

# Common Variants of the Genes Encoding Erythropoietin and Its Receptor Modulate Cognitive Performance in Schizophrenia

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Erythropoietin (EPO) improves cognitive performance in clinical studies and rodent experiments. We hypothesized that an intrinsic role of EPO for cognition exists, with particular relevance in situations of cognitive decline, which is reflected by associations of *EPO* and *EPO* receptor (*EPOR*) genotypes with cognitive functions. To prove this hypothesis, schizophrenic patients ( $N > 1000$ ) were genotyped for 5' upstream-located gene variants, *EPO* SNP rs1617640 (T/G) and *EPOR* STR(GA)<sub>n</sub>. Associations of these variants were obtained for cognitive processing speed, fine motor skills and short-term memory readouts, with one particular combination of genotypes superior to all others ( $p < 0.0001$ ). In an independent healthy control sample ( $N > 800$ ), these associations were confirmed. A matching preclinical study with mice demonstrated cognitive processing speed and memory enhanced upon transgenic expression of constitutively active EPOR in pyramidal neurons of cortex and hippocampus. We thus predicted that the human genotypes associated with better cognition would reflect gain-of-function effects. Indeed, reporter gene assays and quantitative transcriptional analysis of peripheral blood mononuclear cells showed genotype-dependent *EPO/EPOR* expression differences. Together, these findings reveal a role of endogenous EPO/EPOR for cognition, at least in schizophrenic patients.

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## INTRODUCTION

Erythropoietin (EPO), originally described as a hematopoietic growth factor, has been found to improve cognition almost since its first clinical approval for the treatment of renal anemia, but this effect has long been attributed solely to its indirect action via increased hemoglobin

(1). Over the last 15 years, the importance of EPO for nonhematopoietic tissues, particularly the nervous system, has been recognized increasingly. In addition to direct neuroprotective and neuroregenerative functions of the EPO system, its effects on neuroplasticity and cognition have become evident (2–4). EPO treat-

ment has been shown to improve learning and memory functions, not only in disease models, but also in healthy rodents (5–7). In clinical trials on patients suffering from schizophrenia or multiple sclerosis, high-dose rhEPO infusions over several months resulted in better cognitive performance (8,9). Functional magnetic resonance imaging 1 wk after just a single high dose of rhEPO revealed an enhancement of the hippocampal response during memory retrieval in healthy human subjects (10). Importantly, cognitive improvement in these studies was not linked to an increase in hemoglobin. Still-remaining concerns that EPO might exert these cognitive effects via enhanced hemoglobin were further alleviated by the functional separation of hematopoietic and neuro-

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protective actions using EPO variants, devoid of hematopoietic properties (11). In addition, neuroregenerative effects of EPO in insects, lacking hematopoiesis, suggest an intrinsic and phylogenetically ancient significance of EPO for neuroplasticity and cognition (12).

Studies on the role of *EPO* or *EPOR* genotypes have focused on searching for associations of genetic markers in these genes with readouts of the hematopoietic system in healthy individuals and disease states, such as myeloproliferative or myelodysplastic syndromes. The results were mainly negative for *EPO* genotypes (13–18), but several associations for *EPOR* polymorphisms or rare mutations with various forms of familial polycythemia were identified (19,20). Three publications deal with *EPO* genotypes in diabetic retinopathy. One turned out negative (21), whereas the other two found associations, but the results remain contradictory since opposing risk genotypes were described (22,23). Another study reported an association of an *EPO* genotype with age of onset of amyotrophic lateral sclerosis (24). No data are available yet on associations of *EPO* or *EPOR* genotypes with brain functions, including cognition.

We hypothesized that an inherent relevance of EPO for cognitive functioning and neuroplasticity in humans should be reflected by associations of *EPO* and *EPOR* genotypes with selected readouts of cognitive performance and might be uncovered in a disease characterized by cognitive decline. To test these hypotheses, we exploited the GRAS (*Göttingen Research Association for Schizophrenia*) data collection, which provides a unique ground for phenotype-based genetic association studies (PGAS) with information on over 1,000 well-characterized schizophrenic patients (25,26). We show here that in this population, as well as in a healthy replicate sample, *EPO/EPOR* genotypes are associated with several domains of higher cognition. Moreover, on the basis of reporter gene assays and mouse studies, we propose that better performance is linked to higher expression/regulability of the endogenous EPO system.

## MATERIALS AND METHODS

### Subjects

**Disease sample.** The GRAS data collection was approved by the ethics committee of the Georg-August-University (master committee) and the local internal review boards of the collaborating centers. The project complies with the Helsinki Declaration (27). Patients fulfilling DSM-IV criteria for schizophrenia or schizoaffective disorder were included regardless of the stage of the disease (acute, chronic, residual or remitted). All study participants and, if applicable, their legal representatives gave written informed consent (for detailed information on the GRAS sample, see reference 26). A total of  $N = 1,050$  GRAS patients were successfully genotyped for the *EPO* SNP (single nucleotide polymorphism T/G) rs1617640,  $N = 1,054$  for the *EPO* SNP (T/G) rs564449, and  $N = 1,054$  for *EPOR* short tandem repeat, STR(GA)<sub>n</sub>, and are included in the present genetic analyses. Most GRAS patients are of European Caucasian ethnicity (Caucasian 95.4%; other ethnicities 1.8%; unknown 2.8%). Peripheral blood mononuclear cells (PBMCs) were taken from a subsample of the GRAS cohort ( $N = 98$ ) to analyze mRNA expression dependent on genotype.

**Case control sample.** Healthy voluntary blood donors were recruited by the Department of Transfusion Medicine at the Georg-August-University of Göttingen according to national guidelines for blood donation to serve as control subjects. As such, they widely fulfill health criteria, ensured by a broad predonation screening process including standardized health questionnaires, interviews and assessment of hemoglobin concentration, blood pressure, pulse and body temperature. Of the  $N = 1,141$ – $1,142$  successfully genotyped control subjects, 58.9% were male ( $N = 672$ – $673$ ) and 41.1% female ( $N = 469$ – $470$ ). The average age was  $34.61 \pm 12.30$  y (range 18 to 69). The majority of the control subjects were of European Caucasian ethnicity (Caucasian 97.8%; other ethnicities 2%; unknown 0.2%).

**Independent healthy control sample (replicate sample).** Unrelated volunteers of German descent (that is, both parents German) were selected randomly from the general population of Munich, Germany, and contacted by mail (28). To exclude subjects with central neurological diseases and psychotic disorders or individuals who had first-degree relatives with psychotic disorders, several screenings were conducted before volunteers were enrolled in the study. Firstly, subjects who responded were screened initially by phone for the absence of neuropsychiatric disorders. Secondly, detailed medical and psychiatric histories were obtained for both the patients themselves as well as their first-degree relatives by using a semistructured interview. Thirdly, if inclusion criteria were fulfilled, the subjects were invited for a comprehensive interview including the German version of the structured clinical interview for DSM-IV (SCID I and SCID II) (29) to confirm the absence of any lifetime psychotic disorder. Additionally, the family history assessment module (30) was conducted to exclude psychotic disorders among first-degree relatives. Furthermore, a neurological examination was conducted to exclude subjects with current central nervous system impairment. In case of volunteers being older than 60 years, the Mini-Mental State Examination (MMSE; 31) was performed to exclude subjects with possible cognitive impairment. Written informed consent was obtained from all individuals after providing them with a detailed description of the study, which was approved by the local ethics committee and carried out in accordance with the Helsinki Declaration (27).

### Phenotype-Based Genetic Association Study (PGAS)

**Disease sample.** On the basis of findings of our previous EPO treatment trial with schizophrenic patients (9), neuropsychological measures of processing speed (Digit Symbol-Coding [Zahlen-Symbol-Test]), a subtest of German version of Wechsler Adult Intelligence Scale [WAIS; 32]) and perceptual organization (subtests Dotting and Tapping from Mac-

**Table 1.** Association of *EPO* and *EPOR* genotypes with cognitive target variables in schizophrenic patients (GRAS) and healthy individuals.

