was carried out as described [23]. In brief, rat primary hippocampal neurons (on day 6) or cortical astrocytes (on day 10), untreated or treated with TPO (10^{-10}–10^{-9}M) or GCSF (10^{-9}M) for 10 min, were lysed, total protein was prepared and the protein content was determined using bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (6–8 μg/lane for neurons; 15 μg/lane for astrocytes) were separated by electrophoresis on polyacrylamide NuPage precast gel gradients (Invitrogen) and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies overnight at 4°C. Polyclonal rabbit anti-Akt1 (Cell Signalling Technology, MA, USA), polyclonal rabbit anti-phospho-Akt1 (Ser-473) (Cell Signalling), polyclonal rabbit anti-STAT3 (Cell Signalling), polyclonal rabbit anti-phospho-STAT3 (Tyr-705) (Cell Signalling), polyclonal rabbit anti-ERK-1/2 (Sigma, Taufkirchen, Germany) and monoclonal mouse anti-phospho-ERK-1/2 (Sigma) primary antibodies were used. The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG (Sigma). Enhanced chemoluminescence (ECL) advance (for neurons) and ECL plus (for astrocytes) systems were used for detection (Amersham Biosciences, Buckinghamshire, England). Densitometric analysis was performed using the public domain NIH Image 1.62 program.

Double immunofluorescence

Immunofluorescence staining for detection of phoshorylated Akt1, ERK1/2 and STAT3 as well as for cell markers for mature neurons (MAP-2, NF-200) and astrocytes (GFAP) in neuronal and subconfluent astrocyte cultures, untreated or treated with TPO (10^{-10}–10^{-9} M) or GCSF (10^{-9} M) for 10 min, were performed on day 6. After 4% paraformaldehyde (neurons) or methanol (astrocytes) fixation and blocking and penetration with 10% horse serum in 0.2% Triton-X-PBS, cells were washed with 1% horse serum-PBS and incubated overnight at 4°C with a

cocktail of primary antibodies (1:100–1:2000 in 1% horse serum-PBS). They were then washed with PBS and incubated at room temperature in dark with the appropriate secondary antibody cocktail for 1 h. For streptavidin conjugation the cells were first incubated with biotinylated anti-rabbit antibodies for 1 h followed by streptavidin-Texas-red for 1 h. The cells on cover slips were then washed with PBS, dried and mounted using fluorescence mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). In addition to the antibodies used for Western blot analysis, monoclonal mouse anti-microtubule-associated protein-2 (MAP2) (Chemicon International, Temecula, CA, USA), polyclonal rabbit anti-NF-200 (Affiniti, Mamhead, UK), monoclonal mouse anti-GFAP (Nova-Castra, Newcastle upon Tyne, UK) and polyclonal rabbit anti-GFAP (Sigma), used as cell markers for neurons and astrocytes, respectively. Biotinylated, streptavidin-Texas-red, (Vector Laboratories), Cy-2 or Cy-3 (Jackson Immunotools-Dianova, Hamburg, Germany) conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were used.

Statistical comparisons

Data, expressed as mean ± SEM, were compared using SPSS 12.0 statistical analysis software. Paired Student's *t*-tests were performed. Values $P < 0.05$ were considered to be significant.

Results

Neuronal viability upon treatment with TPO and GCSF

Under normoxic conditions, addition of 10^{-10} M TPO to primary cultures of rat hippocampal neurons increased cell death to about 170% compared to untreated control (Fig. 1a). GSCF (10^{-9} M) alone had no effect on neuronal cell death under normoxic conditions (98 ± 3% of control (= 100%), $n = 4$) but it completely abolished the TPO-induced cell killing when the cultures were simultaneously treated with both GCSF and TPO (Fig. 1a). Treatment of the hippocampal neurons with LY294002 (10^{-4} M), an inhibitor of the PI3K/Akt1-pathway, for 15 h slightly reduced neuronal viability under normoxia (Fig. 1a). At this dose LY294002 completely abolished the protective effect of GCSF, indicating its dependence on a functional Akt1 in neurons. A lower dose of LY294002 (10^{-5} M) was not able to block the protective effect of GCSF against TPO-induced cell death (126 ± 7% with LY294002 vs. 116 ± 8% without LY294002, $n = 2$). We have previously shown that LY294002 (10^{-4} M) selectively blocked phosphorylation of Akt1 without affecting the expression of pERK1/2 or pSTAT5 in this culture model of rat primary hippocampal neurons [23].

We next investigated cell viability of hippocampal neurons under hypoxic conditions (< 1% O_2, 15 h). As previously reported [12, 23], cell death under hypoxia was increased compared to normoxic control (Fig. 1b). When neurons were exposed to hypoxia and simultaneously treated with 10^{-9} M GCSF, the increased cell death upon hypoxia was effectively prevented (Fig. 1b). Again, the protective effect of GCSF was completely reversed when the PI3K/Akt1 pathway was inhibited by 10^{-4} M LY294002 (Fig. 1b). LY2924002 (10^{-4} M) alone did not further increase cell death under hypoxia (Fig. 1b). As previously reported [12], TPO under hypoxia did not modify cell viability of hippocampal neurons (data not shown).

Neuronal signalling upon stimulation with TPO and GCSF

To characterize the intracellular phosphorylation pattern of Akt1, STAT3 and ERK1/2 in primary hippocampal neurons upon stimulation with TPO or GCSF, we quantified the protein expression of their phosphorylated (**p**) and total (**t**) forms after treatment with TPO or GCSF. Stimulation with TPO for 10 min had no significant effect on the ratios of **p**Akt1:**t**Akt1 or **p**STAT3:**t**STAT3 in neurons but increased the level of phosphorylation of ERK1/2 to about 117% as compared to control (Fig. 2a).

In sharp contrast to the pattern of phosphorylation elicited by TPO in neurons, stimulation with GCSF led to a rise in Akt1 phosphorylation: the ratio **p**Akt1:**t**Akt1 increased to 160% as compared to control (Fig. 2b). These findings concur with our pharmacological data showing that inhibition of Akt1 erased the neuroprotective effect of GCSF (Fig. 1a, b). GCSF had no significant influence on the level of ERK1/2 and STAT3 phosphorylation in neurons (Fig. 2b).

Astrocytic viability and proliferation in response to TPO and GCSF

We next investigated whether TPO and GCSF influence cell survival of rat cortical astrocytes. We found no difference in astrocyte viability after 15 h treatment with

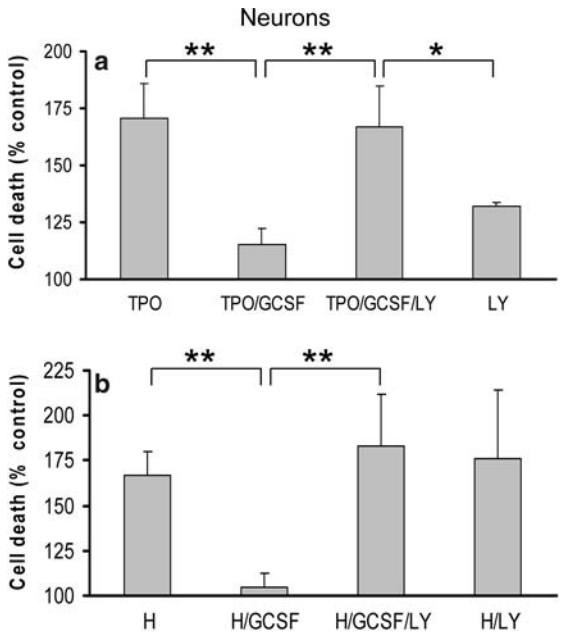

Fig. 1 Effect of TPO and GCSF on cell viability in hippocampal neurons. Primary hippocampal neurons after 5 days in culture were treated **(a)** with 10^{-10} M TPO and/or 10^{-9} M GCSF and/or 10^{-4} M PI3K inhibitor LY294002, as indicated, for 15 h, or **(b)** with 10^{-9} M GCSF and/or 10^{-4} M LY294002, as indicated, and exposed either to hypoxia or normoxia for 15 h. Cell viability on day 6 was determined by Trypan blue dye exclusion method. Cell death in untreated neurons under normoxia (23 ± 2%) was set equal to 100% (= control). Data represent mean ± SEM, n = 4–7, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control; H, hypoxia

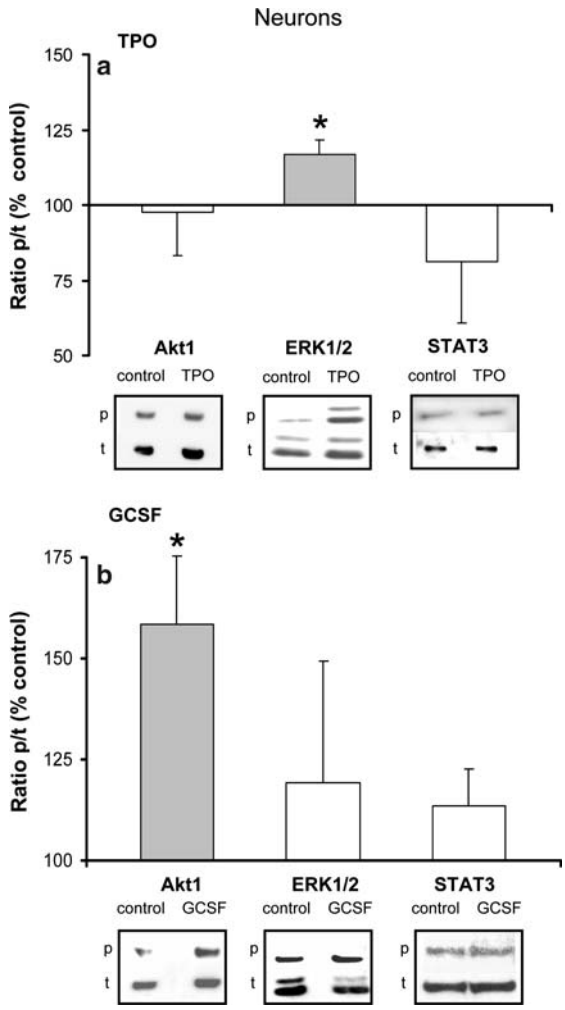

Fig. 2 Effect of TPO and GCSF on activation of intracellular signalling in hippocampal neurons. Primary hippocampal neurons after 6 days in culture were treated **(a)** with 10^{-10} M TPO, or **(b)** with 10^{-9} M GCSF for 10 min. Actual Western blots are shown as insets under each panel. Protein levels were quantified by densitometry after Western blotting with antibodies against either total or phosphorylated forms of Akt1, ERK1/2 or STAT3. The ratio phosphorylated form to total form in unstimulated cells was set equal to 100%. Data represent mean ± SEM, n = 4–6, $^{*}P < 0.05$ compared to control. p, phosphorylated form of protein; t, total form of protein

vehicle or TPO (10^{-9}M) (Fig. 3a). When astrocytes were cultured under hypoxic (H) conditions (<1% O_2 for 15 h), cell death was not different from that observed under normoxia (Fig. 3a) but hypoxia combined with glucose deprivation (oxygen/glucose deprivation, OGD) increased cell death of cortical astrocytes to about 330% as compared to the normoxic control (Fig. 3a). Neither TPO (10^{-9} M) nor GCSF (10^{-9} M) influenced cell viability in astrocyte cultures under conditions of OGD (Fig. 3a).

Since growth factors such as TPO and GCSF are known to stimulate proliferation of bone marrow precursor cells [12, 14, 32], we tested the potential of these factors to influence astrocytic proliferation. On day 6, cultures were treated for 36 h with either vehicle, TPO or GCSF (both 10^{-9} M) under low serum conditions. Under these conditions, cell number per well in the vehicle control was 40000 ± 8000 (n = 8), under TPO treatment 31500 ± 4000 cells/well (n = 8) and under GCSF treatment 23600 ± 5000 cells/well (n = 8), respectively (Fig. 3b). We next tried to block this antiproliferative effect of TPO and GCSF with inhibitors of MEK. The two specific inhibitors tested, PD98059 (10^{-5} M) and U0126 (10^{-5} M) had both a strong antiproliferative effect of their own in our astrocyte cultures and could thus not be used to study the role of ERK 1/2 in the antiproliferative action of TPO and GCSF.

