Molecular Psychiatry (2004) 9, 42–54

www.nature.com/mp

ORIGINAL RESEARCH ARTICLE

Erythropoietin: a candidate compound for neuroprotection in schizophrenia

H Ehrenreich[1], D Degner[1], J Meller[2], M Brines[3], M Béhé[2], M Hasselblatt[1], H Woldt[1], P Falkai[4], F Knerlich[1], S Jacob[1], N von Ahsen[5], W Maier[4], W Brück[6], E Rüther[1], A Cerami[3], W Becker[2,*] and A-L Sirén[1]

[1]*Max-Planck-Institute for Experimental Medicine and Department of Psychiatry, Georg-August-University, Goettingen, Germany;* [2]*Department of Nuclear Medicine, Georg-August-University, Goettingen, Germany;* [3]*The Kenneth S Warren Institute, Kitchawan, NY, USA;* [4]*Department of Psychiatry, Friedrich-Wilhelm-University, Bonn, Germany;* [5]*Department of Clinical Chemistry, Georg-August-University, Goettingen, Germany;* [6]*Department of Neuropathology, Georg-August-University, Goettingen, Germany*

Erythropoietin (EPO) is a candidate compound for neuroprotection in human brain disease capable of combating a spectrum of pathophysiological processes operational during the progression of schizophrenic psychosis. The purpose of the present study was to prepare the ground for its application in a first neuroprotective add-on strategy in schizophrenia, aiming at improvement of cognitive brain function as well as prevention/slowing of degenerative processes. Using rodent studies, primary hippocampal neurons in culture, immunohistochemical analysis of human post-mortem brain tissue and nuclear imaging technology in man, we demonstrate that: (1) peripherally applied recombinant human (rh) EPO penetrates into the brain efficiently both in rat and humans, (2) rhEPO is enriched intracranially in healthy men and more distinctly in schizophrenic patients, (3) EPO receptors are densely expressed in hippocampus and cortex of schizophrenic subjects but distinctly less in controls, (4) rhEPO attenuates the haloperidol-induced neuronal death *in vitro*, and (4) peripherally administered rhEPO enhances cognitive functioning in mice in the context of an aversion task involving cortical and subcortical pathways presumably affected in schizophrenia. These observations, together with the known safety of rhEPO, render it an interesting compound for neuroprotective add-on strategies in schizophrenia and other human diseases characterized by a progressive decline in cognitive performance.
Molecular Psychiatry (2004) **9**, 42–54. doi:10.1038/sj.mp.4001442
Published online 28 October 2003

Keywords: recombinant human erythropoietin; EPO; schizophrenia; clinical; rodent; SPECT

Introduction

The discovery of dopamine antagonists in the middle of the last century represents a major achievement for psychiatry, in general, and for the treatment of schizophrenic psychosis, in particular.[1] Dopamine antagonists delivered a new class of drugs, neuroleptics, which are capable of reducing or eliminating psychotic symptoms. Patients tend to return 'to normal' with respect to their most obvious behavior and their compliance towards treatment. Underneath this 'normalization', however, there is a silently, slowly and nondramatically progressing deterioration of cognitive functioning.[2–5] This deterioration shows gradual differences among patients with the most severely affected individuals representing Kraepelin's 'dementia praecox'.[6] Brain atrophy and ventricular enlargement as morphological substrates of this continuous cognitive decline have been suspected for decades using autopsy material and imaging studies.[5,7,8] A pivotal long-term follow-up study of brain morphology in first episode schizophrenia has revealed *progressive* ventricular enlargement, consistent with a neurodegenerative process.[9] Another recent study, also based on high-resolution magnetic resonance imaging, found dynamic patterns of accelerated gray matter loss in the brains of childhood-onset schizophrenia with earliest defects in the parietal association cortex.[10] Gray matter loss in parietal cortex is known to be strongly associated with environmental factors.[11,12] The deficits described in the study intensified and spread over 5 years of disease progression frontally to the prefrontal cortex and to temporal brain regions, indicative of ongoing

Correspondence: Professor Hannelore Ehrenreich, MD, DVM, Max-Planck-Institute for Experimental Medicine, Hermann-Rein-Str. 3, D-37075 Goettingen, Germany.
E-mail: ehrenreich@em.mpg.de
*Wolfgang Becker died at the time this work had been completed. The authors mourn the passing away of a brilliant young colleague, a kind and caring person who during his short life made invaluable contributions to his field.
Received 18 February 2003; revised 27 August 2003; accepted 17 September 2003

degenerative processes.[10] At the cellular level, these processes may involve an increased postnatal rate of neuronal apoptosis/pruning, metabolic alterations of neurons, loss of axons, and reduced synaptic sprouting/altered neuroplasticity.[5,13,14] With respect to molecular mechanisms, atypical gene expression, for example, of Oct-6[15] or of apolipoprotein L,[16] has been identified in the brains of schizophrenic patients. The accelerating impact of neurotrauma on the development of schizophrenia has already been described by Kraepelin[6] and confirmed later in epidemiological studies.[17–19] Taken together, these findings point to an important role of neurodegenerative components in the pathophysiology of schizophrenia next to the more readily accepted neurodevelopmental aspects of the disease.[5,13,20–22] In fact, it becomes increasingly difficult, and perhaps also unreasonable, to draw a line between neurodevelopmental and neurodegenerative processes in schizophrenia.

Recognition of neurodegenerative components in turn urges for an early application of neuroprotective strategies as 'add-on therapy' to the predominantly symptomatic antipsychotic treatment of schizophrenic psychosis. Unfortunately, there is no satisfying animal model for schizophrenia that would allow sufficient prediction of the efficacy of neuroprotective treatment in man. Existing models at best represent certain aspects of this complex human disease.[23] Some behavioral phenomena resulting from information processing disturbances in schizophrenic patients can also be observed in animals, for example, poor associative learning or disruption of latent inhibition, as measured for instance with the conditioned taste aversion (CTA) paradigm. Latent inhibition is the retardation of associative conditioning resulting from pre-exposure to the conditioned stimulus alone prior to conditioning.[24–26] CTA tests an animal's ability for learning to associate illness with a novel taste stimulus, such that the animals avoid the novel taste upon subsequent re-exposure to the novel stimulus. CTA involves the brain at a variety of cortical and subcortical levels. The association that links ascending and descending information together producing aversive behavior can be either attenuated or strengthened by changes affecting the medial septum[26] or any other of the interconnecting units. For example, the nucleus accumbens is involved in aversive conditioning and this area may be key to psychotic symptoms.[27,28] Agents enhancing CTA-related cognitive functions may have some potential to influence certain aspects of cognitive deterioration positively in schizophrenia and should, therefore, be considered as candidate compounds for neuroprotection in this disease.

Neuroprotection may be defined as the attempt to maintain the highest possible integrity of cellular interactions in the brain, *id est* protection of neural function.[29] A candidate compound, addressing the pathophysiological mechanisms involved in the progression of schizophrenic psychosis, is erythropoietin (EPO). EPO exerts antioxidant, antiapoptotic, anti-inflammatory, neurotrophic, angiogenic and synaptogenic activity.[30–32] Encouraging results on its neuroprotective power in man have been obtained from our recent treatment trial in stroke patients.[33] Thus, neuroprotection in principle *can* work in man. Although ischemia as an acute and schizophrenia as a chronic condition do not compare with respect to etiology and pathogenesis, there are final common pathways, for example, metabolic distress and/or lack of trophic intercellular support that can be expected to respond to a neuroprotective treatment. EPO has been in clinical use for over a decade and has been found to be well tolerated. Its chronic administration over the years has saved lives of thousands of patients suffering from chronic renal failure.[34] Thus, one prerequisite for a candidate compound for neuroprotection in schizophrenia would be fulfilled: safety. In addition, such a compound has to (1) be presumably effective in improving cognition and/or slowing the degenerative process, (2) be capable of crossing the blood–brain barrier, and (3) have receptive structures in the brains of schizophrenic patients. The aim of the present study was to address these prerequisites for EPO-treatment of schizophrenia and thereby to create the basis for a first neuroprotective add-on strategy in this disease.

Materials and methods

Rodent studies

All experiments had received approval by the local Animal Care and Use Committee.

Measurement of EPO immunoreactivity in rat CSF after intraperitoneal administration In order to demonstrate directly that EPO can, in fact, transfer from the systemic circulation into the brain, recombinant human (rh)EPO was injected intraperitoneally (i.p.) into normal rats (5000 U/kg) and serial samples of CSF were obtained from the cisterna magna for EPO determination.[35] The CSF concentrations of EPO were measured by an ELISA (R&D Systems, Minneapolis, MN, USA) using the manufacturer's protocol. The sensitivity of the assay is approximately 2 mU/ml.