	<i>EPO</i> rs1617640			<i>EPOR</i> STR (GA) <sub>n</sub>		<i>EPO</i> and <i>EPOR</i>			Effect (p)	
	GG	GT	TT	Effect (p) <sup>a</sup>	Low sum	High sum	Effect (p)	GG and 21–35 repeats		All other combinations
Disease sample (GRAS), mean ± SD (95% CI) <sup>b,c,d</sup>										
	N = 161–172	N = 408–465	N = 345–381		N = 466–509	N = 448–509		N = 60–63	N = 854–955	
Perceptual organization										
Dotting and Tapping	0.08 ± 1.98 (-0.22–0.38)	0.02 ± 1.83 (-0.14–0.19)	-0.11 ± 1.94 (-0.30–0.09)	1.492 (0.222)	0.01 ± 1.85 (-0.16–0.16)	-0.03 ± 1.94 (-0.20–0.14)	4.402 (0.045)	0.65 ± 1.92 (0.18–1.13)	-0.06 ± 1.88 (-0.18–0.06)	10.259 (0.001)
Processing speed										
Digit Symbol-Coding (WAIS) <sup>e</sup>	40.13 ± 14.19 (38.0–42.3)	37.62 ± 12.73 (36.5–38.8)	36.93 ± 13.42 (35.6–38.3)	8.868 (0.003)	37.80 ± 13.20 (36.7–38.9)	37.77 ± 13.38 (36.6–38.9)	1.893 (0.169)	43.57 ± 14.46 (40.0–47.1)	37.40 ± 13.12 (36.6–38.2)	16.166 ( $<0.001$ )
Cognition composite <sup>f</sup>	0.08 ± 0.94 (-0.07–0.22)	-0.01 ± 0.88 (-0.09–0.08)	-0.07 ± 0.92 (-0.16–0.03)	3.327 (0.036)	-0.00 ± 0.89 (-0.09–0.07)	-0.02 ± 0.93 (-0.11–0.06)	4.274 (0.039)	0.37 ± 0.90 (0.14–0.59)	-0.04 ± 0.90 (-0.10–0.02)	13.891 ( $<0.001$ )
Verbal learning and memory (VLMT) <sup>g</sup>	42.74 ± 13.22 (40.7–44.8)	42.22 ± 12.84 (41.0–43.5)	40.62 ± 12.69 (39.3–42.0)	0.852 (0.356)	42.22 ± 13.00 (41.1–43.4)	41.18 ± 12.82 (40.0–42.4)	5.298 (0.022)	46.72 ± 12.49 (43.6–49.9)	41.36 ± 12.83 (40.5–42.2)	9.063 (0.003)
Healthy individuals, mean ± SD (95% CI) <sup>h</sup>										
	N = 332	N = 1095	N = 859		N = 447	N = 434		N = 50	N = 831	
Perceptual organization										
Block Design (WAIS) <sup>e</sup>	31.78 ± 9.24 (30.8–32.8)	30.32 ± 9.43 (29.8–30.9)	30.32 ± 9.58 (29.7–31.0)	7.263 (0.007)	32.83 ± 9.18 (32.0–33.7)	30.99 ± 8.76 (30.2–31.8)	9.815 (0.002)	33.46 ± 9.23 (30.9–36.0)	31.83 ± 9.00 (31.2–32.4)	1.068 (0.302)
Processing speed										
Digit Symbol-Coding (WAIS) <sup>e</sup>	53.19 ± 13.22 (51.8–54.6)	51.74 ± 12.96 (51.0–52.5)	51.34 ± 13.33 (50.5–52.2)	4.116 (0.043)	54.29 ± 12.08 (53.2–55.4)	54.13 ± 12.12 (53.0–55.3)	0.004 (0.953)	56.56 ± 10.68 (53.6–59.5)	54.07 ± 12.16 (53.2–54.9)	1.678 (0.196)
Cognition composite <sup>f</sup>	0.12 ± 0.88 (0.02–0.21)	-0.01 ± 0.88 (-0.07–0.04)	-0.03 ± 0.89 (-0.09–0.03)	7.768 (0.005)	0.22 ± 0.84 (0.14–0.29)	0.11 ± 0.79 (0.04–0.19)	4.142 (0.043)	0.34 ± 0.81 (0.11–0.56)	0.16 ± 0.82 (0.10–0.21)	2.025 (0.155)

<sup>a</sup>Effects for GG versus T carriers.

<sup>b</sup>Analysis of covariance (ANCOVA) with age, negative symptoms (PANSS), medication status (chlorpromazine equivalents) and duration of disease as covariates, and Blom-transformed single targets.

<sup>c</sup>Owing to missing data upon phenotyping and the exclusion of nonnative German speakers for language-dependent readouts (VLMT), sample size varies between N = 914–1018 in the total GRAS sample.

<sup>d</sup>CI, confidence interval.

<sup>e</sup>Test from German version of WAIS (32).

<sup>f</sup>Cognition composite represents mean of Dotting and Tapping subtests and Digit Symbol-Coding test (both tests Blom transformed).

<sup>g</sup>Exclusion of nonnative German speakers (N = 92).

<sup>h</sup>Analysis of covariance (ANCOVA) with age as covariate and Blom-transformed single targets.

Quarrie Test for Mechanical Ability; 33) were selected from the GRAS database for phenotype–genotype association analyses (target variables). The “Verbal Learning and Memory Test” (Verbaler Lern- und Merkfähigkeitstest [VLMT]; 34) was included as another target variable to cover aspects of short-term memory. Additionally, to demonstrate the specificity of genotype associations with the selected cognitive readouts, sociodemographic (that is, age, gender, level of education) and clinical variables (age at

first episode, duration of disease, medication status, Positive and Negative Syndrome Scale [PANSS; 35] subscales and Global Assessment of Functioning [GAF; 36]) were included in the analysis (see Table 1 for target and Table 2 for sociodemographic and clinical variables).

#### Healthy individuals (replicate sample).

To replicate the phenotype–genotype associations found in GRAS patients in an independent group of healthy individuals, we aimed at covering comparable domains of processing speed and perceptual organ-

ization. Digit Symbol-Coding test as measure of processing speed and Block Design (Mosaik-Test) as test of perceptual organization capacities (both from German version of WAIS; 32) were employed. Unfortunately, neuropsychological tests measuring verbal memory were not available in sufficient numbers for replication.

#### Statistical Analyses

Statistical analyses of phenotype–genotype associations for healthy and schizophrenic individuals were per-

**Table 2.** Sociodemographic and disease-related variables in schizophrenic patients and healthy controls are not associated with EPO/EPOR genotypes.