Astrocytic signalling upon stimulation with TPO and GCSF

Phosphorylation patterns of Akt1, STAT3 and ERK1/2 were next studied in rat cortical astrocyte cultures after TPO or GCSF stimulation (both 10^{-9} M). TPO had no

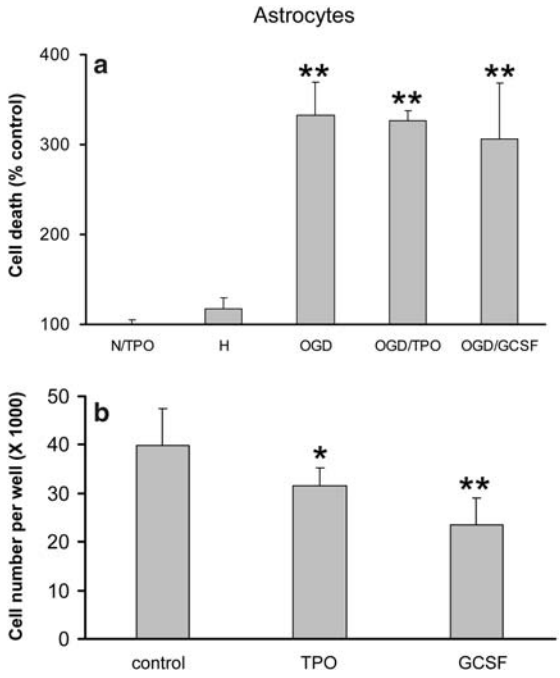

Fig. 3 Effect of TPO and GCSF on cell viability and proliferation in cortical astrocytes. **(a)** Primary astrocytes after 9 days in culture were treated with 10^{-9} M TPO or 10^{-9} M GCSF and exposed either to hypoxia, oxygen/glucose deprivation or normoxia for 15 h, as indicated. Cell viability on day 10 was determined by Trypan blue dye exclusion method. Cell death in untreated astrocytes under normoxia (9 ± 1%) was set equal to 100%. **(b)** Primary astrocytes after 6 days in culture were treated for 36 h with 10^{-9} M TPO or 10^{-9} M GCSF in DMEM supplemented with 1% FCS. Number of trypsinized astrocytes was measured on day 8 in Neubauer chamber. Data are presented as thousands of cells pro one well of a 24-well plate. Data represent mean ± SEM, $n = 8–12$, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control; N, normoxia; H, hypoxia; OGD, oxygen/glucose deprivation

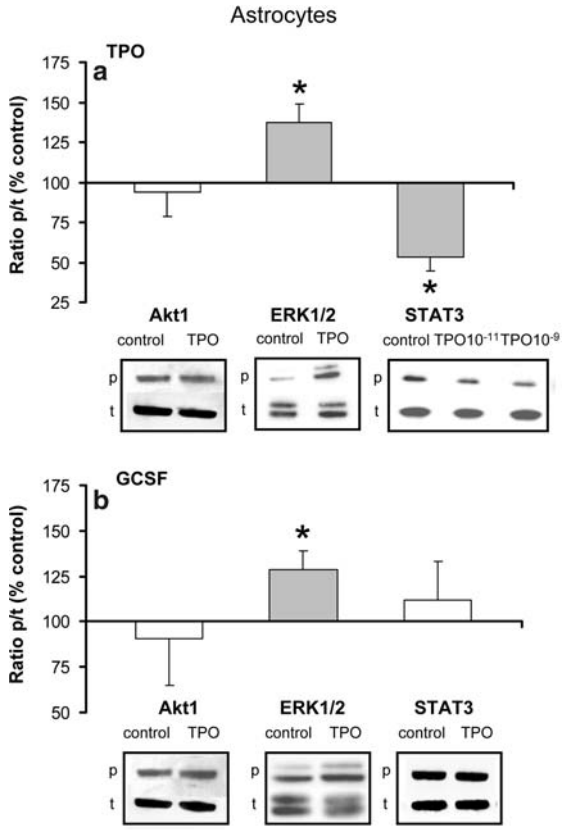

Fig. 4 Effect of TPO and GCSF on activation of intracellular signalling in cortical astrocytes. Primary astrocytes after 10 days in culture were treated **(a)** with 10^{-9} M TPO or **(b)** with 10^{-9} M GCSF for 10 min. Actual Western blots are shown as insets under each panel. Protein levels were quantified by densitometry after Western blotting with antibodies against either total or phosphorylated forms of Akt1, ERK1/2 or STAT3. The ratio phosphorylated form to total form in unstimulated cells was set equal to 100%. Data represent mean ± SEM, $n = 5–10$, $^{*}P < 0.05$ compared to control. p, phosphorylated form of protein; t, total form of protein

effect on the level of Akt1 phosphorylation in astrocytes whereas it increased the ratio **p**ERK1/2:**t**ERK1/2 by about 140% as compared to baseline (Fig. 4a). Remarkably, the level of STAT3 phosphorylation was dose-dependently reduced in astrocytes upon TPO (10^{-11}–10^{-9}M) stimulation (Fig. 4a). In contrast to the powerful Akt1 induction in hippocampal neurons, stimulation of astrocytic cultures with GCSF failed to alter the level of Akt1-phosphorylation but led to a significant increase in ERK1/2 phosphorylation (Fig. 4b). GCSF had no effect on STAT3 activity in astrocytes (Fig. 4b).

Cellular expression of signalling pathways upon stimulation with TPO and GCSF in neurons and astrocytes

Double immunofluorescence staining with antibodies against **p**Akt1, **p**ERK1/2 and **p**STAT3 and specific cell markers for mature neurons (MAP-2, NF-200) and astrocytes (GFAP) confirmed the Western blotting data. Strong expression of neuronal markers was seen in primary hippocampal neuronal cell bodies and processes at day 6 in culture. In agreement with the Western blotting data, expression of **p**Akt1 was very weak in unstimulated or TPO-treated cultures but strongly upregulated upon stimulation with GCSF (Fig. 5a–c). Constitutive expression of **p**ERK1/2 in neurons was seen in all conditions (Fig. 5d–f) but was stronger in TPO-treated cells (Fig. 5e). Expression of **p**STAT3 could not be detected by immunofluoresence in hippocampal neurons (Fig. 5g–i).

In agreement with the results of Western blotting in astrocytic cultures on day 10, we observed a strong constitutive expression of **p**Akt1 in primary astrocytes on day 6 in culture that was not modified by TPO or

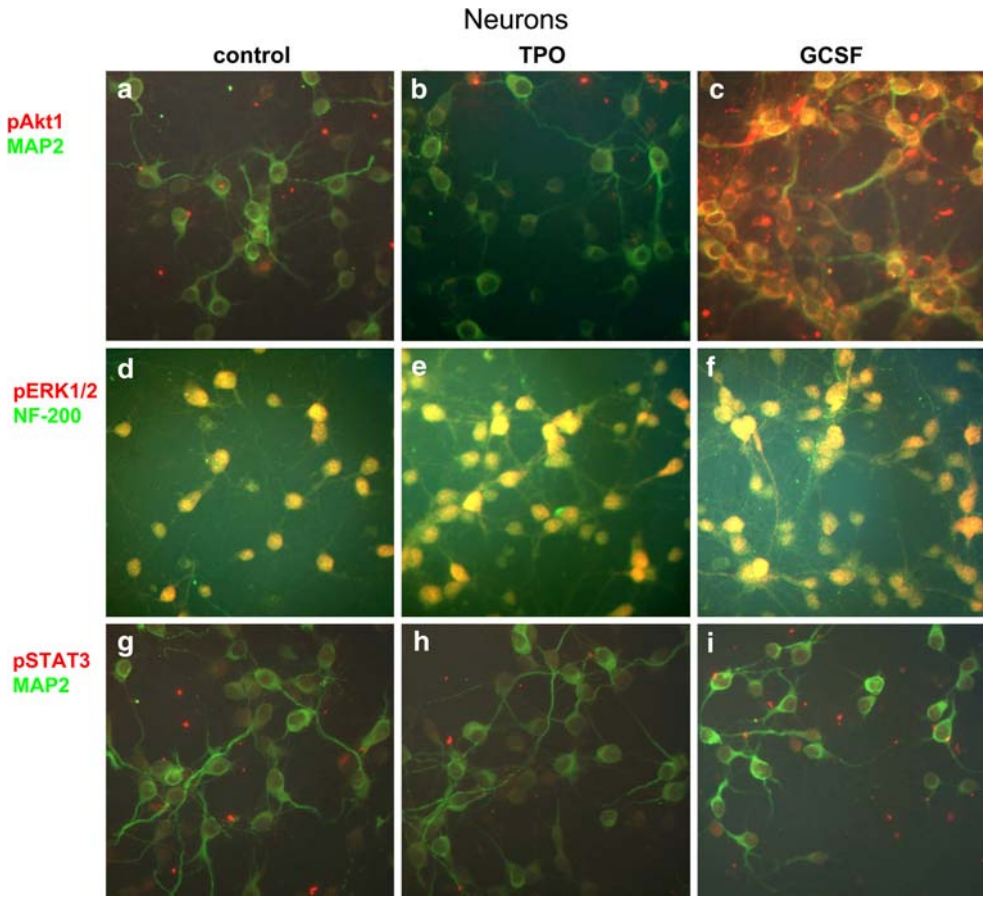

Fig. 5 Immunofluorescence images of pAkt1, pERK1/2 and pSTAT3 in primary hippocampal neurons. **(a–c)** Merged images of double immunofluorescence staining demonstrating pAkt1 in MAP-2 positive neurons at day 6 in culture. (**a)** control, (**b)** after treatment with TPO (10^{-10} M for 10 min), and (**c)** after treatment with GCSF (10^{-9} M for 10 min). Red fluorescence—pAkt1, green fluorescence—MAP2. **(d-f)** Merged images of double immunofluorescence staining demonstrating pERK1/2 in NF-200 positive neurons at day 6 in culture. (**d)** control, (**e)** after treatment with TPO (10^{-10} M for 10 min), and (**f)** after treatment with GCSF (10^{-9} M for 10 min). Red fluorescence—pERK1/2, green fluorescence—NF-200. **(g–i)** Merged images of double immunofluorescence staining demonstrating pSTAT3 in MAP-2 positive neurons at day 6 in culture. (**g)** control, (**h)** after treatment with TPO (10^{-10} M for 10 min), and (**i)** after treatment with GCSF (10^{-9} M for 10 min). Red fluorescence—pSTAT3, green fluorescence—MAP2

GCSF treatments (Fig. 6a–c). Expression of **p**ERK1/2 and **p**STAT3 was also detected in unstimulated astrocytes on day 6 (Fig. 6d, g). In agreement with results using Western blotting, staining for **p**ERK1/2 in GFAP-positive astrocytes was increased after treatment with both TPO and GCSF (Fig. 6d–f). The inhibitory effect of TPO on astrocytic STAT3 activation was verified by immunocytochemistry. After treatment with TPO nuclear staining of **p**STAT3 in astrocytes was dramatically reduced compared to untreated control cultures and to cultures treated with GCSF (Fig. 6g–i).