CTA Female Balb/c mice were trained to limit their total daily water intake to a single 5 min drinking period per day, and learned to drink enough water during this period to remain at equilibrium. Animals were divided into groups and administered either saline or rhEPO (5000 U/kg), injected i.p. 4 h before presentation of a novel saccharin–vanilla liquid. Immediately after finishing drinking the sweet liquid, animals received either saline or an illness-producing dose of lithium (20 mg/kg of a 0.15 M LiCl, delivered i.p.). Thereafter, three groups of animals were followed for 21 days. The first group (*sham*) did not receive lithium after drinking. The second group (*rhEPO*) received both rhEPO and lithium. The third

group (*control*) received saline (without rhEPO) and lithium.

In vitro experiments

Culture of hippocampal neurons Hippocampal neurons were prepared and cultured according to Brewer[36] with slight modifications. Briefly, hippocampi were removed from a newborn Wistar–Imamichi rat into a dish containing Hank's balanced salt solution (HBSS) supplemented with 0.1% glucose, 1 mM $NaHCO_3$, 1 mM pyruvate, 10 mM HEPES and B27 (Invitrogen, Karlsruhe, Germany). Hippocampi were incubated for 30 min with papain (2 mg/ml, Sigma, Taufkirchen, Germany) on a rotating plattform, followed by trituration. Cell suspension was underlayered with a density gradient prepared in four 1-ml steps of 35, 25, 20 and 15% of Optiprep (Nycomed, Oslo, Norway) in HBSS/B27 (v/v). The cell suspension was centrifuged for 15 min at room temperature, 800 *g* (1900 r.p.m.). Neuron-enriched fractions were collected, washed with 5 ml of HBSS/B27, and centrifuged (200 *g*, room temperature, 1 min). The pellet was resuspended in serum-free growth medium (NeurobasalA/B27 (Invitrogen) with 5 ng/ml of basic fibroblast growth factor (bFGF, Invitrogen), 0.5 mM L-glutamine (Sigma), 50 U/ml of penicillin and 50 μg/ml of streptomycin (Boehringer Mannheim, Germany)). Neurons were plated on poly-D-lysine (Sigma) coated 12 mm coverslips in four-well-plates (Nunc, Wiesbaden, Germany), at a density of 50 cells/mm^2. Cultures were grown in a humidified incubator at 37°C, 5% CO_2. For testing the biological (ie protective) effect of labeled rhEPO (see below), DTPA-rhEPO or In-DTPA-rhEPO (10^{-10} M) were added to the cultures on day 5 together with fresh medium (first medium exchange). The 15-h hypoxia condition was started immediately thereafter as described previously.[37] For the haloperidol experiments, neurons were exposed to 10^{-6} or 10^{-7} M haloperidol with or without rhEPO (10^{-10} M) already upon plating, a second, identical amount was added on day 3. A complete medium exchange took place on day 5 with the fresh medium again containing 10^{-6} or 10^{-7} M haloperidol with or without rhEPO (10^{-10} M). Cell death rate was then measured after 15 h. To determine the amount of dead cells, the Trypan blue exclusion method was used.

PCR and expression analysis by quantitative real-time RT-PCR Whole-cell RNA was isolated using the RNeasy® Kit (Qiagen, Hilden, Germany). Total RNA was subsequently used to generate first-strand cDNA by random priming with reagents and protocols used as recommended by the manufacturers (Gibco, Pharmacia, Freiburg, Germany). PCR primers for RT-PCR expression analysis of EPO receptor (EPOR, GenBank Acc. No. NM_017002) were for 5′ TGA GTG TGT CCT GAG CAA CC and rev 5′ CCA GCA CAG TCA GCA ACA GT (200 bp). Elongation factor 2 expression (EF-2, GenBank Acc. No. Y07504) was used for normalization, for 5′ CTC TAT GCC AGT GTG CTG AC, rev 5′ GGA TTC ATT GAC AGG CAG GT (196 bp). PCRs resulted in single products as judged by agarose gel analysis. The PCR product was purified (QIAquick PCR purification kit, Qiagen, Hilden, Germany) and its concentration determined (Genequant, Amersham Biosciences, Freiburg, Germany). Standards were then diluted to the appropriate concentrations for the expression analysis.

PCR reactions were carried out in a final volume of 20 μl in glass capillaries on a LightCycler real-time PCR machine (Roche Biochemica, Mannheim, Germany). The reaction mixture consisted of 1 μl cDNA solution, 1 U *Taq* DNA polymerase (PANScript DNA Polymerase, PAN Biotech, Aidenbach, Germany), 2 μl 10 × PCR buffer (Invitrogen, Karlsruhe, Germany), 0.2 mM each dATP, dCTP, dGTP and dTTP (Roche Biochemica), 3 mM $MgCl_2$, 500 mg/l bovine serum albumin (New England BioLabs, Frankfurt, Germany), 50 ml/l dimethylsulfoxide (Sigma, Munich, Germany) and SYBR Green I (Molecular Probes, Eugene, OR, USA) at a final concentration of 1 : 10 000. Amplification primers were used at 1 μM concentration. PCR grade water was added to 20 μl. Due to the low EPOR expression in neurons under normoxic conditions, this PCR was optimized by the addition of dsDNA fragments to resemble hotstart conditions and improve the detection sensitivity.[38] Fragments 5′ AGC GGA TAA CAA TAT CA and 5′ TGA TAT TGT TAT CCG CT (3 μM) were annealed and incubated for 15 min in the PCR mixture excluding amplification primers to inhibit the polymerase. PCR primers were then added and PCR cycling commenced. The cycling program consisted of 30 s of initial denaturation at 95°C and 45 cycles of 95°C, 0 s; 55°C, 5 s and 72°C, 5 s, with maximum ramp rate. The acquisition temperature was chosen above that of primer dimers to avoid interference with the detection of the specific PCR product. Acquisition temperature for the EPOR PCR was 87 and 84°C for the EF-2 PCR.

Labeling of rhEPO

All chemicals were purchased from Sigma-Aldrich (Dreieich, Germany) if not otherwise indicated. $^{111}InCl_3$ was obtained from Mallinckrodt (Hennef, Germany). Recombinant human (rh) EPO was kindly provided by Janssen Research Foundation (Titusville, NJ, USA).

DTPA derivatization and labeling of rhEPO A measure of 1.7 mg rhEPO was rebuffered with 5 × 5 ml 0.1 M $NaHCO_3$ on a 10 kDa size exclusion filter unit (Millipore Corporation, Bedford, MA, USA) to a final volume of 500 μl. Thereafter, 10 mg of diethylene-triamine-penta-acetic acid anhydride (cDTPA) in 20 μl DMSO were added and incubated for 1 h at room temperature. This step was repeated once. Afterwards, the reaction mixture was rebuffered with 5 × 5 ml 0.5 M sodium acetate, pH 5.4, on the size exclusion filter to a final volume of 200 μl. The

content of DTPA-rhEPO was evaluated by UV-VIS spectrometry at 275 nm to be 8.2 mg/ml.

Cold In-labeling procedure A volume of 500 μl of 10^{-3} M $InCl_3$ in 0.1 M HCl was added to DTPA-rhEPO solution (1 mg/500 μl 0.5 M sodium acetate buffer, pH 5.4) and incubated at 37°C. Quality control was performed over a PD-10 size exclusion column with 0.5 M sodium acetate buffer, pH 5.4, with a fraction size of 500 μl. The In-DTPA-rhEPO was purified and rebuffered with PBS via a 10 kDa filter. The exact amount of rhEPO content was evaluated with UV/VIS spectroscopy.

Hot In-labeling procedure A volume of 500 μl of 2×10^{-6} M $InCl_3$ in 0.1 M HCl with 10 mCi $^{111}InCl_3$ (1.5×10^{-10} mol) was added to 0.41 mg DTPA-rhEPO solution in 500 μl 0.5 M sodium acetate buffer, pH 5.4, and incubated at 37°C. Quality control was performed over a PD-10 size exclusion column with 0.5 M sodium acetate buffer, pH 5.4, with a fraction size of 500 μl. Purification for patient studies was performed in the same way with PBS.

Analysis of In-111 in human cerebrospinal fluid (CSF) and serum samples A volume of 2 ml of CSF or serum was added on a Sephadex G-50 column (1×10 cm) and eluted with PBS at a fraction size of 500 μl. Samples were counted in a gamma counter. For comparison, a standard mixture of In-111-rhEPO and In-111 was measured.