Basic variables	EPO rs1617640				EPOR STR (GA) <sub>n</sub>			EPO and EPOR		
	GG	GT	TT	Effect (p) <sup>a</sup>	Low sum	High sum	Effect (p)	GG and 21-35 repeats	All other combinations	Effect (p)
Disease sample (GRAS) (95% CI) <sup>b,c,d</sup>										
	N = 165-171	N = 452-483	N = 366-388		N = 496-519	N = 484-520		N = 60-63	N = 920-976	
Age, mean ± SD, y	40.32 ± 12.48 (38.4-42.2)	39.15 ± 12.43 (38.0-40.3)	39.69 ± 12.74 (38.4-41.0)	(0.316)	39.67 ± 12.39 (38.6-40.7)	39.42 ± 12.71 (38.3-40.5)	(0.540)	37.34 ± 12.50 (34.23-40.5)	39.69 ± 12.55 (38.9-40.5)	(0.166)
Gender, no. (%), male	110 (63.6%) (54.6-72.6)	332 (67.9%) (62.9-72.9)	260 (67%) (61.3-72.7)	1.002 (0.317)	352 (67.2%) (62.4-72.2)	350 (66.4%) (61.5-71.4)	0.094 (0.759)	43 (68.3%) (54.3-82.2)	659 (66.8%) (63.2-70.4)	0.059 (0.808)
Ethnicity, no. (%), Caucasian <sup>e</sup>	171 (98.8%) (97.2-100.4)	461 (94.3%) (92.2-96.4)	368 (94.8%) (92.6-97.1)	1.916 (0.384)	499 (95.4%) (93.6-97.2)	501 (95.1%) (93.2-97.0)	1.691 (0.429)	63 (100.0%) (-)	937 (94.9%) (93.5-96.3)	1.276 (0.528)
Years of education, mean ± SD <sup>f</sup>	12.40 ± 3.15 (11.9-12.9)	12.11 ± 2.93 (11.8-12.4)	11.95 ± 3.23 (11.6-12.3)	(0.208)	12.16 ± 3.06 (10.7-14.5)	12.05 ± 3.10 (12.0-15.7)	(0.528)	12.75 ± 3.25 (11.9-13.6)	12.06 ± 3.07 (11.9-12.3)	(0.143)
Current occupation, no. (%), unemployed	30 (17.3%) (3.8-30.9)	77 (15.7%) (7.6-23.9)	59 (15.2%) (6.0-24.4)	0.275 (0.600)	75 (14.3%) (6.4-22.3)	91 (17.3%) (9.5-25.0)	1.805 (0.179)	14 (22.2%) (0.4-44.0)	152 (15.4%) (9.7-21.1)	1.915 (0.166)
Age at first episode, mean ± SD, y	26.72 ± 9.30 (25.3-28.1)	25.73 ± 8.21 (25.0-26.5)	26.75 ± 8.94 (25.9-27.6)	(0.742)	26.35 ± 8.71 (25.6-27.1)	26.19 ± 8.66 (25.5-26.9)	(0.654)	25.12 ± 9.30 (22.8-27.4)	26.35 ± 8.64 (25.8-26.9)	(0.101)
Duration of disease (first episode), mean ± SD, y	13.25 ± 11.48 (11.5-15.0)	13.28 ± 10.71 (12.3-14.2)	12.83 ± 10.34 (11.8-13.9)	(0.811)	13.27 ± 10.44 (12.4-14.2)	12.94 ± 10.96 (12.0-13.9)	(0.365)	11.80 ± 11.27 (9.0-14.6)	13.19 ± 10.66 (12.5-13.9)	(0.161)
Smoker status, no. (%), smoker	57 (33.7%) (20.6-40.8)	128 (27.2%) (23.2-31.2)	113 (30.5%) (25.8-35.2)	2.776 (0.250)	148 (29.1%) (25.2-33.0)	154 (30.5%) (26.5-34.5)	0.244 (0.621)	22 (35.5%) (23.6-47.4)	276 (29.1%) (26.2-32.0)	1.135 (0.287)
Chlorpromazine equivalents, mean ± SD	637.1 ± 514.8 (559.5-714.7)	724.29 ± 792.18 (653.6-794.9)	671.7 ± 647.5 (607.0-736.4)	(0.957)	751.5 ± 727.3 (637.9-736.1)	680.6 ± 674.2 (622.5-738.7)	(0.486)	542.6 ± 427.9 (436.9-648.3)	713.1 ± 714.2 (655.3-754.0)	(0.174)
PANSS positive, mean ± SD	13.49 ± 6.17 (12.6-14.4)	14.30 ± 6.75 (13.7-14.9)	13.16 ± 5.75 (12.6-13.7)	(0.669)	13.95 ± 6.45 (13.4-14.5)	13.53 ± 6.16 (13.0-14.1)	(0.355)	13.98 ± 6.59 (12.3-15.6)	13.72 ± 6.30 (13.3-14.1)	(0.805)
PANSS negative, mean ± SD	17.69 ± 7.78 (16.5-18.9)	18.68 ± 8.11 (17.9-19.4)	18.09 ± 7.73 (17.3-18.9)	(0.286)	18.80 ± 8.30 (18.1-19.5)	17.78 ± 7.49 (17.1-18.4)	(0.106)	18.18 ± 7.98 (16.2-20.2)	18.30 ± 7.92 (17.8-18.8)	(0.863)
PANSS general, mean ± SD	33.37 ± 11.17 (31.7-35.1)	34.56 ± 12.51 (33.4-35.7)	32.88 ± 11.28 (31.7-34.0)	(0.809)	34.10 ± 12.43 (33.0-35.2)	33.36 ± 11.25 (32.4-34.4)	(0.610)	33.20 ± 12.29 (30.1-36.3)	33.77 ± 11.83 (33.0-34.5)	(0.570)
PANSS total, mean ± SD	64.34 ± 22.53 (60.9-67.8)	67.43 ± 24.72 (65.2-69.7)	64.03 ± 22.08 (61.8-63.3)	(0.484)	66.89 ± 24.96 (64.7-69.1)	64.38 ± 21.73 (62.4-66.3)	(0.284)	65.30 ± 24.95 (59.0-71.6)	65.67 ± 23.36 (64.2-67.2)	(0.733)
GAF mean ± SD	45.88 ± 18.48 (43.1-48.7)	45.30 ± 17.26 (43.7-46.9)	46.40 ± 16.67 (44.7-48.1)	(0.934)	46.09 ± 17.78 (44.5-47.6)	45.51 ± 16.72 (44.1-47.0)	(0.836)	47.02 ± 18.60 (42.4-51.7)	45.72 ± 17.16 (44.6-46.8)	(0.749)
Healthy individuals (95% CI) <sup>b</sup>										
	N = 335	N = 1,111	N = 869		N = 449	N = 437		N = 50	N = 836	
Age, mean ± SD, y	51.12 ± 16.37 (49.4-52.9)	51.91 ± 15.45 (51.0-52.8)	52.18 ± 15.42 (51.2-53.2)	(0.686)	47.97 ± 14.16 (46.7-49.3)	48.52 ± 14.42 (47.2-49.9)	(0.427)	46.88 ± 15.14 (42.7-51.1)	48.32 ± 14.24 (47.4-49.3)	(0.605)
Gender, no. (%), male	160 (47.8%) (40.0-55.5)	547 (49.2%) (45.1-53.4)	416 (47.9%) (43.1-52.7)	0.088 (0.767)	221 (44.6%) (38.1-51.2)	198 (40.2%) (33.3-47.0)	1.359 (0.244)	23 (46.0%) (25.6-66.4)	396 (47.4%) (42.5-52.3)	0.035 (0.851)
Education, no. (%), low level <sup>g</sup>	60 (20.6%) (11.0-30.1)	287 (25.8%) (20.8-30.9)	224 (25.8%) (20.1-31.5)	4.316 (0.116)	101 (20.4%) (12.5-28.3)	90 (18.3%) (10.3-26.2)	1.108 (0.575)	7 (14.0%) (-11.7-39.7)	184 (22.0%) (16.0-28.0)	3.386 (0.184)

<sup>a</sup>Effects for GG versus T carriers.

<sup>b</sup>Methods used: Mann-Whitney U tests and  $\chi^2$  tests.

<sup>c</sup>Owing to missing data upon phenotyping, sample size varies between N = 771-1,049 in the total GRAS sample.

<sup>d</sup>CI, confidence interval.

<sup>e</sup>Exploratory exclusion of non-Caucasian subjects did not appreciably alter any of the main findings of the paper.

<sup>f</sup>Rating according to graduation/certificate; patients currently in school or in educational training are excluded.

<sup>g</sup>Low level education: equal or less than nine years of academic formation.

formed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA; <http://www.spss.com>). GraphPad Prism, version 5.01 (La Jolla, CA, USA) was used to analyze expression and mouse data.