Discussion

Growth factor expression is critical for undisturbed brain development and functioning. In terminally differentiated cells distinct biological responses to growth factors may depend on cell type specific activation of signalling cascades. As summarized in Fig. 7 we show here that TPO and GCSF exert effects on survival and proliferation via cell type specific intracellular signalling in rat hippocampal neurons and cortical astrocytes. In astrocytes, the most remarkable effect of both TPO and GCSF was inhibition of cell

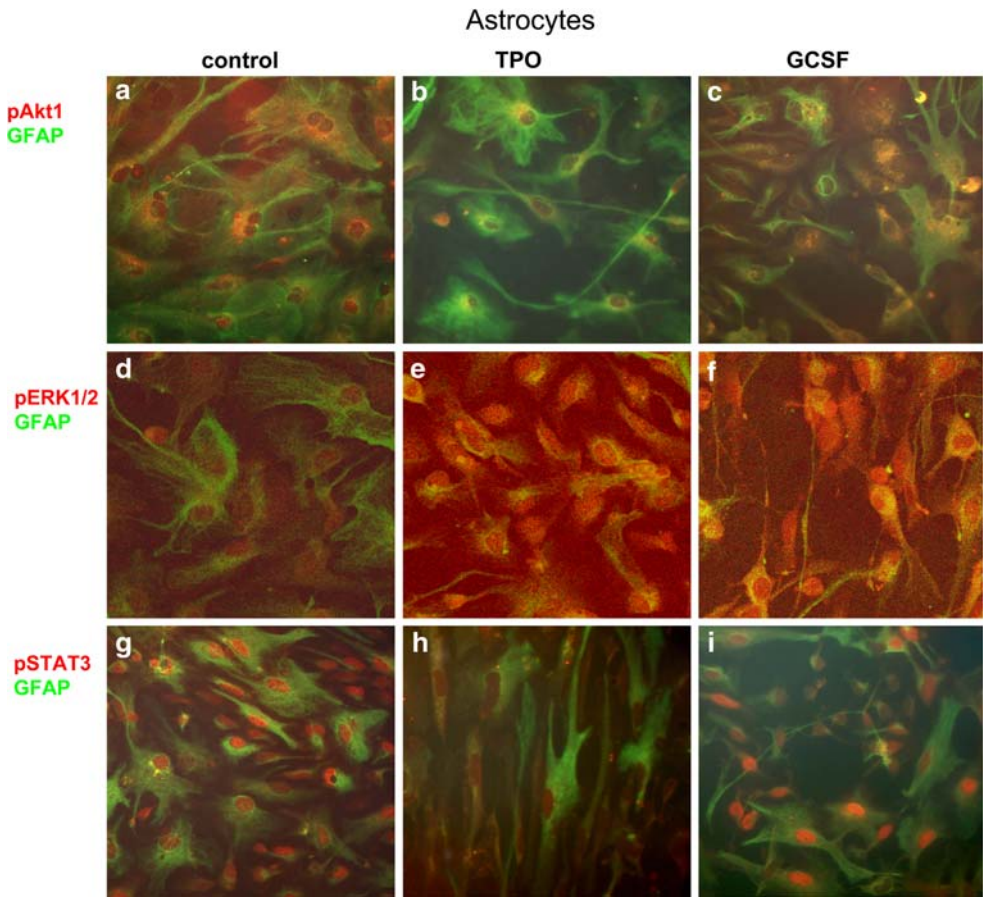

Fig. 6 Immunofluorescence images of pAkt1, pERK1/2 and pSTAT3 in primary cortical astrocytes. **(a–c)** Merged images of double immunofluorescence staining demonstrating pAkt1 in GFAP positive astrocytes at day 6 in culture. (**a**) control, (**b**) after treatment with TPO (10^{-9} M for 10 min), and (**c**) after treatment with GCSF (10^{-9} M for 10 min). Red fluorescence—pAkt1, green fluorescence—GFAP. **(d-f)** Merged images of double immunofluorescence staining demonstrating pERK1/2 in GFAP positive astrocytes at day 6 in culture. (**d**) control, (**e**) after treatment with TPO (10^{-9} M for 10 min), and (**f**) after treatment with GCSF (10^{-9} M for 10 min). Red fluorescence—pERK1/2, green fluorescence—GFAP. **(g-i)** Merged images of double immunofluorescence staining demonstrating pSTAT3 in GFAP positive astrocytes at day 6 in culture. (**g**) control, (**h**) after treatment with TPO (10^{-9} M for 10 min), and (**i**) after treatment with GCSF (10^{-9} M for 10 min). Red fluorescence—pSTAT3, green fluorescence—GFAP

proliferation. Activation of astrocytic ERK1/2 and inhibition of astrocytic STAT3 activity was observed after acute growth factor treatment. In neurons, TPO induced cell death and selectively activated ERK1/2 with no effect on the degree of phosphorylation of either Akt1 or STAT3. Rather in contrast to the effect of TPO, GCSF protected neurons from cell death via selectively activating the neuronal PI3K/Akt1 pathway. A balance between growth factors, their receptors and signalling proteins apparently plays an important role in regulation of neural cell survival and regeneration.

The role of GCSF and TPO in regulation of cell survival has been well characterized in the hematopoietic system. Antiapoptotic activity of these proteins is necessary for the maintenance of a multipotent and undifferentiated state of hematopoietic stem cells [32, 33]. Interestingly, treatment with TPO results in increased expression of the proapoptotic protein Bax in ovarian follicular cells [34]. Since TPO-induced neuronal apoptosis was also blocked by a pan-caspase inhibitor [12], induction of proapoptotic proteins most likely contributed to the cell killing effect of TPO in primary hippocampal neurons.

In the present study we show that TPO increased the level of neuronal ERK1/2 phosphorylation. Ras-ERK1/2 activation seems to be one of the upstream events initiating the proapoptotic cascade upon TPO receptor stimulation in neurons since selective pharmacological inhibition of this pathway completely prevents TPO-induced neuronal death [12]. Paradoxically, activation of ERK1/2 in neurons

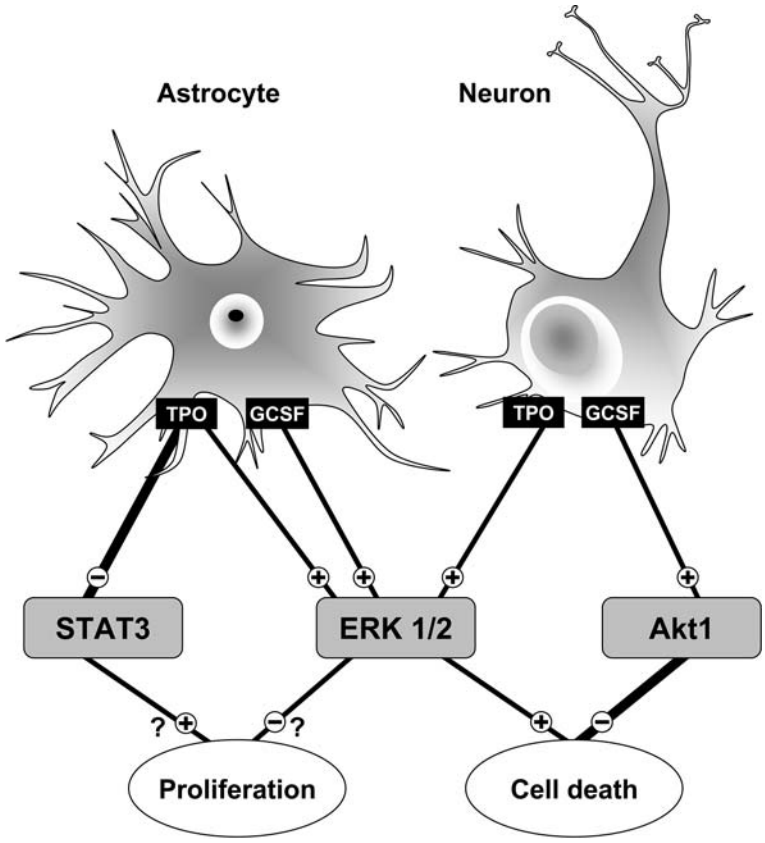

Fig. 7 Summary scheme of the cell type specific signalling pattern of TPO and GCSF in differentiated rat brain cells in culture

has been first linked to cell survival [35] but recent reports have shown the opposite: Blocking ERK1/2 phosphorylation with the MEK inhibitor PD98059 significantly increased cell survival after mechanical trauma and focal cerebral ischemia in vivo [36, 37]. A cell death promoting effect of ERK was also demonstrated in cerebellar granule neurons [38]. In mouse hippocampal and primary cortical neurons oxidative toxicity is associated with persistent activation of ERK1/2 [39, 40] and cell death in this model can be prevented by ERK1/2 inhibition [39, 40].

We previously reported that simultaneous administration of EPO prevented the TPO-induced cell death in cultures of hippocampal neurons [12]. The neuronal PI3K/Akt1 pathway seemed to be fundamental for the neuroprotective action of EPO [12]. In the present study, TPO-induced neuronal death was inhibited by GCSF. Again, this effect was dependent on a functional PI3K/Akt1 signalling pathway (Fig. 1a). Moreover, a robust increase in Akt1 phosphorylation could be seen upon GSCF treatment in neurons (Fig. 2b). In this respect, our data agree with the recent literature demonstrating an increase in Akt1 phosphorylation after an acute 5–15 min treatment with 50 ng/ml ($= 2.7 \times 10^{-9}$ M) GCSF in rat cortical neuronal cultures [13]. These data stress the importance of the PI3K/Akt1 signalling pathway in neuroprotection [12, 13, 23, 24, 41]. Akin to EPO, GCSF protected neurons against hypoxia-induced (Fig. 1b) and glutamate-induced [8, 13] cell death. Similar to our observation in hippocampal neurons, the PI3-kinase inhibitor LY294002 (50 μM) attenuated the protective effect of GCSF against staurosporine-induced death of rat cortical neurons [13]. Both EPO and GCSF have potent neuroprotective properties in vivo as well [8, 13, 42, 43].

This is the first report on the effect of GCSF or TPO on Akt1 phosphorylation in astrocytes. We demonstrate here that both biological effects as well as signalling pathways activated by TPO and GCSF in astrocytes are divergent from those they induced in neuronal cells. TPO or GCSF did not influence astrocytic viability under normoxic or hypoxic conditions, in contrast to their potent actions in neurons. Astrocytes exhibit high level of constitutional Akt1 phoshorylation which could have contributed to the increased hypoxia-resistance of astrocytes as compared to neurons which display low constitutive Akt1 phosphorylation and high vulnerability to hypoxia. The remarkable increase in Akt1 phosphorylation in neurons by GCSF, however, could not be observed in astrocytes. The failure to

activate Akt1 may explain, at least in part, why GCSF was unsuccessful in protecting astrocyte cultures against OGD toxicity.

Previous literature provides controversial evidence for the role of ERK1/2 in astrocytes: Depending on the inducer, cell culture type and condition, activation of astrocytic ERK1/2 has been associated with proliferation [44–46], cell cycle arrest [47], increased apoptosis [48], mitochondrial vacuolation [49] or morphological transformation from flat polygonal to stellate cells [50]. In the present study, TPO and GCSF reduced cell proliferation and activated ERK1/2 in primary rat astrocytes, entailing that activation of ERK1/2 by TPO and GCSF in these primary rat cultures may mediate antiproliferative actions. These findings concur with the observation that a sustained activation of ERK1/2 in primary human astrocytes led to senescence and cell cycle arrest [47]. Interestingly, EPO promoted morphological differentiation and increased astrocyte specific GFAP expression in primary rat astrocyte cultures by inducing ERK1/2 [51]. Elongation of astrocytic processes upon induction of ERK1/2 in rat primary astrocytes has been reported previously [50], and could also be seen in the present study in response to both TPO and GCSF treatment (Fig. 6d–f).

Even if both TPO and GCSF reduced astrocyte proliferation, only TPO inhibited STAT3 activity. STAT3 signalling has been proposed to play a role in reactive astrogliosis after brain injuries [52, 53]. Moreover, STAT3 activation is thought to be important for tumour cell proliferation since inhibition of its activation by JAK inhibitors, antisense oligonucleotides or by overexpression of dominant negative STAT3 led to cell cycle arrest and/or to tumour cell apoptosis in vitro [54–57]. A decrease in STAT3 phosphorylation may thus have contributed to the antiproliferative action of TPO in the present study.

In conclusion, TPO and GSCF differentially regulate cell survival and activation of intracellular signalling pathways in neurons and astrocytes (Fig. 7). The actions of these factors showed remarkable cell type specificity. A balance between growth factors, their receptors and signalling proteins may play an important role in regulation of cell survival in the brain.