Patients and controls
The present study has been approved by the Ethical Committee of the Medical Faculty of the Georg-August-University, Goettingen, as well as by the German Nuclear Regulatory Agency. Five patients and five healthy controls, all male, participated after informed written consent. Patients were 20–35 years old (mean $\pm$ SD=25.0 $\pm$ 5.9 years). Consensus diagnosis of schizophrenia, either primarily paranoid or hebephrenic (according to DSM IV criteria), had been made 3.6 $\pm$ 3.2 (1–9) years ago and has now, for study participation, been confirmed and completed by structured interview and chart reviews. Upon entering the study, the present episode had lasted for 1–3 weeks. The five controls were 23–32 years old (mean $\pm$ SD=27.6 $\pm$ 3.6 years; P=0.43 as compared to patients). Patients and controls did not differ with respect to routine laboratory parameters including white and red blood cell count, hematocrit, hemoglobin and thrombocytes. All patients were on antipsychotic medication at the time of testing (risperidone, olanzapine, flupenthixol or perazine).

In-111-rhEPO-scintigraphy
The In-111-rhEPO scans were performed in the five patients and five controls at 4 time points, i.e. 0.5–1, 4–4.5, 18–24, and 42–45 h postinjection (i.v.) of 40 000 U rhEPO containing 120–185 MBq In-111-rhEPO (ie $\leq$5% of total rhEPO injected). Whole-body imaging was performed with a pre-selected time of 30 min using a dual headed gamma camera (Prism 2000, Marconi Medical Systems (formerly PICKER), Cleveland, OH, USA), fitted with a medium-energy collimator. A 128×128 computer matrix and the 171 and 245 keV photopeaks were used. For single photon emission computed tomography (SPECT) of the skull, the same camera and medium-energy collimator were employed, a 360° circular orbit, 60 stops with 30 s acquisition time each and a 128×128 matrix. Reconstruction was performed by an iterative algorithm (ISA).[39] Following orbitomeatal reorientation, the skull was divided from the base to the vault into 24–32 transversal sections. From these sections, the mid-section was chosen for each subject. A region of interest (ROI) technique was used to generate a quotient between the mean counts per pixel of the intracerebral activity and the mean counts per pixel of the skull (bone marrow) activity in this slice (Q IC/BM) at 4–4.5, 18–24, and 42–45 h postinjection, respectively (Figure 3b).

The biodistribution of the tracer was calculated using the geometric mean method by generating ROIs in anterior and posterior projections over the whole body, liver, spleen, bone marrow and kidneys. The activity of each organ was expressed as percentage of the whole-body activity at the respective time points. Since it is virtually impossible to generate a whole-body bone marrow-ROI, the ROI was determined from the second to fourth lumbar vertebrae and the result was multiplied with 11.23 to correct for the whole-body bone marrow activity. In fact, the hematopoietic bone marrow of the second to fourth lumbar vertebrae in young adults amounts to 8.9% of the whole-body hematopoietic bone marrow.[40]

Immunohistochemical protocol
We had the chance to investigate five autopsy brains of schizophrenic patients (diagnosis based on DSM IV criteria) with a *documented* duration of disease of $>$10 years and to compare them to seven control cases who had died from conditions other than neurological/psychiatric disease. All brains, schizophrenic and control, were normal on routine neuropathological examination (for further case details, see Table 1). Brains were fixed with 4% paraformaldehyde and then processed for paraffin embedding. The sections were deparaffinized in Hemo-De (Fisher Scientific, Schwerte, Germany), three changes (5 min), rehydrated through graded ethanols, washed with distilled water, boiled in citrate buffer, washed in Tris-buffered saline (TBS), incubated in 10% blocking serum in 0.05% Tween-20/TBS at room temperature and exposed to polyclonal rabbit anti-human EPOR antibodies (1 : 200, C-20, Santa Cruz Biotechnology, Heidelberg, Germany) in 2% goat sera/PBS at 4°C overnight. After washes in 0.05% Tween-20/TBS, the sections were covered with biotinylated goat-anti-rabbit antibodies (1 : 100, Vector Laboratories Inc., Burlingame, CA, USA) for 60 min. After TBS washes (3×10 min), amplification of the signal was carried out by the avidin–biotin (ABC) method

Table 1 Case description summary

Case	*Age (years)*	*Gender*	*Cause of death*	*Clinical diagnosis*	*Interval between death and autopsy (h)*
1	85	Male	Cardiorespiratory failure	Chronic paranoid schizophrenia	72
2	79	Female	Lung embolism	Chronic paranoid schizophrenia	24
3	85	Male	Aspiration	Chronic schizophrenia	24
4	83	Male	Cardiorespiratory failure	Chronic paranoid psychosis	24
5	68	Female	Hypovolemic shock	Chronic paranoid schizophrenia	72
Mean ± SD	80 ± 7				43 ± 26
6	68	Female	Gastric bleeding	Breast cancer, duodenal ulcer	22
7	75	Male	Septic shock	Diabetes, chronic heart failure	24
8	77	Female	Cardiorespiratory failure	Chronic heart failure	72
9	80	Male	Acute heart failure	Hypertension, coronary heart disease	72
10	78	Female	Cardiorespiratory failure	Chronic heart failure	48
11	83	Male	Aortic rupture, cardiorespiratory failure	Coronary heart disease, renal failure	72
12	82	Male	Aspiration, cardiorespiratory failure	Chronic heart failure	24
Mean ± SD	78 ± 5				48 ± 24

with the appropriate peroxidase kit (Vectastain Elite, Vector) and the signal visualized by incubation in 0.06% diaminobenzidine (DAB; Sigma Chemical Co., Deisenhofen, Germany) and 0.006% H_2O_2 in 0.1 M Tris-HCl (pH 7.6). The slides were dehydrated, cleared, mounted with Eukitt® (Kindler GmbH, Freiburg, Germany) and coverslipped for investigation under a light microscope. Quantitation of the staining intensity was scored from nonoverlapping high-powered ($\times 400$) light microscopic fields of hippocampus and frontal cortex (10 fields/case) and assigned to four categories of increasing intensity: 'no staining (=0)', 'light staining (=1)', intermediate staining (=2)' and 'strong staining (=3)'. Scoring was performed by a rater blinded to diagnosis. The controls for immunohistochemistry included: (1) preadsorption of the first antibody overnight with $20\times$ excess of control antigen. (2) Staining of an adjacent section with an antibody raised against an unrelated antigen (rabbit anti-cannabinoid CB1 receptor in 1 : 2000 dilution, Cayman Chemical, Ann Arbor, MI, USA). For double-immunofluorescence labeling, selected brain sections were incubated overnight with EPOR antibodies as described above and exposed after washes to Texas-Red-labelled goat-anti-rabbit antibodies (1 : 100, Vector) in a humid chamber (30 min). After washes with 0.05% Tween-20/TBS, the sections were treated with monoclonal mouse anti-neuronal nuclei (NeuN) antibodies (1 : 500, Chemicon Int. Inc., Temecula, CA, USA) or monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibodies (DAKO, Glostrup, Denmark) in 2% horse sera/PBS at $+4^{\circ}$C (24 h), washed in 0.05% Tween-20/TBS, and incubated in a humid chamber with fluorescein (FITC) labeled horse anti-mouse antibodies (1 : 100, Vector) for 30 min. The slides were then removed from the chamber, washed in 0.05% Tween-20/TBS and in TBS, and coverslipped with Vectashield (Vector) fluorescence mounting medium.

Statistical analysis

For statistical analysis of the data, analysis of variance was used. Comparison of individual means was performed using the Mann—Whittney *U*-test or the Student's *t*-test, with $P \leq 0.05$ considered to be statistically significant.

Results

Rodent studies

Recovery of immunoreactive EPO in rat CSF Prerequisite for application of rhEPO in brain disease without regularly disturbed blood–brain-barrier function is its transfer over this barrier. As shown in Figure 1a, intraperitoneal injection of rhEPO (5000 U/kg body weight) in healthy rats led to an increase in CSF EPO immunoreactivity, peaking at 3.5 h.

Cognitive performance in mice upon intraperitoneal treatment with rhEPO This set of mouse studies was performed in order to explore the potential of rhEPO to influence cognitive tasks representing some aspects of cognition compromised in schizophrenia. CTA was measured by determining the reduction in drinking upon subsequent exposure to an illness-producing solution, novel saccharin–vanilla liquid, in three groups of mice treated with (1) rhEPO and lithium (*rhEPO*), (2) saline and lithium (*control*), (3) saline

and saline (*sham*). After a 5-day recovery from the lithium or sham treatment, water-deprived animals were presented again with the same novel saccharin–vanilla liquid. The results plotted for the three groups are shown in Figure 1b. Day 3 represents the baseline consumption of water after habituation to the test cage. On Day 4, animals received an injection of either saline or rhEPO (5000 U/kg i.p.) 4 h before presentation of the novel saccharin–vanilla fluid, followed by treatment with lithium or saline. This treatment resulted in a small decrease in fluid consumption in all groups on Day 4, a previously documented adverse effect of the injection and novelty of the fluid. After recovery for 5 days, the first test for the establishment of a CTA showed no decrease in consumption for controls. However, animals having received lithium demonstrated a virtually complete aversion to the fluid, in spite of being water deprived (Day 9). Continued deprivation of water eventually produced an extinction of the CTA, but was characterized by a markedly delayed recovery by the animals that had received rhEPO, as shown by the filled circles in Figure 1b. The robustness of the CTA established herein is better appreciated by considering the degree of water deficit in lithium treated mice followed on each test day, as the rhEPO-treated animals tolerated a water deficit approximately twice that of control (saline injected) mice (Figure 1c).