**Case control study (disease sample versus healthy blood donors or replicate sample).** For *EPOR* STR (GA)<sub>n</sub>, the sum of repeat lengths of both alleles was analyzed. To account for intraindividual allelic heterogeneity (that is, the degree of heterogeneity between the two alleles), the difference between allelic repeat lengths was calculated. Distributions of single allele lengths, allelic repeat sum, allelic heterogeneity and of *EPO* SNP genotypes (SNPs rs1617640 and rs564449) between schizophrenic subjects and healthy controls were assessed by  $\chi^2$  tests with (*EPOR*) and without (*EPO*) Monte Carlo sampling (1,000 runs).

**PGAS.** For the phenotype–genotype association analysis using the *EPO* SNP rs1617640 as independent variable, T carriers (GT and TT) were aggregated and contrasted with individuals homozygous for the G allele. Group comparisons for the *EPOR* repeat were based on median splits (21–36 versus 37–54; for the first PGAS approach, Tables 1 and 2) or tercile splits (21–35 versus 36–38 versus 39–54; for more detailed subgroup comparisons, see Figure 1F) of allelic repeat sums. Data on cognitive target variables are presented such that higher values always indicate better performance. They were standardized to mean zero and variance one by Blom transformation (37). In language-dependent tests (VLMT), nonnative German speakers (N = 92) were excluded for analyses. A cognition composite score was calculated for each individual representing the mean of the Blom-transformed data for processing speed and perceptual organization. Genotype differences were assessed by analysis of covariance including covariates age (both healthy individuals and disease controls), duration of disease, chlorpromazine equivalents and severity of negative symptoms (PANSS; the latter three covariates only for the disease sample) as they are known to influence performance on neuropsychological

tests. Genotype differences with respect to sociodemographic and clinical readouts were tested using  $\chi^2$  (nominal variables) or Mann-Whitney *U* tests (continuous variables). The impact of *EPO* and *EPOR* genotypes on mRNA levels and expression differences dependent on *EPOR* genotype were tested nonparametrically using Kruskal-Wallis and Mann-Whitney *U* tests in the subsample of patients of whom PBMCs were available (N = 98).

### DNA Extraction and Normalization

**Disease sample and healthy blood donors.** Genomic DNA was purified from whole blood using JETQUICK Blood & Cell Culture DNA Spin Kit (Genomed GmbH, Löhne, Germany) according to the manufacturer's protocol. Resulting DNA samples were aliquoted and stored at –80°C. For further analysis, DNA was normalized to 50ng/μL with an automated robotic platform (Microlab Star, Hamilton, Bonaduz, Switzerland). For quality control, each sample was analyzed with a 0.8% agarose gel.

**Healthy individuals (replicate sample).** DNA extraction was done with the QIAamp Blood Maxi Kit (Qiagen, Hilden, Germany). DNA concentration was adjusted using the PicoGreen quantitation reagent (Invitrogen, Karlsruhe, Germany).

### Genotyping—Analysis of SNPs in the *hEPO* Gene

**Disease sample and healthy blood donors.** The selected SNPs (rs1617640 and rs564449) in the *EPO* gene were analyzed using Simple Probes (TIB Molbiol, Berlin, Germany) and called using the LightCycler 480 Genotyping Software implemented in the LightCycler 480 system (Roche, Mannheim, Germany). The reaction mixture (10 μL) was prepared with 20 ng of DNA in 384 well plates according to standard protocols (Roche). In each run, eight positive controls (hgDNA, Bioline, Luckenwalde, Germany) and negative water blanks were included for quality and internal control purposes. Overall, successfully genotyped markers amounted to 99.7–99.9%.

**Healthy individuals (replicate sample).** The SNP rs1617640 was genotyped using the iPLEX assay on the MassARRAY MALDI-TOF mass spectrometer (Sequenom, Hamburg, Germany). Genotyping call rates were all >95%.

### Genotyping—Analysis of GA Repeats (Both Disease Sample and Healthy Individuals)

The polymorphic GA repeat in the promoter region of *hEPOR* was amplified from genomic DNA by PCR. Primers were chosen from de la Chapelle *et al.*, 1993 (38): *hEPOR*\_(GA)<sub>n</sub> forward: 5'-FAM GGTGA CAGAG CAACA CCCTG-3'; *hEPOR*\_(GA)<sub>n</sub> reverse: 5'-ATCAG CATCT CTTCC CAGCC-3' resulting in a PCR fragment of ~186bp. Due to the presence of GGAA repeats in the same region (20), we likely obtained aggregates of all repeats, that is, GGAA as well as GA. Since we assume that the aggregates as a whole are important for modulating function, we did not further analyze the exact composition of the aggregates. In fact, our data validate this assumption. For each sample, the reaction mixture (20 μL) was prepared in 384 well plates, each containing 20 ng of human genomic DNA, 125 μmol/L dNTPs each, 200 nmol/L FAM-labeled forward primer and the reverse primer and 1U Phire polymerase (Finnzymes, Espoo, Finland). The amplicons were separated using size electrophoresis on the ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For this, samples were diluted 1:50 with 0.3 mmol/L EDTA and 4 μL were mixed with 6 μL LIZ-500 Size Standard (Applied Biosystems). Raw data were processed using the Gene Mapper Software 4.0 (Applied Biosystems).

### Analysis of Peripheral Blood Mononuclear Cells (PBMCs)

Blood was collected in CPDA (citrate phosphate dextrose adenine) tubes from schizophrenic patients with different genotypes at the selected markers. PBMCs were isolated applying the standard Ficoll-Paque Plus isolation procedure (GE Healthcare, München, Ger-

many). RNA was prepared using Qiagen miRNeasy Mini Kit (Qiagen). 1 µg of RNA per sample was used to synthesize cDNA (SuperScriptIII, Invitrogen). The qRT-PCR was performed with the aid of SYBR Green detection on the LightCycler 480 system (Roche). The starting amount of cDNA was 20 ng; the number of PCR cycles was 30–34 for EPO and 27–31 for EPOR. Primers were added at 0.5 pMol. CT (cycle threshold) values for *EPO* and *EPOR* were standardized to CT values of *GAPDH*. *hEPO*\_qRT-PCR forward: 5'-TCCCA GACAC CAAAG TTAAT TTCTA-3'; *hEPO*\_qRT-PCR reverse: 5'-CCCTG CCAGA CTTCT ACGG-3'; *hEPOR*\_qRT-PCR forward: 5'-TTGGA GGACT TGGTG TGTTT C-3'; *hEPOR*\_qRT-PCR reverse: 5'-AGCTT CCATG GCTCA TCCT-3'; *hGAPDH*\_qRT-PCR forward: 5'-CTGAC TTCAA CAGCG ACACC-3'; *hGAPDH*\_qRT-PCR reverse: 5'-TGCTG TAGCC AAATT CGTTG T-3'.

### Cloning and Transfection Studies

**Constructs.** The *EPO* promoter constructs were built according to Tong and coworkers (23). Briefly, the promoter site (1.5 kb) including either G or T at rs1617640 was PCR amplified from respective human samples and cloned into the pGL3 basic vector (Promega, Mannheim, Germany). In addition, constructs including the 3' region of human *EPO*, as this is known to be of major importance for the regulation of *EPO* expression, were designed. For this, the following primers including *XbaI* sites were used: *hEPO\_3'* forward: 5'-GCGTC TAGAC CAGGT GTGTC CACCT-3'; *hEPO\_3'* reverse: 5'-GCGTC TAGAA TGACA ATCTC AGCGC-3'.

All constructs were verified by restriction enzyme digestion and complete bidirectional DNA sequencing.