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References

1. Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609–642
2. Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, Sasaki R (1998) In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci USA 95:4635–4640
3. Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET, Petit E (1999) A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab 19:643–651
4. Scheepens A, Sirimanne ES, Breier BH, Clark RG, Gluckman PD, Williams CE (2001) Growth hormone as a neuronal rescue factor during recovery from CNS injury. Neuroscience 104:677–687
5. Shingo T, Sorokan ST, Shimazaki T, 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
6. Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, Lin CS, Nikodem VM, Hempstead B, Flanders KC, Costantini F, Noguchi CT (2002) Erythropoietin receptor signalling is required for normal brain development. Development 129:505–516
7. Dame C, Wolber EM, Freitag P, Hofmann D, Bartmann P, Fandrey J (2003) Thrombopoietin gene expression in the developing human central nervous system. Brain Res Dev Brain Res 143:217–223
8. Schabitz WR, Kollmar R, Schwaninger M, Juettler E, Bardutzky J, Scholzke MN, Sommer C, Schwab S (2003) Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. Stroke 34:745–751
9. Kleinschnitz C, Schroeter M, Jander S, Stoll G (2004) Induction of granulocyte colony-stimulating factor mRNA by focal cerebral ischemia and cortical spreading depression. Brain Res Mol Brain Res 131:73–78
10. Knabe W, Knerlich F, Washausen S, Kietzmann T, Sirén AL, Brunnett G, Kuhn HJ, Ehrenreich H (2004) Expression patterns of erythropoietin and its receptor in the developing midbrain. Anat Embryol (Berl) 207:503–512
11. Brines M, Cerami A (2005) Emerging biological roles for erythropoietin in the nervous system. Nat Rev Neurosci 6:484–494
12. Ehrenreich H, Hasselblatt M, Knerlich F, von Ahsen N, Jacob S, Sperling S, Woldt H, Vehmeyer K, Nave KA, Sirén AL (2005) A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain. Proc Natl Acad Sci USA 102:862–867
13. Schneider A, Kruger C, Steigleder T, Weber D, Pitzer C, Laage R, Aronowski J, Maurer MH, Gassler N, Mier W, Hasselblatt M, Kollmar R, Schwab S, Sommer C, Bach A, Kuhn HG, Schabitz WR (2005) The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. J Clin Invest 115:2083–2098
14. Geddis AE, Linden HM, Kaushansky K (2002) Thrombopoietin: a pan-hematopoietic cytokine. Cytokine Growth Factor Rev 13:61–73
15. Schindler C, Strehlow I (2000) Cytokines and STAT signaling. Adv Pharmacol 47:113–174

16. Dong F, Qiu Y, Yi T, Touw IP, Larner AC (2001) The carboxyl terminus of the granulocyte colony-stimulating factor receptor, truncated in patients with severe congenital neutropenia/acute myeloid leukemia, is required for SH2-containing phosphatase-1 suppression of Stat activation. J Immunol 167:6447–6452
17. Zhang Y, Sun S, Wang Z, Thompson A, Kaluzhny Y, Zimmet J, Ravid K (2002) Signaling by the Mpl receptor involves IKK and NF-kappaB. J Cell Biochem 85:523–535
18. Ferrari G, Greene LA (1994) Proliferative inhibition by dominant-negative Ras rescues naive and neuronally differentiated PC12 cells from apoptotic death. Embo J 13:5922–5928
19. Yao R, Cooper GM (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003–2006
20. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665
21. Hetman M, Kanning K, Cavanaugh JE, Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem 274: 22569–22580
22. Susen K, Heumann R, Blochl A (1999) Nerve growth factor stimulates MAPK via the low affinity receptor p75(LNTR). FEBS Lett 463:231–234
23. Sirén AL, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, Keenan S, Gleiter C, Pasquali C, Capobianco A, Mennini T, Heumann R, Cerami A, Ehrenreich H, Ghezzi P (2001) Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci USA 98:4044–4049
24. Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, Priller J, Dirnagl U, 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
25. Um M, Lodish HF (2006) Antiapoptotic effects of erythropoietin in differentiated neuroblastoma SH-SY5Y cells require activation of both the STAT5 and AKT signaling pathways. J Biol Chem 281:5648–5656
26. Brewer GJ (1997) Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71:143–155
27. Lewczuk P, Hasselblatt M, Kamrowski-Kruck H, Heyer A, Unzicker C, Sirén AL, Ehrenreich H (2000) Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin. Neuroreport 11:3485–3488
28. Heyer A, Hasselblatt M, von Ahsen N, Hafner H, Sirén AL, Ehrenreich H (2005) In vitro gender differences in neuronal survival on hypoxia and in 17beta-estradiol-mediated neuroprotection. J Cereb Blood Flow Metab 25:427–430
29. Unzicker C, Erberich H, Moldrich G, Woldt H, Bulla J, Mechoulam R, Ehrenreich H, Sirén AL (2005) Hippocampal cannabinoid-1 receptor upregulation upon endothelin-B receptor deficiency: a neuroprotective substitution effect? Neurochem Res 30:1305–1309
30. Ehrenreich H, Kehrl JH, Anderson RW, Rieckmann P, Vitkovic L, Coligan JE, Fauci AS (1991) A vasoactive peptide, endothelin-3, is produced by and specifically binds to primary astrocytes. Brain Res 538:54–58
31. Hasselblatt M, Lewczuk P, Loffler BM, Kamrowski-Kruck H, von Ahsen N, Sirén AL, Ehrenreich H (2001) Role of the astrocytic ET(B) receptor in the regulation of extracellular endothelin-1 during hypoxia. Glia 34:18–26
32. Haas R, Murea S (1995) The role of granulocyte colony-stimulating factor in mobilization and transplantation of peripheral blood progenitor and stem cells. Cytokines Mol Ther 1:249–270
33. Jacobsen SE, Borge OJ, Ramsfjell V, Cui L, Cardier JE, Veiby OP, Murphy MJ Jr, Lok S (1996) Thrombopoietin, a direct stimulator of viability and multilineage growth of primitive bone marrow progenitor cells. Stem Cells 14 (Suppl 1):173–180
34. Sirotkin AV, Sanislo P, Schaeffer HJ, Florkovicova I, Kotwica J, Bulla J, Hetenyi L (2004) Thrombopoietin regulates proliferation, apoptosis, secretory activity and intracellular messengers in porcine ovarian follicular cells: involvement of protein kinase A. J Endocrinol 183:595–604
35. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331
36. Alessandrini A, Namura S, Moskowitz MA, Bonventre JV (1999) MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Proc Natl Acad Sci USA 96:12866–12869
37. Mori T, Wang X, Jung JC, Sumii T, Singhal AB, Fini ME, Dixon CE, Alessandrini A, Lo EH (2002) Mitogen-activated protein kinase inhibition in traumatic brain injury: in vitro and in vivo effects. J Cereb Blood Flow Metab 22:444–452
38. Subramaniam S, Zirrgiebel U, von Bohlen Und Halbach O, Strelau J, Laliberte C, Kaplan DR, Unsicker K (2004) ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. J Cell Biol 165:357–369
39. Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MR, Johnson JW, DeFranco DB (2000) Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem 275:12200–12206
40. Stanciu M, DeFranco DB (2002) Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. J Biol Chem 277:4010–4017
41. Raghupathi R (2004) Cell death mechanisms following traumatic brain injury. Brain Pathol 14:215–222
42. Six I, Gasan G, Mura E, Bordet R (2003) Beneficial effect of pharmacological mobilization of bone marrow in experimental cerebral ischemia. Eur J Pharmacol 458: 327–328
43. Shyu WC, Lin SZ, Yang HI, Tzeng YS, Pang CY, Yen PS, Li H (2004) Functional recovery of stroke rats induced by granulocyte colony-stimulating factor-stimulated stem cells. Circulation 110:1847–1854
44. Teixeira A, Chaverot N, Strosberg AD, Cazaubon S (2000) Differential regulation of cyclin D1 and D3 expression in the control of astrocyte proliferation induced by endothelin-1. J Neurochem 74:1034–1040
45. Pebay A, Toutant M, Premont J, Calvo CF, Venance L, Cordier J, Glowinski J, Tence M (2001) Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. Eur J Neurosci 13:2067–2076
46. Wang H, Ubl JJ, Stricker R, Reiser G (2002) Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. Am J Physiol Cell Physiol 283:C1351–1364
47. Fanton CP, McMahon M, Pieper RO (2001) Dual growth arrest pathways in astrocytes and astrocytic tumors in response to Raf-1 activation. J Biol Chem 276:18871–18877

48. Pascual M, Valles SL, Renau-Piqueras J, Guerri C (2003) Ceramide pathways modulate ethanol-induced cell death in astrocytes. J Neurochem 87:1535–1545
49. Isobe I, Maeno Y, Nagao M, Iwasa M, Koyama H, Seko-Nakamura Y, Monma-Ohtaki J (2003) Cytoplasmic vacuolation in cultured rat astrocytes induced by an organophosphorus agent requires extracellular signal-regulated kinase activation. Toxicol Appl Pharmacol 193:383–392
50. Abe K, Saito H (2000) The p44/42 mitogen-activated protein kinase cascade is involved in the induction and maintenance of astrocyte stellation mediated by protein kinase C. Neurosci Res 36:251–257
51. Lee SM, Nguyen TH, Park MH, Kim KS, Cho KJ, Moon DC, Kim HY, Yoon do Y, Hong JT (2004) EPO receptor-mediated ERK kinase and NF-kappaB activation in erythropoietin-promoted differentiation of astrocytes. Biochem Biophys Res Commun 320:1087–1095
52. Choi JS, Kim SY, Park HJ, Cha JH, Choi YS, Kang JE, Chung JW, Chun MH, Lee MY (2003) Upregulation of gp130 and differential activation of STAT and p42/44 MAPK in the rat hippocampus following kainic acid-induced seizures. Brain Res Mol Brain Res 119:10–18
53. Sriram K, Benkovic SA, Hebert MA, Miller DB, O'Callaghan JP (2004) Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis in vivo? J Biol Chem 279:19936–19947
54. Bowman T, Garcia R, Turkson J, Jove R (2000) STATs in oncogenesis. Oncogene 19:2474–2488
55. Bromberg J (2002) Stat proteins and oncogenesis. J Clin Invest 109:1139–1142
56. Rahaman SO, Harbor PC, Chernova O, Barnett GH, Vogelbaum MA, Haque SJ (2002) Inhibition of constitutively active Stat3 suppresses proliferation and induces apoptosis in glioblastoma multiforme cells. Oncogene 21:8404–8413
57. Konnikova L, Kotecki M, Kruger MM, Cochran BH (2003) Knockdown of STAT3 expression by RNAi induces apoptosis in astrocytoma cells. BMC Cancer 3:23

4. Signalling of EPO and GH in Stat5 deficient neurons

4.1 Overview of project II

Stat5 protein is a member of the Stat family of transcription factors that control cell fate decisions such as differentiation, proliferation and apoptosis (Buitenhuis *et al.* 2004). Stat5 mediates cellular responses to cytokines, growth factors and a number of hormones (Buitenhuis *et al.* 2004). There are two highly conserved Stat5 isoforms – Stat5a and Stat5b (Cui *et al.* 2004). Studies with Stat5 inactivation and gene knockouts revealed multiple aspects of their physiological and pathophysiological functions: Many features of Stat5a/b deficient mice can be explained by impaired signalling of EPO and GH. In particular, double Stat5a/b knockout mice have a severe anaemic phenotype due to ineffective erythropoiesis caused by decreased survival of early erythroblasts (Socolovsky *et al.* 2001; Cui *et al.* 2004). Lack of Stat5a/b also leads to deficits in GH-controlled body growth (muscle and bone development) accompanied by classical dwarf phenotype (Klover and Hennighausen 2006). Since both EPO and GH were shown to influence brain development and neuronal survival (Scheepens *et al.* 2005; Aberg *et al.* 2006; Chen *et al.* 2006) it is highly plausible that $Stat5^{-/-}$ animals would have some neuronal abnormalities.

EPO was demonstrated to have neuroprotective activity and to increase neurogenesis both *in vivo* and *in vitro*. Involvement of PI3K/Akt and Ras/ERK in EPO-promoted neuronal effects has been confirmed by overexpression of inhibitory proteins and by using pharmacological inhibitors (Brines and Cerami 2005). An increased phosphorylation of Stat5 has been associated with the neuroprotective effects mediated by EPO receptor (Sirén *et al.* 2001; Um and Lodish 2006). However, the definitive role of Stat5 in EPO signalling in the brain is not known.