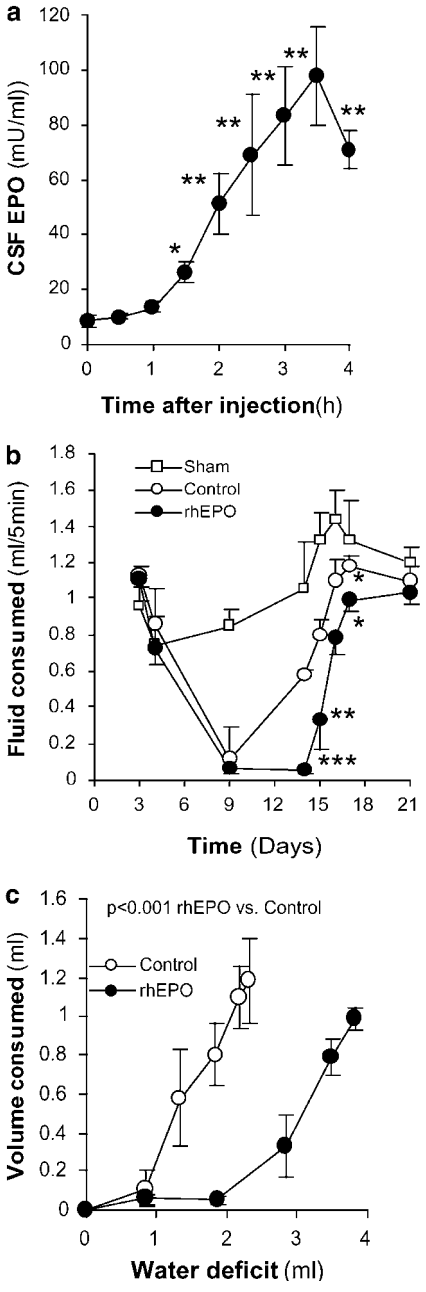

Figure 1 Rodent studies. (a) Time course of EPO concentrations in rat CSF after intraperitoneal administration (5000 U/kg). Data represent mean ± SEM; n=7 per group. Asterisks denote significant difference from basal level (ie before rhEPO administration) (*$P<0.05$; **$P<0.01$). (b) CTA in mice upon intraperitoneal treatment with rhEPO. Time course of the reduction in fluid consumed (ml/5 min) upon subsequent exposure to the illness-producing solution, novel saccharin–vanilla liquid. Day 3 represents the baseline consumption of water after habituation to the test cage. On Day 4, animals received an injection of either saline or rhEPO (5000 U/kg, i.p.) 4 h before presentation of the novel saccharin–vanilla fluid, followed by treatment with lithium or saline. After a 5-day recovery from the lithium or sham treatment, water-deprived animals were presented again with the same novel saccharin–vanilla liquid. The first group (*sham*) was pretreated with saline and did not receive lithium after drinking. The second group (*rhEPO*) received both rhEPO and lithium. The third group (*control*) received saline (without rhEPO) and lithium. Data represent mean ± SEM; n=5–6 per group (*$P<0.05$; **$P<0.01$; ***$P<0.001$ as compared to control). (c) CTA in mice upon intraperitoneal treatment with rhEPO. The degree of water deficit present on each test day in the rhEPO-treated mice (closed circles) and in control mice (open circles) as measured by the volume of water consumed each day. Data represent mean ± SEM; n=5–6 per group. Curves are different on the $P<0.001$ level by repeated measures ANOVA.

Cell culture experiments

Effect of rhEPO on haloperidol-induced neuronal death These experiments have been performed to collect first *in vitro* evidence for a potential interaction of the prototype classical neuroleptic haloperidol and rhEPO on neuronal survival in culture. In agreement with previous reports, haloperidol led to an increased death rate of cultured hippocampal neurons at a concentration of 10^{-6} M.[41] This increased cell death could be significantly reduced by the addition of rhEPO (Figure 2a). Quantitative real-time RT-PCR was then performed to determine whether the protective effect of rhEPO on haloperidol-induced cell death might be explained by an upregulation of EPOR under these circumstances. Quantification of EPOR mRNA copies, however, did not reveal differences between neurons

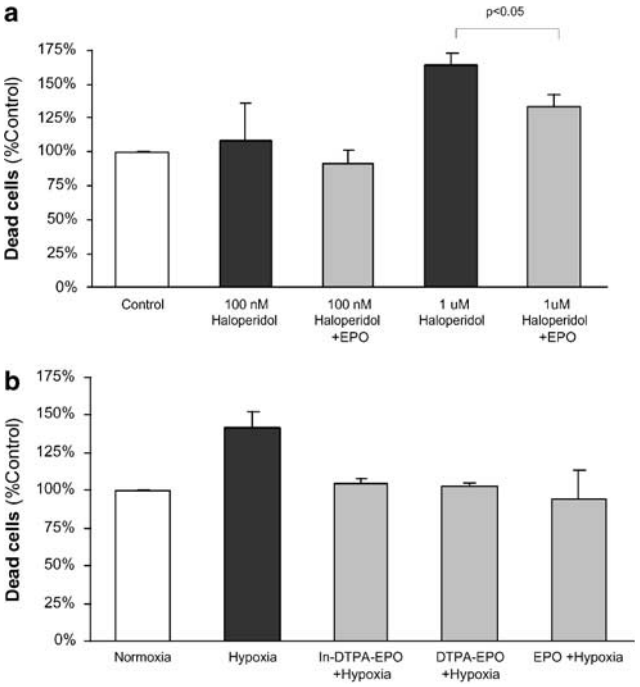

Figure 2 Cell culture studies. (a) The protective effect of rhEPO (100 pM) on survival of hippocampal neurons upon haloperidol-induced cell death. Data (mean ± SEM) are expressed as percentage of cell death from vehicle control (open bar), n=5–13 per group. (b) The protective effect of rhEPO (100 pM) on survival of hippocampal neurons upon hypoxia is not altered by DTPA nor by In-DTPA linked to the rhEPO molecule. Data represent mean ± SEM; n=3 per group.

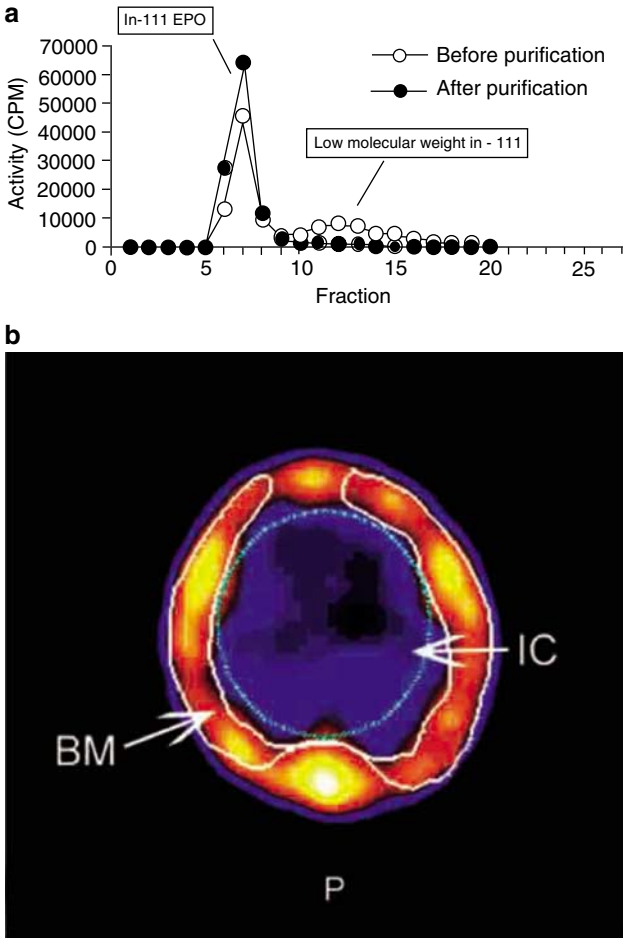

Figure 3 Preparation of human SPECT studies. (a) Chromatogram showing the result of purification of In-111-DTPA-rhEPO. (b) Demonstration of regions of interest (ROI) used to generate a quotient between the mean counts per pixel of the intracerebral activity and the mean counts per pixel of the skull (bone marrow) activity in the SPECT study. IC—intracerebral component, BM—bone marrow, P—posterior.

under basal conditions, exposure to haloperidol (10^{-6} M) or to haloperidol (10^{-6} M) plus rhEPO (10^{-10} M): 93 ± 20, 88 ± 17, and 91 ± 25 copies normalized to EF-2, respectively. Expressed as a percentage of the respective basal level of each experiment, these data were: 100, 114 ± 18, and 134 ± 23% (n=5).