**Luciferase Assays.** Neuro2a (N2a) cells (LGC Standards GmbH, Wesel, Germany) were plated in 96-well cell culture plates (NUNC, Langenselbold, Germany) at 15,000 cells per well in DMEM supplemented with 5% FCS without antibiotics. At 16–18 h after plating, cells were transfected with Lipofectamine 2000 (Invitrogen) using the manufacturer's protocol. Of

the different *EPO* constructs, 30 ng (containing G or T) and 1 ng of pRL-TK-control vector (Promega) were cotransfected. For each treatment, six replicates were performed. At 24 h after transfection, cobalt chloride (CoCl<sub>2</sub>), a well known mimetic of hypoxic *EPO* induction (39,40), was added in different concentrations to induce chemical hypoxia to the transfected cells. Concentrations were selected such that after the CoCl<sub>2</sub> exposure, no signs of toxicity or increased cell death were observed (Trypan blue counts <1% in all conditions, including normoxic controls). At 48 h after transfection, cells in each well were lysed, using 30 µL passive lysis buffer (Promega). The dual-luciferase reporter assay (Promega) was used according to the manufacturer's protocol. Prior to measurement, lysates were transferred into a black plastic microtiter plate. Measurements were performed with the microplate reader Mitras LB940 (Berthold Technologies, Regensdorf, Switzerland) and associated software MicroWin 2000. Firefly values were divided by the corresponding Renilla readings producing values expressed as relative luciferase units (RLU).

### Mouse Studies

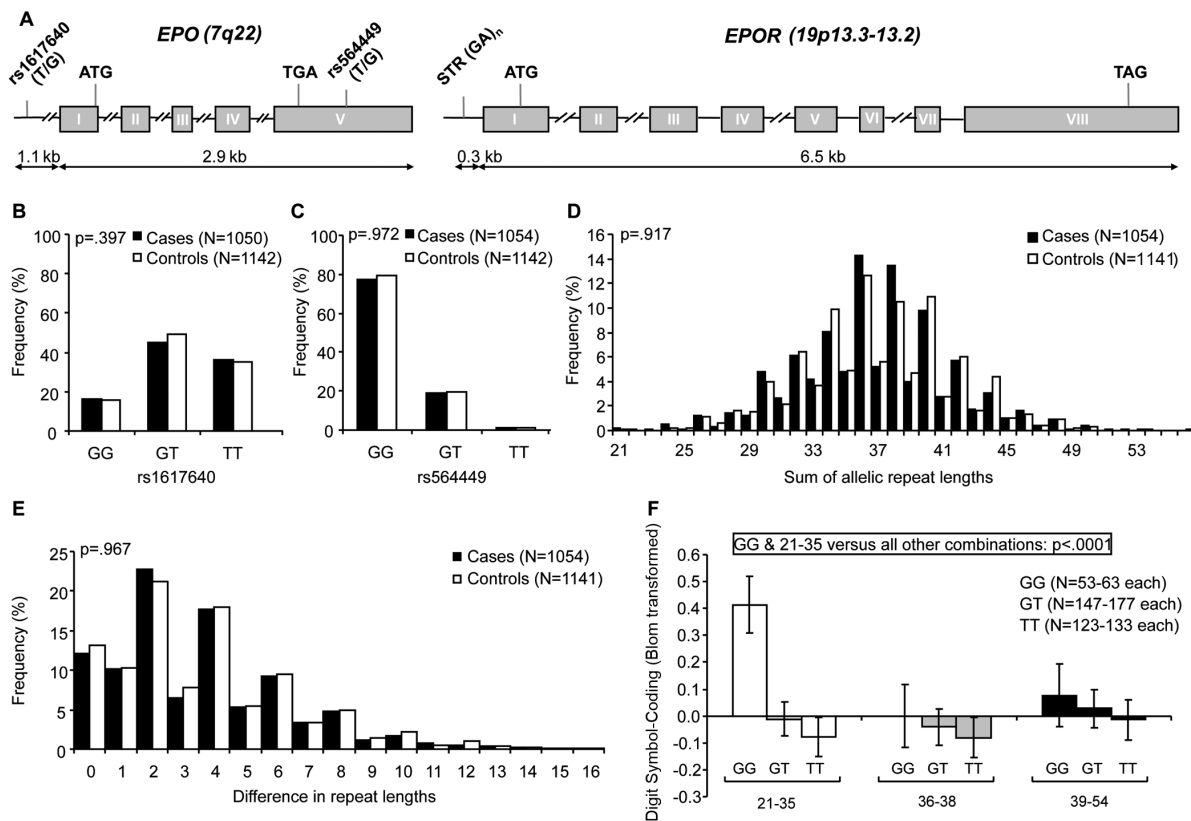
**The generation and characterization of cEPOR transgenic mice** have been reported in detail elsewhere (7). Briefly, EPOR<sup>R129C</sup> (cEPOR) bears a single point mutation at nucleotide 484, that is, in the exoplasmic domain, causing a substitution of cysteine for arginine at codon 129 of the N terminus (R129C). The cDNA sequence of cEPOR, containing a hemagglutinin (HA; YPYDVDPY) tag inserted five residues downstream of the signal peptidase cleavage site (41,42) was excised with *PacI* and *Sall* from the pMX-HA-cEPOR plasmid. The HA-cEPOR cDNA was inserted into pNN265 plasmid, with a modified multiple cloning site, that carries a 5' hybrid intron and a 3' intron plus poly-A signal from SV40 through *PacI* and *Sall* sites. Finally, the entire DNA fragment of HA-cEPOR, flanked by a hybrid intron at the 5' end and a polyadenylation signal from SV40 at the 3' end was cut out from pNN265 vector using *NotI*

and placed downstream of the 8.5kb α-CaMKII promoter. The TG founders were produced by pronuclear injection of the linearized DNA into C57BL6/N (TG1) or FvB/N (TG2) zygotes. The analysis of line TG1 mice was performed after 4–7 backcrosses with C57BL6/N wild type mice (that is, all results reported in this study were obtained from generations 4–7 of the TG1 line). The TG1 line was used (because of its clean C57BL6/N background) for the behavioral experiments presented here. Analysis of line TG2 mice was performed after 8–9 backcrossings to C57BL6/N mice. The genotype of transgenic offspring was analyzed by PCR of tail genomic DNA using primers specific for the 3' end of the α-CaMKII promoter sequence (5'-GGGAG GTAGG AAGAG CGATG-3') and the 5' end of the HA-cEPOR cDNA sequence (5'-CACCC TGAGT TTGTC CATCC-3') yielding a 769 bp product. PCR amplification of the tail DNA was carried out with the following conditions: 2 min at 94°C (1 cycle); 30 s at 94°C, 30 s at 60°C and 1 min at 72°C (35 cycles), followed by final extension at 72°C for 10 min.

### Behavioral Testing

All experiments were approved by the local animal care and use committee in accordance with the German Animal Protection Law. For behavioral testing, mice were housed in groups of 3–5 in standard plastic cages, food and water *ad libitum* (except for the 5-choice [water deprivation] and T-maze [food deprivation] training periods). The temperature in the colony room was maintained at 20°–22°C, with a 12 h light:dark cycle (light on at 7 AM). Behavioral experiments were conducted during the light phase of the day (between 8 AM and 5 PM). Mouse data were analyzed using repeated measures analysis of variance (ANOVA) and Mann-Whitney *U* tests.

**Five-choice serial reaction time task (5-CSRTT).** The 5-CSRTT measures higher brain functions, ranging from various discrete learning/memory to attentional paradigms (43,44). A detailed description of the procedure and training steps is given



**Figure 1.** *EPO* and *EPOR* genotype analyses in schizophrenic patients of the GRAS data collection and healthy controls (blood donors). (A) Genetic overview of *EPO/EPOR* including analyzed genetic markers. (B,C) A case control study reveals comparable distribution of *EPO* SNP genotypes in schizophrenic and healthy control subjects, thus excluding *EPO* genotypes as risk factors for schizophrenia. (D,E) Case control analysis of *EPOR* STR ( $(GA)_n$ ) repeat lengths shows comparable results for both samples, again excluding a risk constellation of *EPOR* genotypes. (F) Grouping of genotype combinations with respect to Digit Symbol-Coding test performance uncovers one genotype highly superior to all others: GG&21–35 (lowest) repeat sum. Mean  $\pm$  standard error of the mean (SEM) given;  $\chi^2$  tests and analysis of covariance (ANCOVA) applied.