In vivo evidence suggests that GH has neuroprotective effects in the cerebral cortex, hippocampus and thalamus after hypoxic-ischemic injury (Aberg *et al.* 2006). Only a limited number of studies have addressed possible protective effects of GH on neuronal cells and the role of GH in neurite outgrowth or neuronal differentiation *in vitro* (Scheepens *et al.* 2005; Aberg *et al.* 2006). The GH-mediated pathways remain to be insufficiently characterized in the CNS (Aberg *et al.* 2006). Stat5 pathway is one of a few

pathways shown to be directly activated by GH in neurons (Bennett *et al.* 2005) but the role of its activation is not known.

In this study we elucidated the role of Stat5 in EPO and GH signalling in neuronal survival (neuroprotective activity) and neurite outgrowth (trophic activity) (see also Focus of the present work). We found that EPO utilized different signalling pathways for its neuroprotective (PI3K/Akt) as compared to its trophic (Stat5) effects. In regard to GH, Stat5 was essential both for its protective and trophic activities and at least for its neuroprotective effect Stat5 signalling seems to be upstream of the PI3K/Akt pathway. The understanding of exact molecular mechanisms underlying activities of such multifunctional factors as EPO and GH is expected to have a substantial impact on clinical neurobiology, offering attractive possibilities in therapies for traumatic and degenerative diseases of the CNS.

4.2 Original publication:

Byts N, Samoylenko A, Ivanisevic M, Hennighausen L, Ehrenreich H & Sirén A-L Essential role for Stat5 in the neurotrophic but not in the neuroprotective effect of erythropoietin. Submitted as *Letter-to-the-Editor* to “Cell Death and Differentiation” on March 1, 2007.

Personal contribution:

I established the mouse hippocampal neuronal cultures, performed all culture work including toxicity and neurite outgrowth assays and Western blotting. I performed data analysis and wrote the first draft of the manuscript.

Essential role for Stat5 in the neurotrophic but not in the neuroprotective effect of erythropoietin

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Running head: Role of functional Stat5 in neurons

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ABSTRACT

The transcription factors signal transducer and activator of transcription 5a and 5b (Stat5) are activated by the neuroprotective and neurotrophic growth factors, erythropoietin (EPO) and growth hormone (GH). Here we show a dissociation of the EPO-induced intracellular pathway for cell death protection from that needed for its neurotrophic activity in hippocampal neuronal cultures from Stat5a/b knockout ($Stat5^{-/-}$) mice. The importance of functional Stat5 for neuroprotective effects of EPO and GH was investigated as their ability to protect neurons against glutamate-induced cell death**.** EPO counteracted glutamate-induced cell death both in $Stat5^{+/+}$ and $Stat5^{-/-}$ neurons. In contrast, GH effectively protected only $Stat5^{+/+}$ cells but had no effect on glutamate toxicity in $Stat5^{-/-}$ neurons. LY294002, an inhibitor of phosphatidylinositol-3' kinase, completely abolished the protective effect of EPO in $Stat5^{+/+}$ and $Stat5^{-/-}$ neurons and reduced the neuroprotective action of GH in $Stat5^{+/+}$ neurons. Both EPO and GH stimulated neurite outgrowth in $Stat5^{+/+}$ neurons but had no trophic effect in $Stat5^{-/-}$ cells. We conclude that EPO utilizes Akt to induce neuroprotection but requires Stat5 for its neurotrophic activity, while the pathways mediating neuronal actions of GH appear to be overlapping with Stat5 being upstream of Akt and executing GH signaling.

Keywords: EPO, growth hormone, glutamate, Ara-C, neurite outgrowth, hippocampal neuronal culture, Stat5a/b knockout mouse

Abbreviations: Ara-C, cytosine β-D-arabinofuranoside; EPO, erythropoietin; EPOR, EPO receptor; ERK, extracellular regulated kinase; GH, growth hormone; GHR, GH receptor; PI3K, phosphatidylinositol-3' kinase; STAT, signal transducer and activator of transcription.

Dear Editor,

The transcription factors signal transducer and activator of transcription 5a and 5b (Stat5) are activated by the neuroprotective and neurotrophic growth factor, erythropoietin (EPO) in neurons and neural stem cells[1-5]. Stat5 has been suggested to mediate protective effects of EPO since activation of Stat5 in neurons accompanies the antiapoptotic effect of EPO[4, 5]. To directly test whether activation of Stat5 in neurons is essential for EPO actions we examined the neuroprotective and neurotrophic effects of EPO in hippocampal neuronal cultures from Stat5a/b knockout ($Stat5^{-/-}$) mice[6]. Since Stat5 has been shown to be crucial for intracellular signaling and the somatotrophic effects of the EPO-related cytokine, growth hormone (GH)[7, 8], we studied in parallel its effects in the $Stat5^{-/-}$ neurons.

$Stat5^{-/-}$ mice are severely anaemic, growth retarded and the vast majority dies perinataly[6]. Hippocampal neurons from embryonal day 18 (E18) $Stat5^{-/-}$ mice and control littermates were cultured and the basal cell death rate on day 9 (DIV9) were similar (5.7±0.8%, n=17 and 4.1±0.5%, n=18, respectively). This demonstrates that Stat5, similar to EPO receptor (EPOR)[9] and Akt[10, 11], is not essential for basal neuronal survival. However, similar to findings in $EPOR^{-/-}$ [9] and $Akt^{-/-}$ [10, 11] cells, neuronal survival of $Stat5^{-/-}$ cultures was greatly reduced when the cells were cultured until DIV9 in the continued presence of a proapoptotic agent. After treatment with cytosine β-D-arabinofuranoside (Ara-C, 3 μM, added 48h after plating) $Stat5^{-/-}$ neurons were severely damaged in contrast to relatively mild damage of $Stat5^{+/+}$ neurons (Fig. 1a). Downregulation of phosphatidylinositol-3' kinase (PI3K)/Akt and Ras/ERK pathways have been reported to accompany the proapoptotic effect of Ara-C[12]. Here we show that Stat5 deficiency augmented Ara-C toxicity in hippocampal neurons. Inhibition of DNA repair has been shown to underlie apoptosis of postmitotic neurons upon Ara-C exposure[13]. Since DNA damaging insults cause more severe damage in immature neurons lacking functional DNA repair mechanisms than in mature neurons[13, 14], an increased vulnerability to Ara-C in neurons lacking Stat5 might reflect a delay in neuronal maturation. We next examined glutamate induced toxicity in $Stat5^{-/-}$ neurons. Glutamate, the main excitatory neurotransmitter in the mammalian brain, is in high concentrations toxic through activation of its receptors and subsequent intracellular Ca^{2+} overload[15]. In

contrast to the decreased sensitivity of maturing neurons to Ara-C toxicity[13, 14], the vulnerability to glutamate increases with neuronal maturation[15]. In the present study we observed reduced susceptibility of Stat5$^{-/-}$ hippocampal neurons to glutamate (Fig. 1a). There was less cell death 24h after glutamate exposure (200 µM, 15 min on DIV8 followed by return to conditioned medium) in Stat5$^{-/-}$ as compared to Stat5$^{+/+}$ neurons, with 39±2% dead cells in Stat5$^{-/-}$ (n=13) vs 51±4% in Stat5$^{+/+}$ (n=15) cultures ($p<0.02$). Since the Stat5 pathway is commonly associated with cell survival[3-5], a Stat5 mediated toxicity upon glutamate treatment seems unlikely. The increased vulnerability of Stat5$^{-/-}$ cells to Ara-C-induced apoptosis but their increased tolerance to glutamate toxicity may point to a disturbed neuronal maturation in the absence of Stat5.

To explore the importance of functional Stat5 for the neuroprotective and neurotrophic effects of EPO[1], we investigated its ability to protect neurons against glutamate-induced cell death in primary hippocampal neuronal cultures from Stat5$^{-/-}$ and Stat5$^{+/+}$ mice**.** Before glutamate addition cells were pretreated for 16 h with EPO or GH at concentrations known to provide protection against neurotoxic agents[16, 17]. EPO at concentrations of 1, 3 and 10 nM counteracted glutamate-induced cell death both in Stat5$^{+/+}$ and Stat5$^{-/-}$ neurons (Fig. 1b). In contrast, GH at identical neuroprotective[19] concentrations effectively protected Stat5$^{+/+}$ cells against glutamate toxicity but had no effect on glutamate toxicity in Stat5$^{-/-}$neurons (Fig. 1c).

Since EPO protected both Stat5$^{+/+}$ and Stat5$^{-/-}$ neurons from glutamate-induced cell death, we next elucidated whether the antiapoptotic PI3K/Akt pathway[1, 4, 5] plays a role in its neuroprotective effect. The PI3K-inhibitor, LY294002 (30 µM) completely blocked Akt phosphorylation up to 16 h after its administration, while the lower dose of 10 µM induced only a transient inhibition of Akt activity (Supplemental Fig.). LY294002 (30 µM on DIV7) slightly increased baseline cell death on DIV9 but did not further enhance cell death in glutamate treated neurons (Fig. 1d). The protective effect of EPO was completely abolished both in Stat5$^{+/+}$ and Stat5$^{-/-}$ cells after treatment with LY294002 (Fig. 1d). LY294002 also reduced the neuroprotective action of GH in Stat5$^{+/+}$ neurons (Fig. 1d). These data indicate that Akt, but not the Stat5 pathway, is necessary for the neuroprotective effect of EPO and that both Stat5 and Akt pathways are involved in GH-induced neuroprotection.

We next studied the potential of EPO and GH to stimulate neurite outgrowth in primary hippocampal cultures isolated from E15 Stat5$^{-/-}$ and Stat5$^{+/+}$ fetuses. The neurotrophic effect of EPO and GH was determined by counting the number of primary processes per cell on DIV3 after beta-tubulin III staining (see Supplemental Materials and Methods). The number of primary neurites was not significantly different between untreated Stat5$^{-/-}$ and Stat5$^{+/+}$ cultures (1.56±0.14, n=5 versus 1.34±0.12 neurites/cell, n=7, respectively). Treatment with EPO at 10 nM concentration stimulated neurite outgrowth in Stat5$^{+/+}$ but not in Stat5$^{-/-}$ cells (Fig. 1e, f). The growth promoting effect of GH on primary neurites was apparent at all concentrations in Stat5$^{+/+}$ neurons whereas it was completely lost in Stat5$^{-/-}$ cells (Fig. 1e, g) indicating that Stat5 signaling is needed for both EPO and GH stimulated neurite outgrowth in primary hippocampal neurons. Stat5 may play a role in neuronal plasticity also in other systems as shown recently by the ability of a constitutively activated Stat5 construct to reduce axonal outgrowth defects in spinal muscular atrophy like motor neurons[18]. The neurotrophic activity of EPO[19, 20] was described before its cytoprotective and antiapoptototic effects were discovered in neuronal cells[1, 4, 5, 9, 16]. Here we show a clear dissociation of the EPO-induced intracellular pathway for cell death protection (Akt) from that needed for its neurotrophic activity (Stat5) (Fig. 1h). In regards to the findings with GH our data agree with a previous report demonstrating reduced brain size and sparser dendritic arborisation in GH receptor deficient (GHR$^{-/-}$) mice[21]. In this context the pathways appear to be overlapping with Stat5 being upstream of Akt and executing GH signaling (Fig. 1h).