Purification and biological activity of In-DTPA rhEPO The labeling yield of In-DTPA rhEPO was about 69% and the radiochemical purity after purification was over 95% (Figure 3a). In order to determine whether primary hippocampal neurons in culture would be protected against hypoxia-induced cell death by In-DTPA-labeled rhEPO in a fashion comparable to native rhEPO, we applied highly purified In-DTPA rhEPO as well as DTPA-rhEPO to cultures exposed to hypoxia for 15 h.[31,37] Figure 2b shows that the protective effect of rhEPO is not altered by labeling with DTPA or In-DTPA.

Human studies

No side effects and no complaints of the participating patients and controls were identified throughout the study. Two schizophrenic patients missed one SPECT session each and one of them missed one session of whole-body imaging due to a scheduling conflict. All other parts of the study could be completed by all participants.

Determination of ROIs for estimating the relative amount of radioactivity in the brain In order to estimate the relative amount of In-111 rhEPO in the brain, ROIs were determined as shown in Figure 3b. These ROIs allowed the calculation of the ratio between the mean counts per pixel of the intracerebral activity and the mean counts per pixel of the skull (bone marrow). In Figure 4, representative and comparable sections, one per subject, are presented that are taken from the 24-h time point. The line-graphs next to the sections show the ratio, determined as described above, of radioactivity at the 4–4.5-, 18–24- and 42–45-h time points. From the screening of the sections and even more so from the calculation of these ratios, it became evident that the relative activity in the brains of schizophrenic

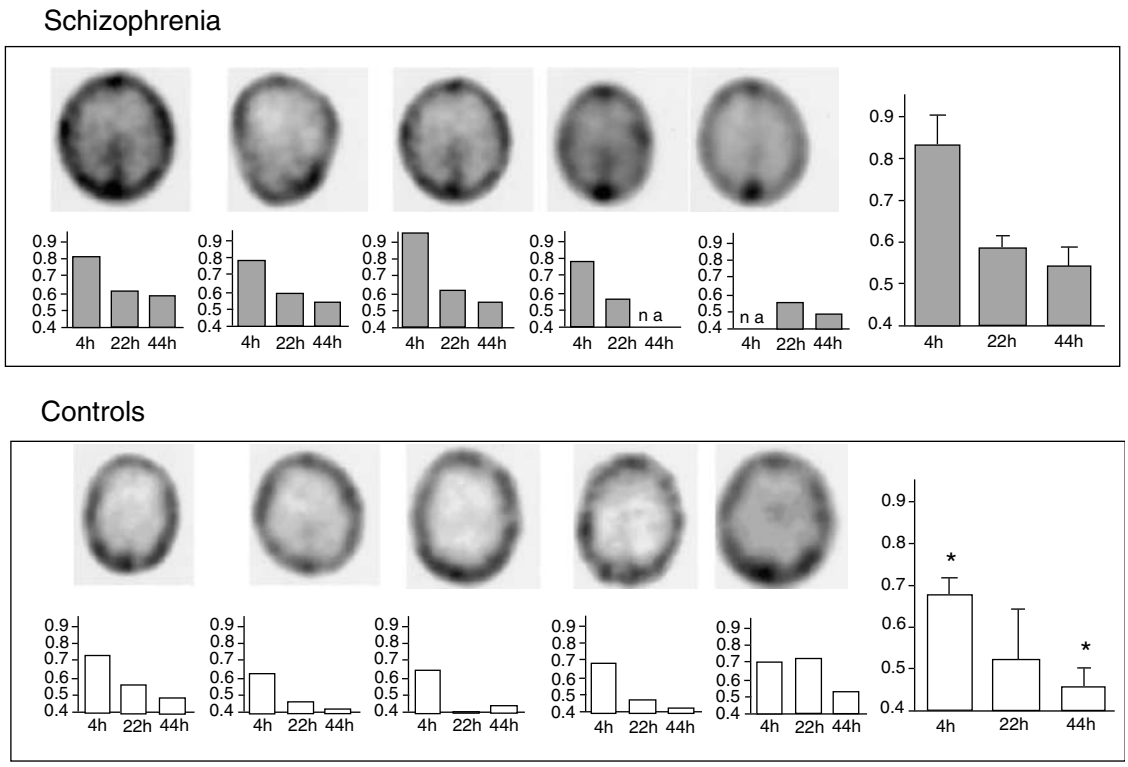

Figure 4 Human SPECT studies: Images illustrating In-111-DTPA-rhEPO radioactivity in the bone marrow of the skull (BM) and intracerebral activity (IC) as well as the time course of the ratio IC/BM. Representative and comparable sections, one per subject, are presented for illustration, all taken from the 22-h time point. The line-graphs next to the sections show the ratio IC/BM of radioactivity at the 4–4.5-, 18–24- and 42–45-h time points. Asterisks denote statistical significance between the healthy control group and the schizophrenic group (*$P<0.05$). *na*—data point *not available*.

patients was higher at all time points as compared to healthy controls. For the 4–4.5- and 42–45-h time points, these differences reached statistical significance.

Profile of In-111 labeled rhEPO and its metabolites in CSF and plasma Figure 5b displays the chromatogram of the CSF from a patient who could be lumbar punctured 36 h after injection of In-111 rhEPO. There is a considerable amount of radioactivity co-eluting with the In-111 rhEPO standard peak. A smaller part of the radioactivity co-elutes with the free In-111 position of the standard (low molecular weight In-111 fractions). Interestingly, there is another peak between these two standard positions that may represent an unknown major metabolite of In-111 rhEPO in the CSF. The serum activity (Figure 5a) is nearly 100 times higher and consists predominantly of the In-111-rhEPO peak. This dominating peak may hide the presence of relatively minor amounts of metabolites.

Distribution of In-111-rhEPO in whole body of healthy and schizophrenic young men Table 2 shows the relative distribution pattern of radioactivity in healthy subjects and schizophrenic patients at 0.5–1-, 4–4.5-, 18–24- and 42–48-h time points after the injection of In-111-DTPA rhEPO. At all time points, there is also a distinct radioactivity found in the bone marrow of the vertebrae. Interestingly, the relative radioactivity in the bone marrow of schizophrenic patients is higher at all time points (significant at 18–24 and 42–48 h) than in controls, whereas the percentage of radioactivity in the other organs is comparable in both groups. Hematocrit, hemoglobin and red blood cell count were normal in all subjects and did not differ among groups (data not shown).

Immunohistochemical staining for EPOR in the brains of schizophrenic patients vs controls To see whether the central nervous system of schizophrenic subjects contains receptors for EPO, brains from schizophrenic patients who died under 'non-brain-related' circumstances, were examined (Table 1). As shown in representative slides in Figure 6a, there is a distinct staining of neurons in the hippocampus of schizophrenic patients. Double immunofluorescence using the neuronal marker NeuN reveals that EPOR immunoreactivity is localized to neurons (Figure 6b). Moreover, GFAP-positive astrocytes (Figure 6f) of schizophrenic patients show a distinct immunohistochemical staining for EPOR (Figure 6e). In contrast, healthy controls show only moderate staining for EPOR (Figure 6c) in neurons (Figure 6d) in comparable areas, whereas glial cells were negative (data not shown). Upon quantification of staining

intensity, these differences reached statistical significance (Figures 6g and h). The specificity of these findings was underlined by determination of the staining intensity and distribution of an unrelated antigen, the cannabinoid type 1 (CB1) receptor, in neighboring sections of the same brains. Employing CB1 antibody resulted in an equally strong neuronal staining in hippocampal and cortical sections from both control and schizophrenic patients.