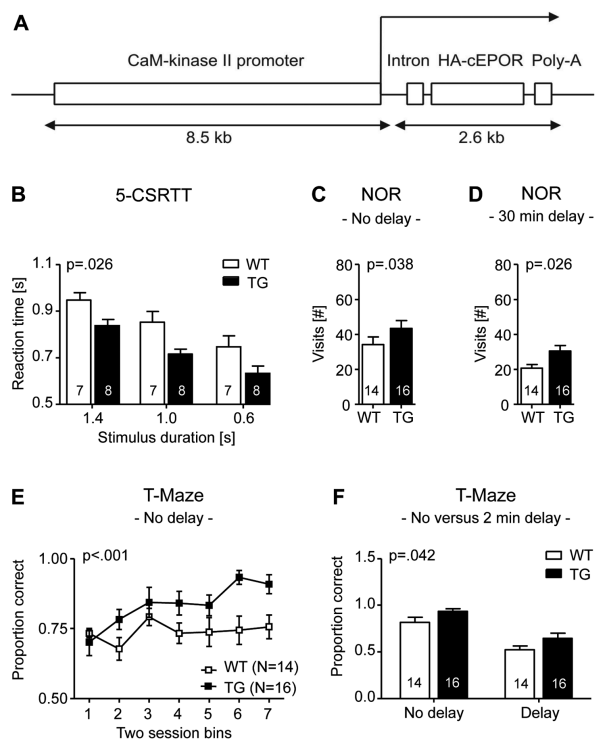
elsewhere (7). In the present study, we report only the results obtained in intervention phase 3 (variable, short stimulus duration, indicative of speed of processing).

**Novel object recognition task.** Briefly, mice are habituated for 20 min to a gray plastic arena (45  $\times$  45 cm, 35 cm high) with no objects. Next, for the training session, an object is placed in the arena, and the time the mouse spends exploring the object (that is, nose  $\leq$  15 mm to the object) is recorded for 10 min (video tracking software Viewer 2; Biobserve, Bonn, Germany). Next, a new, second object is added to the arena and exploration recorded for 10 min (testing session). The whole procedure is repeated several days later with a different set of objects and a 30 min interval between training and

testing session. We use plastic objects of similar size (around 3 cm in diameter) but different shape, texture, and color (pilot experiments had confirmed that mice show no spontaneous preference for any of the objects).

**T-maze.** The T-maze consists of three arms (clear plexiglas, 7 cm wide, 12.5 cm high with the start arm 43.5 cm long, and the goal arms 32.5 cm each). The goal arms contain distinct visual cues on the outer side of the walls; the start arm is plain. A plastic pellet cup (2.2 cm high, 3.5 cm in diameter) is situated in the rear of each goal arm. To have equal olfactory reward cues in both arms, we use pellet cups consisting of two parts separated by a perforated floor: the lower part contains 12 food pellets, which are inaccessible for

mice; the accessible upper part holds one food pellet as reward. Before starting the habituation procedure, mice are food deprived (1 h/day access to food in the home cage) for 3 d. Another 3 d of habituation to apparatus and reward (5 min of exposure to the maze each day, with reward placed at both ends of the T-maze) are followed by training for 12 d, six trials/day. On the first (sample) run of each trial, both goal arms are baited, but the mouse is forced to choose one of the goal arms (the other being closed by a removable wooden block: 16 cm  $\times$  6.9 cm). After entering the preselected goal arm, the mouse is allowed to consume the reward for 20 s and then placed back in the start box. On the second (choice) run, which during the acquisition phase of training follows 20 s



**Figure 2.** Mice with transgenic expression of constitutively active EPOR (cEPOR) driven by the  $\alpha$ -calcium/calmodulin-dependent protein kinase II ( $\alpha$ -CaMKII) promoter demonstrate highly superior cognitive performance compared with their wild type littermates. (A) Construct used for transgenic expression. (B) Significant reduction of reaction time in the attentional testing part of the 5-Choice Serial Reaction Time Task (5-CSRTT) reflects superior speed of cognitive processing in transgenic mice. (C–F) Transgenic mice perform better in Novel Object Recognition (NOR) (depicted is the number of visits of the new object) and T-maze tests with or without delay, illustrating their supremacy in memory tasks. Exact N numbers of all experiments are given directly in the bars or the line graph; all male mice, 5–8 month old at the time point of testing; mean  $\pm$  standard error of the mean (SEM) presented; two-way ANOVA for repeated measures and Mann-Whitney *U* tests applied.

after the sample run, both goal arms are open, and the mouse is rewarded for choosing the previously unvisited arm. The location of the sample arm (left or right) is varied pseudorandomly across trials so that mice received equal numbers of left and right presentations, but no more than two consecutive trials with the same sample location. The mice are run in squads of 6–8 (including both transgenic and littermate control animals) to minimize variation in intertrial intervals (12 min for all mice throughout the 12 d of training). *No-delay trials*: Mice are trained for 14 d in sample and choice runs with 20-s interval (10 trials on d 1; 6 trials/day from d 2–14). *Delay trials*: After finishing

the no-delay procedure, mice are tested in the delay procedure using two different time intervals between sample and choice run: 2 min and 6 min (3 d per delay; six trials/day containing three delay and again three no-delay trials each—in an alternating manner—to rule out motivational decrement and to obtain an internal control condition).

Note: All experiments in this manuscript were conducted in a blinded fashion that is, with the respective investigator/rater being unaware of sample assignment.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).