ACKNOWLEDGEMENTS:

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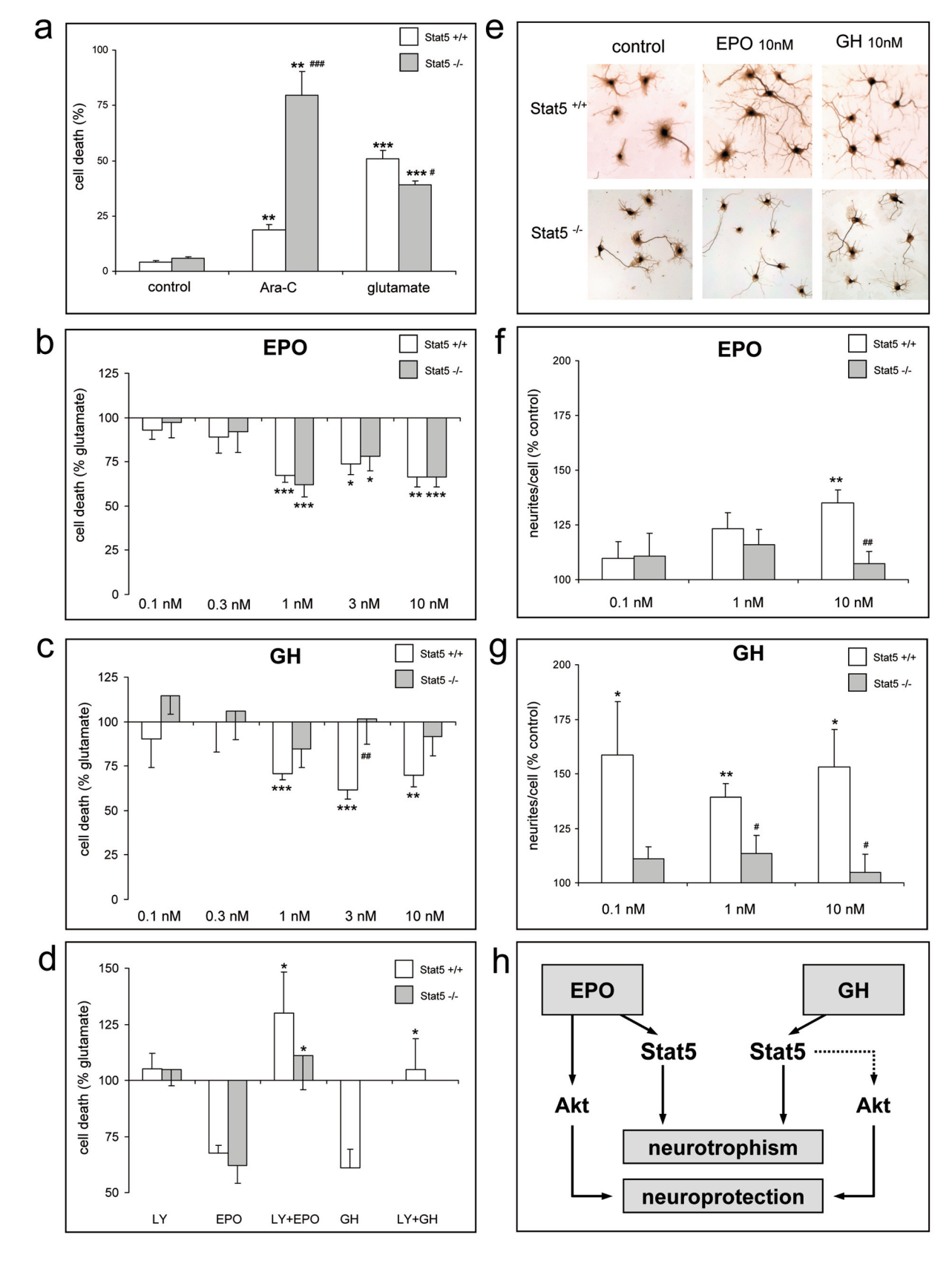
REFERENCES

1. Brines, M. & Cerami, A. (2005) *Nat Rev Neurosci* **6,** 484-494.
2. Liu, J.*, et al.* (2005) *Stroke* **36,** 1264-1269.
3. Shingo, T.*, et al.* (2001) *J Neurosci* **21,** 9733-9743.
4. Sirén, A. L.*, et al.* (2001) *Proc Natl Acad Sci U S A* **98,** 4044-4049.
5. Um, M. & Lodish, H. F. (2006) *J Biol Chem* **281,** 5648-5656.
6. Cui, Y.*, et al.* (2004) *Mol Cell Biol* **24,** 8037-8047.
7. Klover, P. & Hennighausen, L. (2006) *Endocrinology* Dec 7 Epub ahead of print.
8. Kofoed, E. M.*, et al.* (2003) *N Engl J Med* **349,** 1139-1147.
9. Yu, X.*, et al.* (2002) *Development* **129,** 505-516.
10. Tschopp, O.*, et al.* (2005) *Development* **132,** 2943-2954.
11. Yang, Z. Z.*, et al.* (2005) *Mol Cell Biol* **25,** 10407-10418.
12. Leeds, P.*, et al.* (2005) *Neurochem Int* **46,** 61-72.
13. Courtney, M. J.&Coffey, E. T. (1999) *Eur J Neurosci* **11,** 1073-1084.
14. Romero, A. A.*, et al.* (2003) *J Neurochem* **84,** 1275-1287.
15. King, A. E.*, et al.* (2006) *J Comp Neurol* **498,** 277-294.
16. Morishita, E.*, et al.* (1997) *Neuroscience* **76,** 105-116.
17. Silva, C.*, et al.* (2003) *Ann Neurol* **54,** 605-614.
18. Ting, C. H.*, et al.* (2007) *Hum Mol Genet* Jan 12 Epub ahead of print.
19. Konishi, Y.*, et al.* (1993) *Brain Res* **609,** 29-35.
20. Tabira, T.*, et al.* (1995) *Int J Dev Neurosci* **13,** 241-252.
21. Ransome, M. I.*, et al.* (2004) *Eur J Neurosci* **19,** 2069-2079.

FIGURE LEGENDS

Fig. 1 a, Baseline cell death and sensitivity to Ara-C or glutamate in E18 DIV9 primary hippocampal neurons from $Stat5^{+/+}$ and $Stat5^{-/-}$ mice. Cell death was determined on DIV9 after treatment with vehicle, 3 μM Ara-C (added 48 h after plating, n=4) or glutamate (200 μM for 15 min on DIV8, n=13-15). **p<0.01 and ***p<0.001, as compared to vehicle-treated condition; $^{\#}$p<0.05, $^{\#\#\#}$p<0.001 between $Stat5^{-/-}$ and $Stat5^{+/+}$; **b,c** Effect of EPO (n=5-11) or GH (n=4-8) on glutamate toxicity in $Stat5^{+/+}$ or $Stat5^{-/-}$ E18 DIV9 hippocampal neurons. Growth factors in indicated concentrations or vehicle were added to the medium 16 h prior to glutamate (200 μM for 15 min on DIV8). Cell death was evaluated 24 h later. Glutamate-induced cell death was set equal to 100%; *p<0.05, **p<0.01 and ***p<0.001 as compared to vehicle-treated condition; $^{\#\#}$p<0.01, between $Stat5^{+/+}$ and $Stat5^{-/-}$ neurons; **d** Role of Akt in EPO and GH-mediated protection from glutamate toxicity. $Stat5^{+/+}$ and $Stat5^{-/-}$ hippocampal neurons were treated for 20 min with the selective PI3K inhibitor, LY294002 (30 μM) or vehicle before addition of growth factors (EPO/GH) or vehicle on DIV7. After 16 h incubation cells were exposed to glutamate (200 μM for 15 min). Cell death was evaluated 24 h later. Glutamate-induced cell death was set equal to 100%. Maximal neuroprotective dose of EPO (10 nM) and GH (3 nM) was used. (n=3-8); *p<0.05, as compared to growth factor-treated condition. **e**, Representative examples of beta-tubulin-III staining in DIV3 E15 $Stat5^{+/+}$ and $Stat5^{-/-}$ hippocampal neurons; **f-g**, Neurotrophic effects of EPO (n=5) and GH (n=5-7) on DIV3 E15 hippocampal cells. Vehicle, EPO or GH in indicated concentrations were added to cells 1 h after plating and then replenished at 24 h and 48 h. Cells were fixed at 72 h and stained with beta-tubulin-III. Number of primary neurites per cell was counted. Neurite outgrowth in vehicle treated condition was set equal to 100%. *p<0.05 and **p<0.01, as compared to vehicle-treated condition; $^{\#}$p<0.05 and $^{\#\#}$p<0.01, between $Stat5^{+/+}$ and $Stat5^{-/-}$ neurons; **h**, Proposed mechanism of neuroprotective and neurotrophic actions of EPO and GH in hippocampal neurons. Data in each graph are expressed as mean ± SEM; for more detailed description of methods see Supplemental Material and Methods.


a
Stat5 +/+
Stat5 -/-
cell death (%)
control
Ara-C
glutamate
b
EPO
cell death (% glutamate)
0.1 nM
0.3 nM
1 nM
3 nM
10 nM
c
GH
d
LY
EPO
LY+EPO
GH
LY+GH
e
control
EPO 10nM
GH 10nM
Stat5 +/+
Stat5 -/-
f
EPO
neurites/cell (% control)
g
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Stat5
Akt
Akt
neurotrophism
neuroprotection

SUPPLEMENTAL FILES

MATERIALS AND METHODS

All experiments were approved by and conducted in accordance with the regulations of the local Animal Care and Use Committee.

Chemicals

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers: rhEPO (Janssen-Cilag, Neuss, Germany), rhGH (Immuno Tools, Friesoythe, Germany), PI3K inhibitor LY294002 (Biomol, Plymouth Meeting, PA, USA), L-glutamic acid sodium salt hydrate (Sigma, Taufkirchen, Germany).

Breeding and genotype analysis

Stat5 hemizygous and wild-type mice colonies were raised in a C57BL/6 background from a pair of $Stat5^{+/-}$ mice kindly provided by Prof. Hennighausen (Bethesda, MD, USA). The wild-type Stat5a/b allele was detected by PCR analysis using primer 1 (5'-GAA AGC ATG AAA GGG TTG GAG-3') and primer 2 (5'-AGC AGC AAC CAG AGG ACT AC-3') as a recombinant band of 450 bp, while the deleted Stat5a/b allele was determined by using a pair of primer 2 and primer 3 (5'-CCC ATT ATC ACC TTC TTT ACA G-3') as a band of 500 bp. $Stat5^{-/-}$ fetuses appeared pale and smaller then their littermates. Genotype of each $Stat5^{-/-}$ fetus used for cultures was confirmed by PCR.

Cell culture

For cell survival studies DIV9 primary hippocampal neuronal cultures derived from embryonic day 18 (E18) $Stat5^{+/+}$ or $Stat5^{-/-}$ mice were used. Briefly, after complete removal of meninges, hippocampi were dissected in warm HBSS solution (Invitrogen, Karlsruhe, Germany) supplemented with penicillin and streptomycin and trypsinized. After mechanical trituration cells were plated on poly-D-lysin-coated glass cover slips in 4-well plates in a density of 13 000 cells/cm^2. Neurons were cultured in MEM/ B27 medium (Invitrogen) supplemented with sodium bicarbonate, sodium pyruvate, L-glutamine, penicillin, streptomycin and 0.6% glucose. Cultures were incubated at 37°C under 5% CO_2/ 95% air and 90% humidity without medium

exchange up to 9 days. Contamination with glial fibrillary acidic protein positive astrocytes was consistently less than 8%.

For differentiation assay primary hippocampal cells were isolated from E15 mice as described above for E18 mice. Cells were seeded in a low density of 6 000 cells/cm^2 and cultured up to 3 days.

Neurotoxicity assay

Ara-C induced neurotoxicity: Ara-C (cytosine β-D-arabinofuranoside, 3 µM) or vehicle (PBS) was added directly to the culture medium 48 h after plating. Cells were grown without medium exchange up to 9 days in the continued presence of agent. On day *in vitro* 9 (DIV9) cell death was estimated by Trypan blue dye exclusion method. Approximately 700-1200 cells per condition in six distinct, non-overlapping fields of 2 different cover slips were counted directly under the microscope. The percentage of neuronal cell death was determined as the ratio between the number of Trypan blue incorporating (non-viable) cells and the total number of cells.

Glutamate induced neurotoxicity: Primary neurons on DIV7 were treated with EPO, GH in the concentration range of 10^{-10}-10^{-8} M or vehicle (PBS). In some experiments cultures were pre-incubated for 20 min with 30 µM LY294002 or vehicle (0.03% DMSO) 16 h after addition of growth factors the cells were exposed to glutamate (200 µM L-glutamic acid) for 15 min, returned to conditioned medium and cultured for additional 24 h in the continued presence of growth factors. Cell death on DIV9 was determined by Trypan blue dye exclusion method (see above).