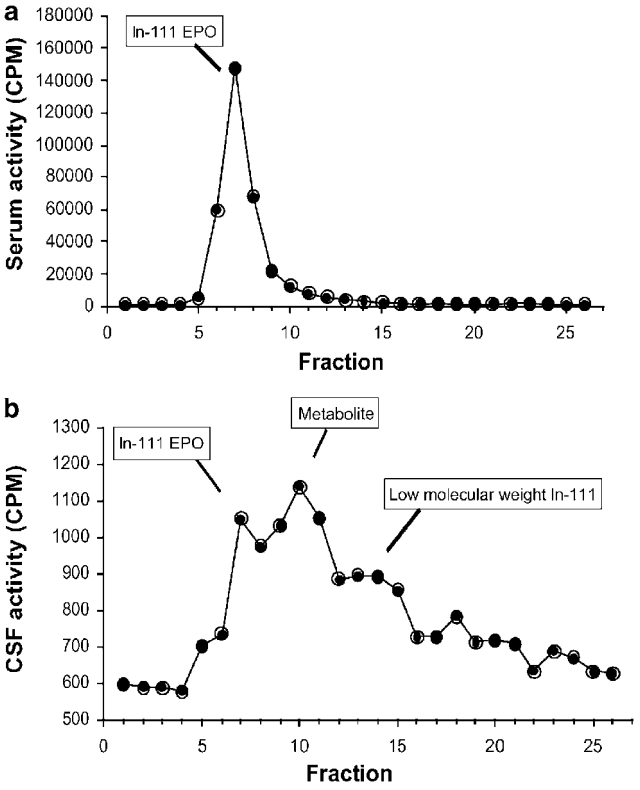

Figure 5 Recovery of In-111-DTPA-rhEPO in human serum and CSF. (a) In-DTPA-rhEPO activity (c.p.m.) in serum as eluted over a Sephadex G50 column; (b) In-DTPA-rhEPO activity (c.p.m.) in CSF as eluted over a Sephadex G50 column.

Discussion

Collectively, these data demonstrate that (1) peripherally applied rhEPO is able to penetrate efficiently into the brain even across an intact blood–brain barrier both in rat and in humans, (2) it is enriched intracranially in healthy men and even more distinctly in schizophrenics, where a remarkable EPOR expression in hippocampus and cortex is found, (3) rhEPO counteracts haloperidol-induced neuronal death *in vitro*, and (4) peripherally administered rhEPO enhances cognitive functioning in mice in the context of an aversion task involving cortical and subcortical pathways presumably affected in schizophrenia.

The present work has addressed the question whether rhEPO might be suitable as a neuroprotective treatment (prophylactic as well as therapeutic) of schizophrenia. For this severely disabling disease, predominantly affecting young people, no satisfying animal model is available.[23] This justifies the use of well-tolerated candidate compounds in humans. Based on positive results of well-designed *proof-of-principle* trials in schizophrenia, it may be worthwhile and even necessary to go back to animal studies employing available animal models[23,42,43] to explore mechanisms of action of the neuroprotectant in a particular setting that reflects defined aspects of this devastating human disease. The animal model applied here addresses such a defined aspect. CTA, an associative learning task known for its robustness, reveals that intraperitoneally applied rhEPO enhances the ability of mice to remember, and learn to avoid, despite excessive water deficit/thirst, an unpleasant taste sensation. As a form of classical conditioning, the strength of CTA is determined by a large number of variables including novelty of the oral stimulus (non-novel stimuli cannot be aversively conditioned), degree of 'illness' produced (toxicity), number of repetitions (training), countering drives (such as thirst) to name a few. Although a wide variety of chemical and physical agents can produce CTA in a dose-dependent manner, lithium chloride reliably produces malaise and anorexia. Like a naturally

Table 2 Relative distribution of In-111-rhEPO in whole body and peripheral organs of healthy and schizophrenic young men

	Whole body (%)	*Liver (%)*	*Spleen (%)*	*Kidney (%)*	*Bone marrow (%)*
Patients					
0.5–1 h	100	8.7±1.0	1.7±0.6	5.5±0.4	25.3±5.2
4–4.5 h	95.0±4.2	7.9±0.7	2.2±0.2	5.7±0.1	27.1±3.7
18–24 h	78.3±3.1	7.0±1.0	2.3±0.2	7.2±1.1	30.4±1.4**
42–45 h	64.8±7.1	7.6±0.7	2.1±0.1	7.2±0.9	28.0±1.8*
Controls					
0.5–1 h	100	7.5±1.4	2.2±0.6	6.6±1.0	22.9±3.5
4–4.5 h	89.4±2.9	6.9±0.9	1.8±0.2	7.2±0.4	22.2±3.6
18–24 h	72.5±4.3	7.3±0.8	1.8±0.2	7.1±1.5	23.6±3.5
42–45 h	61.8±5.0	8.0±0.9	1.8±0.2	7.5±1.3	24.1±2.9

$^{**}P<0.01$, $^{*}P=0.05$ *vs* controls.

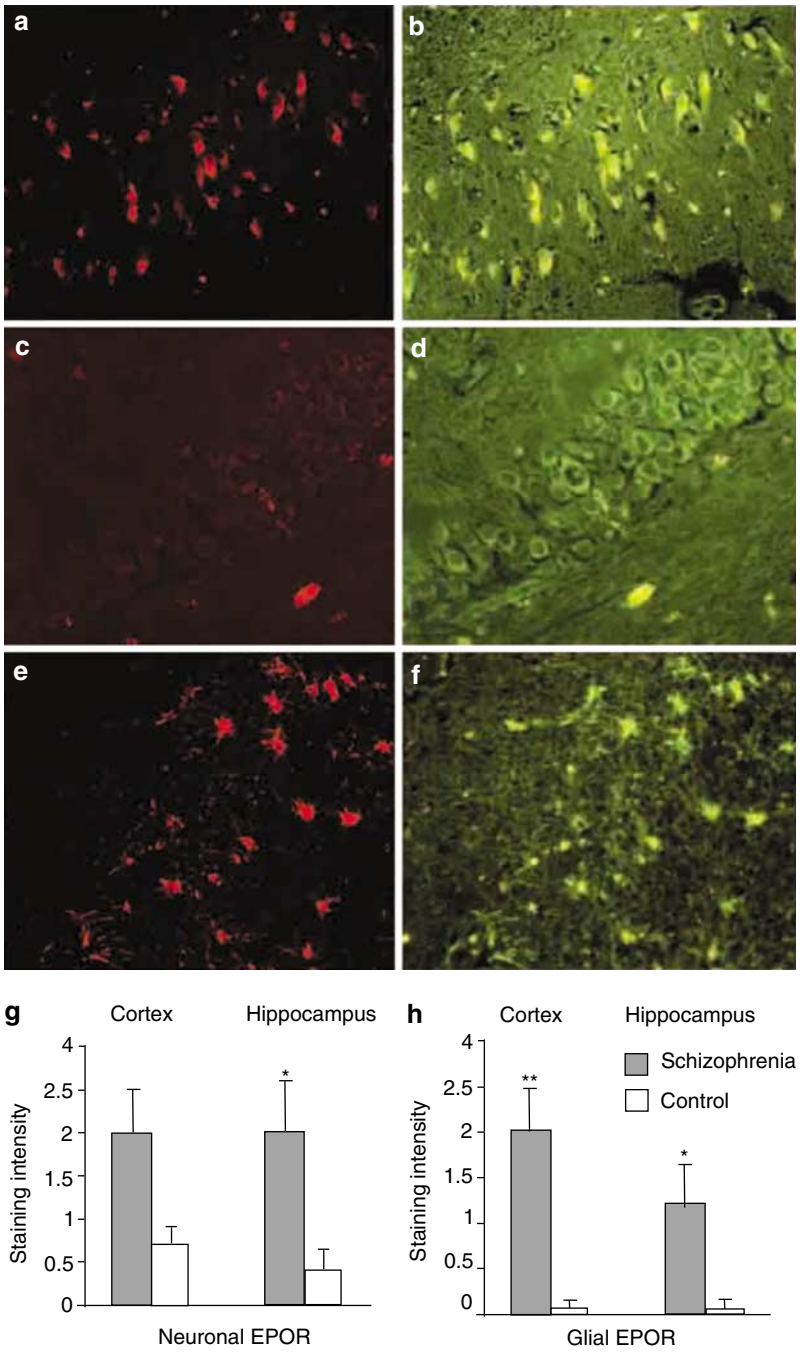

Figure 6 Expression of EPOR immunoreactivity in the brain tissue. Double immunofluorescence demonstrating EPOR in hippocampal neurons of schizophrenic patients (a, b) *vs* controls (c, d) and in astrocytes of schizophrenic patients (e, f). Same magnification ($\times 400$) was used in each panel. Quantitative analysis of neuronal (g) and glial (h) EPOR expression in frontal cortex and hippocampus. Data represent mean $\pm$ SEM. Asterisks ($^{*}P<0.05$; $^{**}P<0.01$) denote statistical significance between the control group ($n=7$) and the schizophrenic patients ($n=5$).

occurring illness, lithium produces a CTA by stimulating cortical and subcortical pathways, including cytokine release.[24,26] The positive results obtained with rhEPO in this context encourage pilot trials of rhEPO in schizophrenia since comparable properties are thought to be affected in this disease.[25,27]

A chronic disease like schizophrenia most likely requires chronic application of a neuroprotectant. In this regard, the positive experience with long-term application of rhEPO in patients suffering from renal anemia[34,44] alleviates the concern that the use of rhEPO in schizophrenia might cause major side effects. An increase in hematocrit, the main hematopoietic effect of EPO, can be corrected by bloodletting. Potentially relevant side effects to be considered based on long-term observations in renal

failure and cancer patients would include hypertension[45] and, as extensively investigated more recently, formation of neutralizing anti-EPO antibodies harboring the risk of developing erythroblastopenia. This latter phenomenon has been reported in a total of about 170 cases, all suffering from renal anemia, all on long-term treatment, all having had subcutaneous applications of EPO[46] (and information from the manufacturer).