## RESULTS

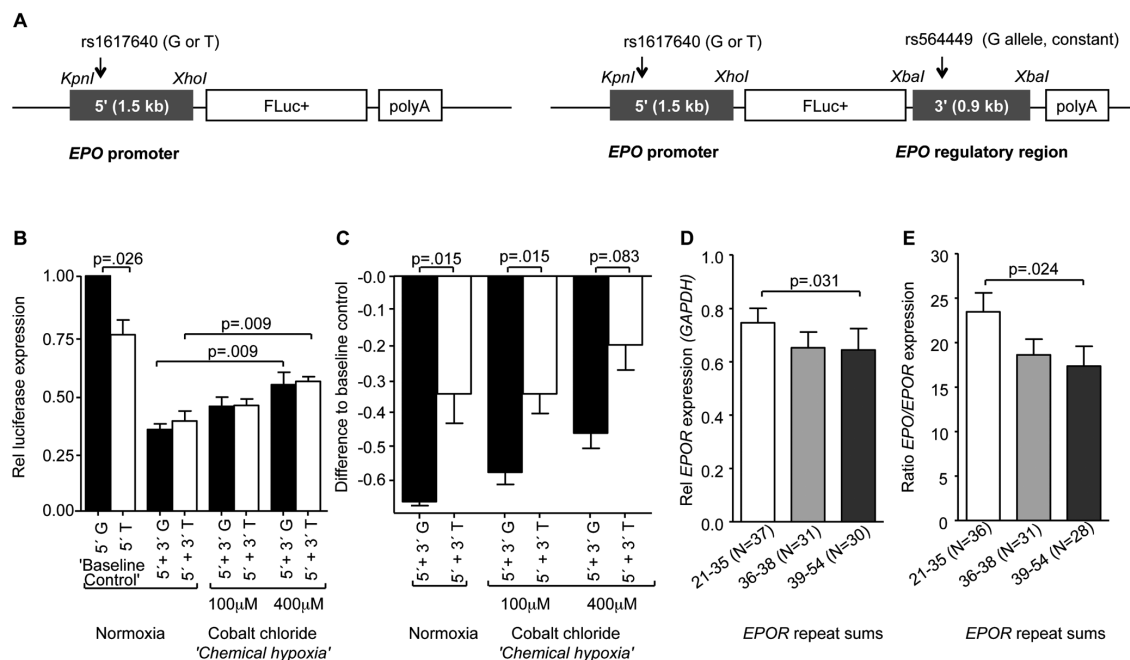
### Case Control Study

*EPO* SNPs rs1617640 (T/G) and rs564449 (T/G), as well as *EPOR* STR ( $GA$ )<sub>n</sub>, are not associated with schizophrenia. When conducting phenotype-based genetic association studies (PGAS) to evaluate the contribution of certain genotypes to defined subphenotypes, a potential role of these genotypes as genetic risk factors for schizophrenia should first be explored. Therefore, we performed a case control study on SNP rs1617640 (T/G) and SNP rs564449 (T/G), located in the 5' upstream region and in the 3' regulatory region of the *EPO* gene, respectively, as well as the STR ( $GA$ )<sub>n</sub> in the *EPOR* 5' upstream area (Figure 1A). To assess the *EPOR* genotype, the repeat lengths sum of both alleles was employed. No significant difference in the distribution of *EPO* genotypes (Figures 1B, C;  $\chi^2 = 1.897$  and  $p = 0.397$ ;  $\chi^2 = 0.058$  and  $p = 0.972$ ) or of *EPOR* repeat lengths sum (Figure 1D;  $\chi^2 = 23.85$ ,  $p = 0.917$ , evaluated with Monte Carlo sampling on 1,000 runs) between cases ( $N = 1,050$ – $1,054$ ) and healthy controls (blood donor sample;  $N = 1,141$ – $1,142$ ) was found (for details see Figure 1 and Supplementary Table S1). An association analysis of single allele repeat lengths instead of allelic repeat lengths sum between cases and controls also failed to yield significant distribution differences ( $\chi^2 = 32.15$ ,  $p = 0.114$ , evaluated with Monte Carlo sampling on 1,000 runs). Furthermore, the intraindividual difference of repeat lengths as a measure of marker heterogeneity did not vary between cases and controls (Figure 1E;  $\chi^2 = 8.54$ ,  $p = 0.967$ , 1000 Monte Carlo simulations). Thus, we could not find any evidence for a role of *EPO/EPOR* genotypes regarding the risk to develop schizophrenia. For the following phenotype analyses, the *EPO* SNP rs564449 (T/G) had to be excluded due to its low minor allele frequency (MAF~11%).

### Phenotype-Based Genetic Association Study (PGAS)

*EPO* SNP rs1617640 (T/G) and *EPOR* STR ( $GA$ )<sub>n</sub> are associated with higher





**Figure 3.** Genotype-dependent *EPO/EPOR* expression differences using reporter gene assays and PBMCs. (A) Reporter gene constructs: 5' upstream region of the *EPO* gene with either G or T at rs1617640 (left) and in addition with the 3' regulatory region of *EPO* (right). (B) G at SNP rs1617640 leads to significantly higher basal gene expression than T (baseline control). Addition of the 3' regulatory region to the construct induces downregulation of gene expression. This suppression is stepwise alleviated by increasing doses of CoCl<sub>2</sub> (100 μmol/L and 400 μmol/L). (C) In all conditions shown in (B), the suppressability (regulability) of gene expression compared with the baseline control (that is, normoxic control condition of B) is highest for the G genotype. (D) *EPOR* mRNA levels in peripheral blood mononuclear cells (PBMCs) of patients, determined by qRT-PCR and normalized to GAPDH as the housekeeper, show that the lowest *EPOR* STR (GA)<sub>n</sub> repeat length sum is associated with the highest *EPOR* expression. Gender distribution among the three repeat groups is well balanced (males/all: 24/37, 18/31, 20/30;  $\chi^2$  test  $p = 0.628$ ). (E) Even when considering the individual *EPO* mRNA levels (which by themselves do not reveal differences; data not shown) in form of an *EPO/EPOR* expression ratio, the significant difference between different STR length carriers remains. N = 28–37 per group; mean  $\pm$  standard error of the mean (SEM) presented; Mann-Whitney *U* tests applied.

**cognition in schizophrenia.** In a previous treatment trial, we showed that high-dose EPO, infused weekly over 12 wks, improves cognitive functions and reduces cortical gray matter loss in chronic schizophrenic patients. The domains most prominently influenced by EPO were speed of cognitive processing and short-term memory (Repeatable Battery for the Assessment of Neuropsychological Status (RBANS<sup>TM</sup>): subtests coding, digit span and figure recall) (9). Assuming that an influence of genetic variation within the EPO system on cognitive function would be detectable targeting these domains, we selected of the tests available within the GRAS database those closest to the above (same test or similar test regarding domain or loading on the same factor, that is, tests leading to similar results in an indi-

vidual due to joint variation in response to the same unobserved latent variable): Digit Symbol-Coding test, VLMT, and Dotting/Tapping (45). Indeed, significant associations were detected for both, *EPO* SNP rs1617640 (T/G) and *EPOR* STR(GA)<sub>n</sub> low versus high repeat sum (Table 1): carriers of G at *EPO* SNP rs1617640 and of *EPOR* STR(GA)<sub>n</sub> low repeat sum showed superior performance. In contrast, none of the relevant sociodemographic or basic disease variables revealed any significant associations (Table 2). To see whether certain combinations of genotypes of the *EPO* and *EPOR* genes would lead to better performance in the sense of a potential interaction effect, we grouped them accordingly. For *EPOR* genotypes, we assigned all individuals to three equally sized groups of allelic repeat sum carriers from

low to high (Figure 1F). Surprisingly, we found one particular genotype combination, GG&21–35 (lowest) repeat sum, to be highly superior compared with all others with respect to performance in Digit Symbol-Coding test ( $p < 0.0001$ ; see Figure 1F) but also in the other tests, where associations had been found for either *EPO* or *EPOR* or both (all  $p \leq 0.003$ ; Table 1).

In contrast to the clear associations with cognitive parameters found here, and in agreement with previous work by others, for example (14), we did not see any evidence of a potential association of *EPO/EPOR* genotypes with blood indices. In fact, we screened a total of 94 patients (Göttingen participants of the GRAS study) where comprehensive information on blood data was available. Repeated determinations over the year in these pa-

tients (between 2–48 times each) allowed a first step to substantiate each individual's normal values (considering also the reason for hospitalization, for example, exclusion of values obtained after blood loss due to a suicide attempt). These in turn delivered the basis of the mean values given in Supplementary Table S2. As shown there, all parameters are highly similar among genotypes. Interestingly, in this small cohort of individuals (N = 94) with information on blood indices, N = 6 subjects carried the GG&21–35 (low) repeat sum genotype and could be checked against all other genotype combinations. Group comparison by Mann-Whitney *U* test did not reveal any difference between groups for hemoglobin levels ( $p = 0.413$ ), whereas the cognitive composite score already yielded a nearly significant result ( $p = 0.093$ ) (Supplementary Figure S2).

#### cEPOR Expression in Pyramidal Neurons of Cortex and Hippocampus Increases Speed of Cognitive Processing and Memory Functions in Mice

On the basis of the above findings, we speculated that the best performing genotype combination should be characterized by higher EPO/EPOR expression. This hypothesis is further supported by (i) the cognition-improving effect of high-dose EPO in clinical trials (8,9) and in mouse studies (5,6) and (ii) our previous data on mice with transgenic expression of constitutively active EPOR (cEPOR) driven by the  $\alpha$ -calcium/calmodulin-dependent protein kinase II ( $\alpha$ -CaMKII) promoter (Figure 2A). These mice show better performance in higher cognitive tasks (7). We reexamined these mice and specifically conducted/analyzed tests measuring speed of cognitive processing and short-term memory, analogous to our neuropsychological findings in humans. In fact, we found a clearly reduced reaction time in the phase addressing selective attention in the 5-CSRTT (2-way ANOVA for repeated measures,  $F_{(1,14)} = 6.159$ ;  $p = 0.026$ ; Figure 2B) as well as superior performance in short-term memory tasks, that is, novel object recognition (no-delay trials  $p =$

0.038; 30-min delay trials  $p = 0.026$ ; Mann-Whitney *U* test) and T-maze (2-way ANOVA for repeated measures, no-delay trials  $F_{(1,22)} = 19.61$ ,  $p = 0.0002$ ; 2-min delay trials  $F_{(1,22)} = 4.668$ ,  $p = 0.042$ ; Figures 2C–F). These data further support the hypothesis of higher expression/activity of the EPO system being associated with better cognitive functioning.