Western blot

Primary wild-type neuronal cultures on DIV7 were treated with 10nM EPO or vehicle after pre-incubation for 20 min with 10-30 µM LY294002 or vehicle (0.03% DMSO) and proteins extracted after 10 min or 16 h. Proteins (60 µg) were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed overnight at 4°C with anti-Akt (1:200; Cell Signalling Technology, MA, USA), anti-phospho-Akt (1:200; Ser-473, Cell Signalling), anti-ERK1/2 (1:10000; Sigma) or anti-phospho-ERK1/2 (1:2000; Sigma) antibodies. Antigens were detected by enhanced chemiluminescence kit (Amersham Bioscience, Freiburg, Germany) after incubation with appropriate horseradish peroxidise-IgG

conjugates (Sigma). Densitometric analysis was performed using the public domain ImageJ program.

Neurotrophic assay

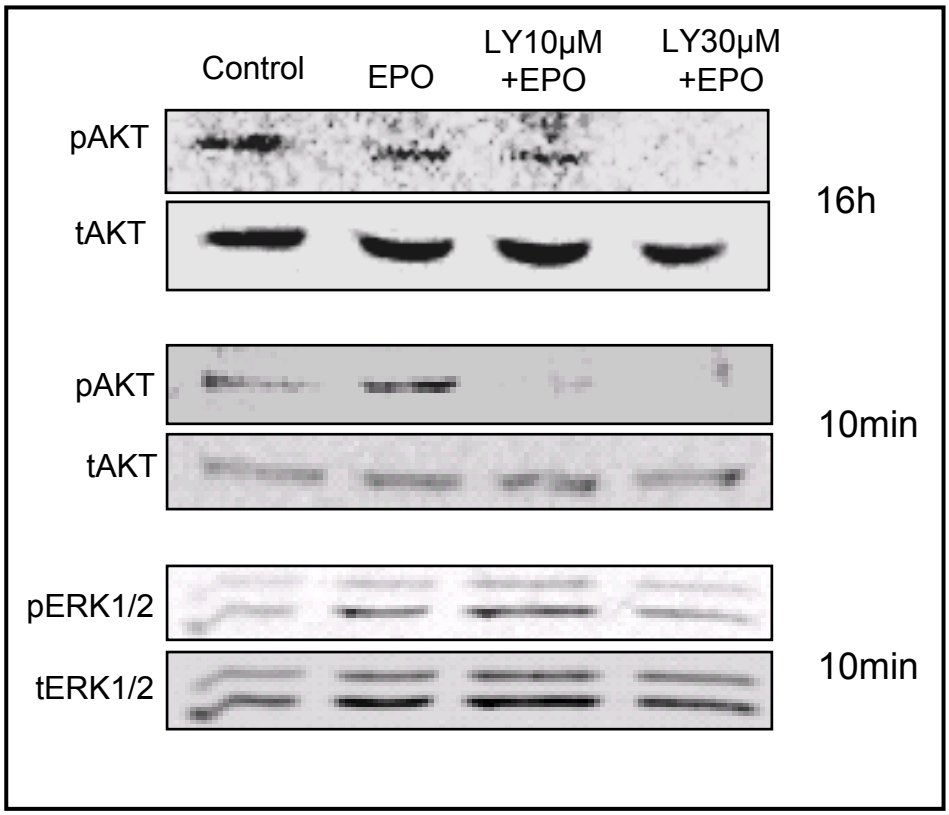
EPO, GH (both at 10^{-10}-10^{-8}M) or vehicle were added directly to the culture medium 1 h after seeding (on DIV0) and then every 24 h. On DIV3 (72 h after seeding) cultures were immunostained for β-tubulin III, a marker of neuronal processes. Briefly, cultures were fixed with 4% paraformaldehyde and treated with 10% normal horse serum in 0.2% Triton X100-PBS. After overnight incubation with anti-β-tubulin III antibody (1:1000 in 1% normal horse serum-PBS; Sigma) at 4°C cells were labelled with biotinylated anti-mouse IgG (1:200 in 1.5% normal horse serum-PBS; Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. Following incubation with ABC kit reagents (mixure of avidin DH and biotinylated horseradish peroxidase H; Vector) immunoreactivity was detected by peroxidase-mediated deposition of diaminobenzidine (Sigma). 100-150 neurons in 8 non-overlapping fields from two cover slips per condition were photographed using Axiophot 2 (Zeiss) microscope. Virtually all (99%) of the cells in our cultures were immunoreactive for β-tubulin III. Because of the young age of dissociation and the short time *in vitro*, cells did not reach maturity; nevertheless most of them form primary neurites and have dendrites distinguishable from axons. To determine the effect of growth factors on neurite outgrowth the number of primary processes per cell was counted.

Statistical comparisons

Data, expressed as mean ± SEM, were compared using SPSS 12.0 Statistical analysis software. Values $p<0.05$ were considered to be significant.

Supplemental Figure Representative Western blots. Wild type E18 hippocampal neurons were treated on DIV7 with EPO (10 nM) or vehicle after 20 min pre-incubation with 10-30 μM LY294002 or vehicle. Cells were harvested either 16 h (*upper panel*) or 10 min (*middle panel*) after EPO treatment and the levels of phosphorylated and total Akt were detected by Western blot analysis. Specificity of LY294002 to inhibit only PI3K/Akt signalling, phosphorylated and total ERK1/2 levels were detected 10 min after EPO addition (*lower panel*).

Supplemental Figure



5. Summary

In the first original publication we investigated effects and signalling pathways of TPO and GCSF in cultured hippocampal neurons and cortical astrocytes.

We have shown that TPO induced neuronal cell death via ERK1/2 activation without influence on Stat3 and PI3K/Akt pathways. In agreement with our results the involvement of Ras/ERK signalling in regulation of neuronal death has been reported previously (Ehrenreich *et al.* 2005; Subramaniam and Unsicker 2006). GCSF counteracted neuronal death induced either by TPO or by hypoxia. This neuroprotective effect was blocked by treatment with a selective inhibitor of PI3K, LY294002. GCSF also induced Akt phosphorylation in our neuronal cultures. Therefore, TPO and GCSF provided opposite effects on neuronal survival not via differential regulation of the same pathway but influencing distinct signalling cascades. Our data are in line with the previously reported role of PI3K/Akt pathway in antiapoptotic action of GCSF against camptothecin-induced cell death in cortical neurons (Schneider *et al.* 2005). Although GCSF neuroprotection was shown before to be accompanied by Stat3 activation (Schneider *et al.* 2005; Solaroglu *et al.* 2006), in our experimental model GCSF did not influence Stat3 phosphorylation.

In contrast to neurons, TPO influenced cell survival of astrocytes neither under normoxic conditions nor after oxygen-glucose deprivation. It is known that in toxic environment astrocytes can survive and display reactive changes while neurons in their surrounding die (Nedergaard and Dirnagl 2005). The inability of TPO to kill glial cells stresses neuron- specific action of this agent. Selectivity of the proapoptotic effect of TPO was reported in regard to neuronal maturation stage as well (Ehrenreich *et al.* 2005). While being a strong protective agent in neurons, GCSF had neither protective activity against oxygen-glucose deprivation nor an influence on phosphorylation pattern of Akt in astrocytes. Interestingly, *in vivo* GCSF was shown to reduce number of caspase-3 positive astrocytes in the cortex following middle cerebral artery occlusion (Solaroglu *et al.* 2006).

Here we have demonstrated that both TPO and GCSF downregulated proliferation of cortical astrocytes. To study which intracellular pathways can execute these effects we tested whether TPO / GCSF influenced phosphorylation patterns of Akt,

Stat3 or ERK1/2 in astrocytes. We detected upregulation in ERK1/2 and downregulation in Stat3 activities after TPO treatment and upregulation of ERK1/2 after GCSF treatment. Sustained ERK1/2 activation was previously reported to lead to cell cycle arrest in normal human astrocytes (Fanton *et al.* 2001), while Stat3 activation is typical for astrocytic tumors (gliomas) (Bowman *et al.* 2000). Nevertheless, in our study we were not able to prove the relation between effects on proliferation and signalling induced by TPO/ GCSF in astrocytes: Due to a strong intrinsic antiproliferative effect of ERK1/2 inhibitors (PD98059 and U0126) as well as toxic side effects of Stat3 inhibitor peptide, the use of these inhibitors in our glial cultures was not possible.

Taken together, TPO and GCSF promoted opposite effects on neuronal survival, activating distinct intracellular pathways (Ras/ERK and PI3K/Akt respectively). TPO and GCSF had similar effects on astrocytic proliferation, activating Ras/ERK pathway. The present study is one of the first shedding light on the complex regulation of different brain cell types by TPO and GCSF. Additional work is needed to clarify the precise mechanisms of TPO/ GCSF interplay in the brain.

In the second study we investigated the involvement of Stat5 in EPO-promoted neurotrophic and neuroprotective effects.

We tested EPO-induced neuroprotection against glutamate exitotoxicity in wild type ($Stat5^{+}/^{+}$) and Stat5 deficient ($Stat5^{-}/^{-}$) mice. EPO protected both $Stat5^{-}/^{-}$ and $Stat5^{+}/^{+}$ neurons to the same extend. However, its effect was completely abolished in the presence of the PI3K inhibitor, LY294002. Therefore our data argue against the involvement of Stat5 in EPO-dependent neuroprotection. In line with the previous studies (Sirén *et al.* 2001; Rodgers and Theibert 2002) our data show that the PI3K/Akt cascade is the major protective pathway used by EPO in hippocampal neurons.

EPO was shown to stimulate neurite outgrowth in mouse primary neuronal cultures from the septum (Tabira *et al.* 1995) as well as to enhance neurite regrowth in cultured retinal ganglionic cells following optic nerve lesion (Kretz *et al.* 2005) and in rodent models of Parkinson's disease *in vivo* (McLeod *et al.* 2006). Here we demonstrated the ability of EPO to stimulate neurite formation in primary embryonic hippocampal cells. Involvement of Stat5 pathway in EPO-promoted trophic effects was not described before. Though Stat5 activation after exposure to EPO was detected in

neural stem cells (NSC) cultures, its relation to EPO effects remained undefined (Shingo *et al.* 2001). In our study EPO-stimulated neurite outgrowth was lost in Stat5 deficient cells indicating an essential role of Stat5 in this effect. The involvement of PI3K/Akt pathway was not possible to investigate in our experimental model due to toxicity of LY 294002.

The last aim of this study was to compare EPO-induced effects and intracellular signalling in neurons with those induced by GH. Protection mediated by GH was lost in $Stat5^{-/-}$ neurons. However, treatment with LY294002 reduced GH-mediated protection in $Stat5^{+/+}$ neurons suggesting that both Stat5 and PI3K/Akt pathways were necessary for GH actions. While it is largely accepted that upon GH treatment Stat5 is directly phosphorylated by JAK2 (Carter-Su *et al.* 2000), the precise molecular mechanism of PI3K activation remains unclear. Our data support the hypothesis that PI3K/Akt pathway is activated downstream of Stat5. One possibility of such consequent activation could be a local production of insulin like growth factor 1 (IGF-1) by GH in neurons (Aberg *et al.* 2006). Recent studies have suggested the involvement of Stat5 in GH stimulated IGF-1 production (Woelfle *et al.* 2003). In such scenario PI3K/Akt pathway is activated downstream to IGF-1 receptor (Aberg *et al.* 2006). In our experimental model we have not distinguished between direct and IGF-1- mediated effects of GH due to relatively long period of incubation with growth factor.

In the present study we have shown that GH enhanced neurite outgrowth in mouse hippocampal cells isolated from E15 embryos. The effect of GH on neurite formation is poorly studied. In one study GH did not influence neurite formation in dissociated cultures of neural stem cells (Scott *et al.* 2006). In line with our results, *in vivo* data showed that in GH receptor knockout mice dendritic branching of pyramidal neurons appeared sparser and the brain size was smaller (Ransome *et al.* 2004). Since we have detected GH-stimulated neurite formation in $Stat5^{+/+}$ but not in $Stat5^{-/-}$ neurons we conclude that Stat5 is essential for neurotrophic effects of GH.