Interestingly, cognitive improvement in renal failure patients upon rhEPO treatment had been observed long before the EPO system has been detected in the brain. This cognitive amelioration was attributed to increases in hematocrit.[34,44] Controlled studies addressing this topic are lacking. The fact, however, that rhEPO induces cognitive improvement before an increase in hematocrit is noted, makes it very likely that ameliorated cognition reflects a direct effect of rhEPO on the central nervous system.[47] A molecular separation of these two properties of EPO could be demonstrated by recent findings of potent neuroprotective effects of desialylated EPO (asialoEPO), which is devoid of any hematopoietic activity.[48] Hereby, the antioxidant properties of EPO, rapidly improving the metabolic/trophic situation in the brain may play an essential role.[49,50] Additional long-term beneficial effects of an increased hematocrit, however, cannot be excluded.

In-111-DTPA rhEPO was developed and employed for the first time to investigate the distribution of intravenously applied rhEPO in brain and whole body of healthy *vs* schizophrenic patients. This method should assist in estimating the degree of penetration of rhEPO into the brain via an intact blood–brain barrier. Using this approach, an unexpectedly high amount of radioactivity was detected in the brains of both, healthy and schizophrenic patients. This high amount became evident already at the 4-h time point and persisted for at least 2 days. The nature of radioactivity could be attributed by chromatographic examination of CSF to approximately 50% intact EPO. Although there was, expectedly, a much higher concentration detectable in serum, the distribution of rhEPO into the brain, comparable to that seen in stroke patients,[33] may be sufficient for exerting its neuroprotective activity also in chronic conditions. Considering the high molecular weight of rhEPO ($>34\,000$ Da), its rapid penetration across an intact blood–brain barrier could be explained by a transport mechanism. In fact, it has previously been demonstrated that few hours after intraperitoneal application, biotinylated rhEPO densely surrounds cerebral microvessels of mice, supportive of an active translocation process.[32,51]

The relative amount of radioactivity in the bone marrow of schizophrenic patients was found to be higher as compared to controls despite comparable hematological parameters and comparable distribution in liver, spleen and kidney. At present, the reason for this discrepancy is a matter of speculation. One possibility may be that chronic treatment with dopamine antagonists increases the density of EPOR in bone marrow and brain. All patients, those who participated in the SPECT study and those from whom post-mortem brain samples were analyzed for EPOR immunoreactivity, have been on neuroleptic medication; the former on atypical, the latter on classical antipsychotics. Thus, it cannot be excluded that dopamine antagonists enhance the transfer of rhEPO across the blood–brain barrier and/or increase rhEPO binding to brain cells. In accordance with the finding of a higher accumulation of radioactivity in the brains of schizophrenic patients, a higher density of immunoreactive EPOR in schizophrenic brains was demonstrated by immunohistochemistry. Chronic therapeutic dopamine antagonism as potential explanation of augmented rhEPO binding in schizophrenic patients is currently under investigation in our laboratory. If there is a causal connection responsible for the observed phenomenon, it certainly is a complex one. The preliminary data presented here using quantitative real-time RT-PCR to measure EPOR mRNA expression of neurons *in vitro* upon exposure to haloperidol did not uncover any change at the message level. Such change, however, can be observed upon another cell death-promoting stimulus, hypoxia.[37] In any case, the protective effect of rhEPO on haloperidol-induced neuronal death *in vitro* may further support the use of rhEPO as neuroprotective add-on therapy in schizophrenia.

In addition to a potential role of chronic dopamine antagonism, the coincidence of a higher accumulation of radioactivity both in brain and bone marrow of schizophrenic men as compared to controls would at least allow one alternative explanation: Persistent metabolic distress in schizophrenia may induce a chronic stimulation of stem cells that could explain a higher density of EPOR in stem cell providing tissues. Such constant stem cell activation would be consistent with the neurodevelopmental hypothesis of schizophrenia.[5,13,20–22] Interestingly, the EPO-system appears to play a major role during embryonic brain development.[52,53] A recent paper shows that EPO regulates the *in vitro* and *in vivo* production of neuronal progenitors by mammalian forebrain neural stem cells.[53]

Considering that pathophysiological processes in schizophrenia appear to be composed of neurodevelopmental as well as of neurodegenerative aspects,[5,22] it may be speculated that also metabolic disturbances due to neurodegenerative processes stimulate the endogenous EPO system[30] leading to a higher density of EPOR expression not only in neurons but also in glial cells. Various metabolic disturbances in schizophrenia have been shown by several groups.[54,55] Interestingly, glial cells appear to be more and more recognized as important players in schizophrenia pathophysiology.[56,57]

Although the situation in ischemic stroke is not directly comparable to the situation of neurodegeneration in schizophrenia, it is interesting to note that blocking of the endogenous EPO system by using

soluble EPOR leads to a dramatic increase in lesion size in an experimental stroke model.[32] Should the-perhaps relatively deficient-endogenous EPO system in schizophrenics profit from exogenous substitution? It is encouraging that the Goettingen EPO-Stroke Trial has demonstrated for the first time the efficiency of a neuroprotective strategy in humans using EPO.[33] In animal models, EPO has been able to improve the outcome in both acute and chronic disorders.[32,51,58] The conditions tested range from various animal models of cerebral ischemia/hypoxia[32,51] and neurotrauma[51] to epilepsy[51], Parkinson syndrome[58] or experimental autoimmune encephalitis.[51]

These observations together with the results of the present study render EPO an interesting compound for neuroprotective add-on strategies in schizophrenia and other human diseases characterized by a decline in cognitive performance. A multicenter *proof-of-concept* trial in schizophrenia has started in Germany in April 2003.

Acknowledgements

The study has received research grant support from the Max-Planck Society, from Lundbeck and from Ortho Biotech Inc.