#### Mechanistic Insight: Genotype-Dependent EPO/EPOR Expression Differences

To better understand a potential influence of the EPO SNP rs1617640 on gene expression, we used reporter gene assays based on the 5' upstream region of the EPO gene with G or T at the respective position. Additionally, we designed constructs including the 3' regulatory region of human EPO, as this is known to be highly homologous between species and thus of major importance for the complex regulation of EPO gene expression (39,46) (Figure 3A). As illustrated in Figure 3B, G at SNP rs1617640 leads to significantly higher basal gene (luciferase) expression than T. Upon addition of the 3' regulatory region to the construct, a remarkable downregulation of gene expression can be observed that is likely due to the interaction with the 5' region which is essential for hypoxia-related EPO regulation (39,40). This suppression is stepwise alleviated by increasing doses of CoCl<sub>2</sub> as an inducer of chemical hypoxia. In all conditions, the suppressability (regulability) of gene expression as compared with baseline is highest for the G genotype (Figure 3C).

To explore the role of the EPOR STR (GA)<sub>n</sub> repeat length sum on gene expression, we determined EPOR and EPO mRNA in peripheral blood mononuclear cells. The results show that the lowest repeat sum is associated with the highest EPOR expression as well as with the highest EPO/EPOR ratio as readout of the interplay between the two genes (Figures 3D, E). EPO mRNA levels per se did not differ significantly between EPOR repeat sum groups (data not shown). The exact mechanisms explaining the observed effects on quantitative

gene expression are still unclear. Both, EPO and EPOR gene variants investigated here are located in the promoter areas of the respective genes, where they may, for instance, directly or indirectly influence transcription factor binding. Indeed, regulation of these genes is highly complex and involves many different transcription and cofactors, the roles of which are still widely obscure (46,47).

#### Replication of the EPO and EPOR Genotype Associations with Cognitive Performance in a Healthy Control Sample

Having obtained associations of genotypes in the EPO system with cognition in a disease population, that is, individuals suffering from schizophrenia with known disease-typical cognitive deterioration, we wanted to know whether similar findings would be obtained in healthy controls. Fortunately, a phenotyped control population from Munich was available for comparison. Again, a case control study on EPO SNP rs1617640 (T/G) as well as EPOR STR (GA)<sub>n</sub> did not reveal differences between these healthy controls and the GRAS subjects (Supplementary Table S1, Supplementary Figure S1). This healthy control population, however, has several confounders with respect to the GRAS sample: (1) Of the relevant cognitive domains, only one has been evaluated with the same test, the other one just with a similar test loading on the same factor (that is, producing similar results in an individual based on a common underlying source of variance); (2) The population is considerably older on average; (3) The sample has a different gender distribution (Table 2). Despite these limitations, the associations are essentially reproduced (Table 1), pointing to very robust effects. Interestingly, having the GG&21–35 repeat sum genotype does not yield an advantage for healthy individuals who generally perform much better on all cognitive tests as compared with schizophrenic individuals (Table 1). This observation suggests that only subjects with an inferior cognitive performance may benefit from this specific genotypic constella-

tion. We wondered whether the superiority of the GG&21–35 group would be revealed when selecting the subgroup of healthy individuals with an average performance equal to the mean performance of the schizophrenic sample on the Digit Symbol-Coding test. Indeed, the low performer group of healthy individuals (Digit Symbol-Coding test performance threshold  $\leq 50$ ) displays a similar tendency of genotype superiority ( $p = 0.089$ ) which is not detectable in the high performer group (Digit Symbol-Coding test performance threshold  $> 50$ ,  $p = 0.809$ ). These results suggest that the GG&21–35 genotype may disclose its benefits particularly in situations of reduced cognitive capacity or (relative) cognitive impairment (Supplementary Figure S3).

## DISCUSSION

In the present hypothesis-driven study, we identified novel associations of *EPO* and *EPOR* genotypes with cognition, namely speed of processing, short-term memory and tasks requiring distinct fine motor components, both in schizophrenic patients and in a healthy control population (replicate sample). On a molecular/cellular level, we demonstrate that the cognitively more beneficial genotypes are associated with higher expression/stronger regulability of expression. In supporting preclinical experiments, we show that mice with transgenic expression of constitutively active *EPOR* in pyramidal neurons of cortex and hippocampus (7) perform better in cognitive domains reminiscent of those influenced by *EPO/EPOR* genotypes in humans.

Interestingly, in the schizophrenic population that is defined by overall reduced cognitive performance, one discrete genotype combination (GG&21–35 repeat sum) achieves highly superior cognitive outcome, whereas this same combination in healthy individuals has only the tendency of an advantage. This advantage is restricted to subjects with lower cognitive capacity. Thus, higher *EPO/EPOR* activity appears to be most beneficial in situations of compromised function. We note that in disease states, such benefit is

likely achieved by the upregulation of the endogenous *EPO* system in the brain, as seen for example, in stroke, schizophrenia or Alzheimer's disease (48–50). In fact, this *EPO/EPOR* upregulation is further potentiated by rhEPO treatment, resulting in lasting cognitive improvement (8,9,51,52).

The selection of the cognitive domains reported here to show associations with *EPO/EPOR* genotypes strictly followed hypotheses derived from the results of earlier rhEPO treatment studies (9). Therefore, multiple testing issues do not apply here. On the other hand, also for a purely hypothesis-driven, exploratory study, it is reassuring to obtain replication of the results in an independent sample, in the present paper, a healthy control population.

Limitations of the present work are particularly (1) the incomplete availability of identical neuropsychological tests in the two populations studied (forcing to use tests loading on the same factor, that is, resulting in highly correlated readouts in a given individual due to shared variance produced by a common underlying variable), and (2) the different age/gender distribution. Despite all these shortcomings, a similar pattern of associations arose, pointing to robust effects and confirming the significance of *EPO/EPOR* genotypes for higher cognition.

We are aware that the use of transfection studies/reporter gene assays and of PBMCs cannot answer all questions related to genotype-dependent brain expression of the *EPO* system. Especially PBMCs are a heterogeneous population of cells. There may be variations in the composition of mononuclear cells such as circulating erythroid progenitor or precursor cells, megakaryocytes, mast cells and macrophages, lymphocytes, and endothelial progenitor cells from sample to sample. Many of these cell types potentially may serve as source of *EPO* expression in the peripheral blood. We cannot exclude, however, that our data on *EPO* and *EPOR* mRNA mainly derive from a small fraction of erythroid progenitors that express these genes. Nevertheless, the pragmatic

approach to analyze PBMCs was the only choice presently available for us to study quantitative gene expression in a reasonable number of humans with defined genotype. To obtain further support for increased expression/activation of the *EPO* system leading to better cognition, we additionally performed mouse studies in a model of targeted upregulation of *EPOR* in neuronal populations that are known to play a pivotal role in the functions of interest (7). The superiority of these mice included essentially the same cognitive domains as those found to be influenced by *EPO/EPOR* genotypes in human populations.

## CONCLUSION

To conclude, we identified an intrinsic role of the *EPO* system for higher cognition, reflected by associations of *EPO/EPOR* genotypes with cognitive performance, which may be of particular significance in disease states. These findings further suggest the *EPO* system as target for treating human brain diseases that are characterized by cognitive decline.

## ACKNOWLEDGMENTS

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## DISCLOSURE

H Ehrenreich has submitted/holds user patents for *EPO* in stroke, schizophrenia and MS. Apart from that, the authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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