6. Literature

Aberg, N. D., K. G. Brywe and J. Isgaard (2006). Aspects of growth hormone and insulin-like growth factor-I related to neuroprotection, regeneration, and functional plasticity in the adult brain. *ScientificWorldJournal* 6:53-80.

Bennett, E., L. McGuinness, E. F. Gevers, G. B. Thomas, I. C. Robinson, H. W. Davey and S. M. Luckman (2005). Hypothalamic STAT proteins: regulation of somatostatin neurones by growth hormone via STAT5b. *J Neuroendocrinol* 17:186-194.

Bowman, T., R. Garcia, J. Turkson and R. Jove (2000). STATs in oncogenesis. *Oncogene* 19:2474-2488.

Brines, M. and A. Cerami (2005). Emerging biological roles for erythropoietin in the nervous system. *Nat Rev Neurosci* 6:484-494.

Buitenhuis, M., P. J. Coffer and L. Koenderman (2004). Signal transducer and activator of transcription 5 (STAT5). *Int J Biochem Cell Biol* 36:2120-2124.

Carter-Su, C., L. Rui and J. Herrington (2000). Role of the tyrosine kinase JAK2 in signal transduction by growth hormone. *Pediatr Nephrol* 14:550-557.

Cattaneo, E., L. Conti and C. De-Fraja (1999). Signalling through the JAK-STAT pathway in the developing brain. *Trends Neurosci* 22:365-369.

Chen, Z. Y., R. Warin and C. T. Noguchi (2006). Erythropoietin and normal brain development: receptor expression determines multi-tissue response. *Neurodegener Dis* 3:68-75.

Cui, Y., G. Riedlinger, K. Miyoshi, W. Tang, C. Li, C. X. Deng, G. W. Robinson and L. Hennighausen (2004). Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Mol Cell Biol* 24:8037-8047.

Ehrenreich, H., D. Degner, J. Meller, M. Brines, M. Behe, M. Hasselblatt, H. Woldt, P. Falkai, F. Knerlich, S. Jacob, N. von Ahsen, W. Maier, W. Bruck, E. Ruther, A. Cerami, W. Becker and A. L. Sirén (2004). Erythropoietin: a candidate compound for neuroprotection in schizophrenia. *Mol Psychiatry* 9:42-54.

Ehrenreich, H., M. Hasselblatt, C. Dembowski, L. Cepek, P. Lewczuk, M. Stiefel, H. H. Rustenbeck, N. Breiter, S. Jacob, F. Knerlich, M. Bohn, W. Poser, E. Ruther, M. Kochen, O. Gefeller, C. Gleiter, T. C. Wessel, M. De Ryck, L. Itri, H. Prange, A. Cerami, M. Brines and A. L. Sirén (2002). Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol Med* 8:495-505.

Ehrenreich, H., M. Hasselblatt, F. Knerlich, N. von Ahsen, S. Jacob, S. Sperling, H. Woldt, K. Vehmeyer, K. A. Nave and A. L. Sirén (2005). A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain. *Proc Natl Acad Sci U S A* 102:862-867.

Fanton, C. P., M. McMahon and R. O. Pieper (2001). Dual growth arrest pathways in astrocytes and astrocytic tumors in response to Raf-1 activation. *J Biol Chem* 276:18871-18877.

Geddis, A. E., H. M. Linden and K. Kaushansky (2002). Thrombopoietin: a pan-hematopoietic cytokine. *Cytokine Growth Factor Rev* 13:61-73.

Hasselblatt, M., H. Ehrenreich and A. L. Sirén (2006). The brain erythropoietin system and its potential for therapeutic exploitation in brain disease. *J Neurosurg Anesthesiol* 18:132-138.

Huang, E. J. and L. F. Reichardt (2003). Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* 72:609-642.

Kaushansky, K. (2006). Lineage-specific hematopoietic growth factors. *N Engl J Med* 354:2034-2045.

Klover, P. and L. Hennighausen (2006). Postnatal body growth is dependent on the transcription factors Stat5a/b in muscle: a role for autocrine / paracrine IGF-1. *Endocrinology*.

Kretz, A., C. J. Happold, J. K. Marticke and S. Isenmann (2005). Erythropoietin promotes regeneration of adult CNS neurons via Jak2/Stat3 and PI3K/AKT pathway activation. *Mol Cell Neurosci* 29:569-579.

McLeod, M., M. Hong, K. Mukhida, D. Sadi, R. Ulalia and I. Mendez (2006). Erythropoietin and GDNF enhance ventral mesencephalic fiber outgrowth and capillary proliferation following neural transplantation in a rodent model of Parkinson's disease. *Eur J Neurosci* 24:361-370.

Nedergaard, M. and U. Dirnagl (2005). Role of glial cells in cerebral ischemia. *Glia* 50:281-286.

Ransome, M. I., Y. Goldshmit, P. F. Bartlett, M. J. Waters and A. M. Turnley (2004). Comparative analysis of CNS populations in knockout mice with altered growth hormone responsiveness. *Eur J Neurosci* 19:2069-2079.

Rodgers, E. E. and A. B. Theibert (2002). Functions of PI 3-kinase in development of the nervous system. *Int J Dev Neurosci* 20:187-197.

Schabitz, W. R., R. Kollmar, M. Schwaninger, E. Juettler, J. Bardutzky, M. N. Scholzke, C. Sommer and S. Schwab (2003). Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. *Stroke* 34:745-751.

Scheepens, A., T. A. Moderscheim and P. D. Gluckman (2005). The role of growth hormone in neural development. *Horm Res* 64:66-72.

Schneider, A., C. Kruger, T. Steigleder, D. Weber, C. Pitzer, R. Laage, J. Aronowski, M. H. Maurer, N. Gassler, W. Mier, M. Hasselblatt, R. Kollmar, S. Schwab, C. Sommer, A. Bach, H. G. Kuhn and W. R. Schabitz (2005). The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J Clin Invest* 115:2083-2098.

Schweizer, U., J. Gunnersen, C. Karch, S. Wiese, B. Holtmann, K. Takeda, S. Akira and M. Sendtner (2002). Conditional gene ablation of Stat3 reveals differential signaling requirements for survival of motoneurons during development and after nerve injury in the adult. *J Cell Biol* 156:287-297.

Scott, H. J., M. J. Stebbing, C. E. Walters, S. McLenachan, M. I. Ransome, N. R. Nichols and A. M. Turnley (2006). Differential effects of SOCS2 on neuronal differentiation and morphology. *Brain Res* 1067:138-145.

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

Sirén, A. L. and H. Ehrenreich (2001). Erythropoietin--a novel concept for neuroprotection. *Eur Arch Psychiatry Clin Neurosci* 251:179-184.

Sirén, A. L., M. Fratelli, M. Brines, C. Goemans, S. Casagrande, P. Lewczuk, S. Keenan, C. Gleiter, C. Pasquali, A. Capobianco, T. Mennini, R. Heumann, A. Cerami, H. Ehrenreich and P. Ghezzi (2001). Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci U S A* 98:4044-4049.

Smithgall, T. E. (1998). Signal transduction pathways regulating hematopoietic differentiation. *Pharmacol Rev* 50:1-19.

Socolovsky, M., H. Nam, M. D. Fleming, V. H. Haase, C. Brugnara and H. F. Lodish (2001). Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. *Blood* 98:3261-3273.

Solaroglu, I., V. Jadhav and J. H. Zhang (2007). Neuroprotective effect of granulocyte-colony stimulating factor. *Front Biosci* 12:712-724.

Solaroglu, I., T. Tsubokawa, J. Cahill and J. H. Zhang (2006). Anti-apoptotic effect of granulocyte-colony stimulating factor after focal cerebral ischemia in the rat. *Neuroscience* 143:965-974.

Subramaniam, S. and K. Unsicker (2006). Extracellular signal-regulated kinase as an inducer of non-apoptotic neuronal death. *Neuroscience* 138:1055-1065.

Tabira, T., Y. Konishi and F. Gallyas, Jr. (1995). Neurotrophic effect of hematopoietic cytokines on cholinergic and other neurons in vitro. *Int J Dev Neurosci* 13:241-252.

Um, M. and H. F. Lodish (2006). Antiapoptotic effects of erythropoietin in differentiated neuroblastoma SH-SY5Y cells require activation of both the STAT5 and AKT signaling pathways. *J Biol Chem* 281:5648-5656.

Woelfle, J., J. Billiard and P. Rotwein (2003). Acute control of insulin-like growth factor-I gene transcription by growth hormone through Stat5b. *J Biol Chem* 278:22696-22702.

7. Short description of current project and list of publications

Initial characterization of Stat5$^{-/-}$ neurons was performed in regard to the role of Stat5 in the signalling of EPO and the data obtained were included to the second original publication. A more general characterization of Stat5 signalling in the brain is ongoing. Currently we test the hypothesis that Stat5 is important for neuronal maturation. We investigate the activity, expression and distribution of different developmentally regulated glutamate receptor subunits and study the expression and distribution of specific pre- and postsynaptic markers in primary neuronal cultures isolated from wild type (Stat5$^{+/+}$) and Stat5 knockout (Stat5$^{-/-}$) mice using confocal imaging, Western blotting and pharmacological tools such as ifenprodil (a specific antagonist of the glutamate NMDA receptor containing the NR2B subunit).

Publications:

1) **Byts NV**, Khyzhniak SV, Ukr Biokhim Zh 2004; 76:17-22.
Expression of the lipopolysaccharide receptors in the rat liver cells under inflammatory and normal conditions.
2) **Byts N**, Samoylenko A, Woldt H, Ehrenreich H, Sirén A-L, Neurochem Res 2006; 31: 1219-1230.
Cell type specific signaling by hematopoietic growth factors in neural cells
3) **Byts N**, Samoylenko A, Ivanisevic M, Hennighausen L, Ehrenreich H, Sirén A-L Essential role for Stat5 in the neurotrophic but not in the neuroprotective effect of erythropoietin.(submitted)
4) Samoylenko A, **Byts N**, Ehrenreich H, Sirén A-L
Thrombopoietin inhibits nerve growth factor-induced neuronal differentiation and Ras/ERK1/2 signalling. (in preparation)

8. Curriculum vitae

April 17, 1980 Born in Kiev, Ukraine

Citizenship Ukraine

May 1997 Graduated from Kiev Physical and Mathematical Lyceum Nr. 145.

September 1, 1997 Entered Kiev National Taras Shevchenko University, biological faculty.

June 27, 2001 Graduated from Kiev National Taras Shevchenko University (biological faculty, department of biochemistry), obtained the basic high education in specialty ”Biology”, having the Bachelor Diploma (honoured) and a qualification of a bachelor-biologist and teacher of biology. Defended graduation theses in theme “Characterization of the effects of ionizing irradiation on the structure of the membranes of mitochondria from small intestine enterocytes”.

State Exam: “developmental biology” with excellent mark, June 2001.

June 27, 2002 Graduated from Kiev National Taras Shevchenko University (biological faculty, department of biochemistry), obtained the full high education in specialty ”Biochemistry”, having the Specialist Diploma (honoured) and a qualification of biologist-biochemist and teacher of biology. Defended graduation theses in theme “Characterization of structural changes in the membranes of mitochondria from small intestine enterocytes after treatment with ionising irradiation and cadmium ions” is with an excellent mark.

State Exam: “Biochemistry “with excellent mark, June 2002

January 15, 2003 Became a PhD student in GRK 335 “Klinische, Zelluläre und Molekulare Biologie Innere Organe” on the project “LPS-signal transduction in rat Kupffer cells and hepatocytes. Involvement of different putative LPS receptors: CD14, Toll-like receptor-4 and CD11/CD18” under supervision of Dr. Schieferdecker.

Left GRK due to departure of supervisor.

October 1, 2003 Became a PhD student in GRK 632 “Neuroplasticity: from Molecules to Systems” on the project “Signalling of hematopoietic growth factors in mammalian neural cells” under supervision of Prof. Dr. Dr. Ehrenreich.