References

1 Carlsson A. The current status of the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* 1988; **1**: 179–186.
2 Andreasen NC, Olsen S. Negative v positive schizophrenia. Definition and validation. *Arch Gen Psychiatry* 1982; **39**: 789–794.
3 Kremen WS, Seidman LJ, Faraone SV, Tsuang MT. Intelligence quotient and neuropsychological profiles in patients with schizophrenia and in normal volunteers. *Biol Psychiatry* 2001; **50**: 453–462.
4 Haefner H, Hambrecht M, Loffler W, Munk-Jorgensen P, Riecher-Rossler A. Is schizophrenia a disorder of all ages? A comparison of first episodes and early course across the life-cycle. *Psychol Med* 1998; **28**: 351–365.
5 Lieberman JA. Is schizophrenia a neurodegenerative disorder. A clinical and neurobiological perspective. *Biol Psychiatry* 1999; **46**: 729–739.
6 Kraepelin E. In: Robertson GM (ed). *Dementia Praecox and Paraphrenia.* E&S Livingstone: Edinburgh, Scotland, 1919 (Endogenous dementias. In: Barclay RM (transl). *Text Book of Psychiatry*, Vol iii, Part ii, 8th edn. Chicago Medical Book Company: Chicago, 1919 (in German) (Translation of Chapter IX 'Die endogenen Verblödungen' from 1913a in English)).
7 Henn FA, Braus DF. Structural neuroimaging in schizophrenia. An integrative view of neuromorphology. *Eur Arch Psychiatry Clin Neurosci* 1999; **249**: 48–56.
8 Vogeley K, Schneider-Axmann T, Pfeiffer U, Tepest R, Bayer TA, Bogerts B *et al.* Disturbed gyrification of the prefrontal region in male schizophrenic patients: a morphometric postmortem study. *Am J Psychiatry* 2000; **157**: 34–39.
9 Lieberman J, Chakos M, Wu H, Alvir J, Hoffman E, Robinson D *et al.* Longitudinal study of brain morphology in first episode schizophrenia. *Biol Psychiatry* 2001; **49**: 487–499.
10 Thomson PM, Vidal C, Giedd JN, Gochman P, Blumenthal J, Nickolson R *et al.* From the cover: mapping adolescent brain change reveals dynamic wave of accelerated gray matter loss in very early-onset schizophrenia. *Proc Natl Acad Sci USA* 2001; **98**: 11650–11655.
11 Resnick SM, Pham DL, Kraut MA, Zonderman AB, Davatzikos C. Longitudinal magnetic resonance imaging studies of older adults: a shrinking brain. *J Neurosci* 2003; **23**: 3295–3301.
12 Cannon TD, Thompson PM, van Erb TG, Toga AW, Poutanen VP, Huttunen M *et al.* Cortex mapping reveals regionally specific patterns of genetic and disease-specific gray-matter deficits in twins discordant for schizophrenia. *Proc Natl Acad Sci USA* 2002; **99**: 3228–3233.
13 McGlashan TH, Hoffman RE. Schizophrenia as a disorder of developmentally reduced synaptic connectivity. *Arch Gen Psychiatry* 2000; **57**: 637–648.
14 Mirnics K, Middleton FA, Lewis DA, Levitt P. Analysis of complex brain disorders with gene expression microarrays: schizophrenia as a disease of the synapse. *Trends Neurosci* 2001; **24**: 479–486.
15 Ilia M, Beasley C, Meijer D, Kerwin R, Cotter D, Everall I *et al.* Expression of Oct-6, a POU III domain transcription factor in schizophrenia. *Am J Psychiatry* 2002; **159**: 1174–1182.
16 Mimmack ML, Ryan M, Baba H, Navarro-Ruiz J, Iritani S, Faull RL *et al.* Gene expression analysis in schizophrenia: reproducible up-regulation of several members of the apolipoprotein L family located in a high-susceptibility locus for schizophrenia on chromosome 22. *Proc Natl Acad Sci USA* 2002; **99**: 4680–4685.
17 Achte K, Jarho L, Kyykka T, Vesterinen E. Paranoid disorders following war brain damage. Preliminary report. *Psychopathology* 1991; **24**: 309–315.
18 Malaspina D, Goetz RR, Friedman JH, Kaufmann CA, Faraone SV, Tsuang M *et al.* Traumatic brain injury and schizophrenia in members of schizophrenia and bipolar disorder pedigrees. *Am J Psychiatry* 2001; **158**: 440–446.
19 McAllister TW. Traumatic brain injury and psychosis: what is the connection? *Semin Clin Neuropsychiatry* 1998; **3**: 211–223.
20 Marenco S, Weinberger DR. The neurodevelopmental hypothesis of schizophrenia: following a trail of evidence from cradle to grave. *Dev Psychopathol* 2000; **12**: 501–527.
21 Lewis DA, Levitt P. Schizophrenia as a disorder of neurodevelopment. *Annu Rev Neurosci* 2002; **25**: 409–432.
22 Velakoulis D, Wood SJ, McGorry PD, Pantelis C. Evidence for progression of brain structural abnormalities in schizophrenia: beyond the neurodevelopmental model. *Aust NZ J Psychiatry* (2000); **34**(Suppl): S113–S126.
23 Lipska BK, Weinberger DR. To model a psychiatric disorder in animals: schizophrenia as a reality test. *Neuropsychopharmacology* 2000; **23**: 223–239.
24 Conti LH, Palmer AA, Vanella JJ, Printz MP. Latent inhibition and conditioning in rat strains which show differential prepulse inhibition. *Behav Genet* 2001; **31**: 325–333.
25 Ellenbroek BA, Budde S, Cools AR. Prepulse inhibition and latent inhibition: the role of dopamine in the medial prefrontal cortex. *Neuroscience* 1996; **75**: 535–542.
26 Turgeon SM, Kegel G, Davis MM. Electrolytic lesions of the medial septum enhance latent inhibition in a conditioned taste aversion paradigm. *Brain Res* 2001; **890**: 333–337.
27 Weiner I. The 'two-headed' latent inhibition model of schizophrenia: modeling positive and negative symptoms and their treatment. *Psychopharmacology* 2003; **168**, online DOI 10.1007.
28 Fenu S, Bassareo V, Di Chiara G. A role for dopamine D1 receptors of the nucleus accumbens shell in conditioned taste aversion learning. *J Neurosci* 2001; **21**: 6897–6904.
29 Ehrenreich H, Sirén A-L. Special issue-editorial: neuroprotection—what does it mean—what means do we have? *Eur Arch Psychiatry Clin Neurosci* 2001; **251**: 149–151.
30 Sirén A-L, Ehrenreich H. Erythropoietin—a novel concept of neuroprotection. *Eur Arch Psychiatry Clin Neurosci* 2001; **251**: 179–184.
31 Sirén A-L, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P *et al.* Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci USA* 2001; **98**: 4044–4049.
32 Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M *et al. In vivo* evidence that erythropoietin protects neurons

from ischemic damage. *Proc Natl Acad Sci USA* 1998; **95**: 4635–4640.
33 Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, Stiefel M *et al.* Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol Med* 2002; **8**: 495–505.
34 Jelkmann W. Use of recombinant human erythropoietin as an antianemic and performance enhancing drug. *Curr Pharm Biotechnol* 2000; **1**: 11–31.
35 Frankmann SP. A technique for repeated sampling of CSF from the anesthetized rat. *Physiol Behav* 1986; **37**: 489–493.
36 Brewer GJ. Isolation and culture of adult rat hippocampal neurons. *J Neurosci Methods* 1997; **71**: 143–155.
37 Lewczuk P, Hasselblatt M, Kamrowski-Kruck H, Heyer A, Unzicker C, Sirén AL *et al.* Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin. *Neuroreport* 2000; **11**: 3485–3488.
38 Kainz P, Schmiedlechner A, Strack HB. Specificity-enhanced hot-start PCR: addition of double-stranded DNA fragments adapted to the annealing temperature. *Biotechniques* 2000; **28**: 278–282.
39 Luig H, Eschner W, Bahre M, Voth E, Nolte G. An iterative strategy for determining the source distribution in single photon tomography using a rotating gamma camera (SPECT). *Nuklearmedizin* 1988; **27**: 140–146.
40 Ellis RE. The distribution of active bone marrow in the adult. *Phys Med Biol* 1961; **5**: 255–258.
41 Behl C, Rupprecht R, Skutella T, Holsboer F. Haloperidol-induced cell death—mechanism and protection with vitamin E *in vitro*. *Neuroreport* 1995; **7**: 360–364.
42 Mohn AR, Gainetdinov RR, Caron MG, Koller BH. Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. *Cell* 1999; **98**: 427–436.
43 Grace AA, Moore H, O'Donnell P. The modulation of corticoaccumbens transmission by limbic afferents and dopamine: a model for the pathophysiology of schizophrenia. *Adv Pharmacol* 1998; **42**: 721–724.
44 Nissenson AR. Epoetin and cognitive function. *Am J Kidney Dis* 1992; **20**: 21–24.
45 Bohlius J, Langensiepen S, Schwarzer G, Engert A. Epoetin in the treatment of malignant disease: a comprehensive meta-analysis. *Blood* 2002; **100**: 3430A.
46 Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P *et al.* Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med* 2002; **346**: 469–475.
47 Ehrenreich H, Sirén A-L. Benefits of recombinant human erythropoietin on cognitive function. *Erythropoiesis* 2001; **11**: 35–39.
48 Erbayraktar S, Grasso G, Sfacteria A, Xie QW, Coleman T, Kreilgard M *et al.* Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity *in vivo*. *Proc Natl Acad Sci USA* 2003; **100**: 6741–6746.
49 Chattopadhyay A, Choudhury TD, Bandyopadhyay D, Datta AG. Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. *Biochem Pharmacol* 2000; **59**: 419–425.
50 Genc S, Akhisaroglu M, Kuralay F, Genc K. Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production *in vitro*. *Neurosci Lett* 2002; **321**: 73–76.
51 Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C *et al.* Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 2000; **97**: 10526–10531.
52 Kietzmann T, Knabe W, Schmidt-Kastner R. Hypoxia and hypoxia inducible factor modulated gene expression in brain: involvement in neuroprotection and cell death. *Eur Arch Psychiatry Clin Neurosci* 2001; **251**: 170–178.
53 Shingo T, Sorokan ST, Shimazaki T, Weiss S. Erythropoietin regulates the *in vitro* and *in vivo* production of neuronal progenitors by mammalian forebrain neural stem cells. *J Neurosci* 2001; **21**: 9733–9743.
54 Do KQ, Trabesinger AH, Kirsten-Kruger M, Lauer CJ, Dydak U, Hell D *et al.* Schizophrenia: glutathione deficit in cerebrospinal fluid and prefrontal cortex *in vivo*. *Eur J Neurosci* 2000; **12**: 3721–3728.
55 Harada S, Tachikawa H, Kawanishi Y. Glutathione *S*-transferase M1 gene deletion may be associated with susceptibility to certain forms of schizophrenia. *Biochem Biophys Res Commun* 2001; **281**: 267–271.
56 Coyle JT. The nagging question of the function of *N*-acetylaspartylglutamate. *Neurobiol Dis* 1997; **4**: 231–238.
57 Benes F, Coyle JT. *Deja vu* all over again. *Biol Psychiatry* 1998; **43**: 781–782.
58 Genc S, Kuralay F, Genc K, Akhisaroglu M, Fadiloglu S, Yorukoglu K *et al.* Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. *Neurosci Lett* 2001; **298**: 139–141.