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**Recombinant human erythropoietin delays loss
of gray matter in chronic schizophrenia**

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Running title: Erythropoietin delays cortical atrophy in schizophrenia

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Abstract

Neurodevelopmental abnormalities together with neurodegenerative processes contribute to schizophrenia, an etiologically heterogeneous, complex disease phenotype which has been difficult to model in animals. The neurodegenerative component of schizophrenia is best documented by magnetic resonance imaging (MRI), demonstrating progressive cortical gray matter loss over time. No treatment exists to counteract this slowly proceeding atrophy. The hematopoietic growth factor erythropoietin (EPO) is neuroprotective in animals. Here we show by voxel-based morphometry in 32 human subjects in a placebo-controlled study that weekly high-dose EPO for as little as 3 months halts the progressive atrophy in brain areas typically affected in schizophrenia, including hippocampus, amygdala, nucleus accumbens, and several neocortical areas. Specifically, gray matter protection is highly associated with improvement in attention and memory functions. These findings suggest that a neuroprotective strategy is effective against common pathophysiological features of schizophrenic patients, and strongly encourage follow-up studies to optimize EPO treatment dose and duration.

Key words:

Hematopoietic growth factor, neuroregeneration, neuroprotection, voxel-based morphometry, brain atrophy, cognition, schizophrenia

Introduction

Schizophrenia is the common term of an etiologically and pathophysiologically heterogeneous group of diseases, characterized by psychotic symptoms, loss of affect, social withdrawal and cognitive dysfunction. Neurodegenerative processes on top of neurodevelopmental abnormalities contribute to disease manifestation and progression. The neurodegenerative component of schizophrenia is best reflected by progressive cortical gray matter density (GMD) loss over time^{e.g. 1}. This loss is prominent in the early phase of schizophrenia, reaching a slower steady state progression once the disease process has turned into the chronic phase. In this phase of schizophrenia, approximately 0.5% of gray matter are lost yearly compared to 0.2% in healthy individuals², and this loss goes hand in hand with increasing functional deficits, in particular cognitive decline, comprising deficits in processing speed, attention, memory, and executive functioning.

No treatment exists to date to counteract these neurodegenerative processes. Clinical studies using various different first and second generation antipsychotics yielded inconsistent results regarding progression of GMD loss^{e.g. 3, 4}. Greater GMD loss upon first generation antipsychotics was found transiently in the recovery phase from a first schizophrenic episode despite parallel symptomatic relief comparable to second generation drugs^{e.g. 4}. Importantly, in the majority of studies, GMD changes were neither correlated with clinical nor with neurocognitive readouts^{e.g. 3, 4}. Cognitive improvements were small and unlikely to be greater than placebo or practice effects^{5, 6}. Also, less adverse consequences of an antipsychotic

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drug on neurocognition in comparison to its respective comparator drug have been discussed as 'false positive' effects⁷.

To explore a neuroprotective strategy in schizophrenia, for which adequate animal models do not exist, we performed a clinical trial with recombinant human EPO. Originally established for the treatment of anemia, this drug is well tolerated, clinically safe, crosses the blood-brain-barrier⁸ and possesses multifaceted direct neuroprotective properties^{9, 10}. EPO is able to prevent brain atrophy following parietal cortical lesion of juvenile mice¹¹ or middle cerebral artery occlusion in mice¹², and to improve cognitive performance in various disease models as well as in healthy rodents^{8, 13, 14}.

We show here that treatment of schizophrenic men over 3 months with weekly high-dose EPO reduces GMD loss in discrete brain regions typically affected in schizophrenia, and that this reduction in GMD loss is strongly associated with improved attention and memory functions.

Patients and Methods

Study design and patients

The protocol for the present double-blind, placebo-controlled randomised study was approved as part of a larger multicenter trial protocol¹⁵ by the ethical committees of the Georg-August-University of Göttingen as well as of the Christian-Albrechts-University, Kiel. Total duration of the study was 2 years. After

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written informed consent, own and/or authorized legal representatives, chronic schizophrenic men (N = 32, aged 29-54 years) with a disease duration of ≥ 10 years, stable medication and disease state (no acute episode within 6 months before start of the study) were included. They had to have a defined cognitive and psychopathological deficit (Repeatable Battery for the Assessment of Neuropsychological Symptoms¹⁶ = RBANS ≥ 1 SD below normal; Positive and Negative Syndrome Scale = PANSS¹⁷, negative ≥ 20 , general ≥ 48 , positive ≤ 28). Thus, patients with severe positive symptoms (according to their PANSS score) have been excluded in this first study on EPO in schizophrenia. The reasons for this rigorous selection (considering the small number of patients to be included) were: (1) High positive PANSS scores would have severely compromised the evaluation of cognitive outcomes; (2) Acute psychosis with its unpredictable change in state, often also requiring additional intervention, would have made cognitive and perhaps also MRI follow-up testing unreliable; (3) Strict PANSS (all subscales) and RBANS limits were set to ascertain a highly homogenous sample of chronic schizophrenic patients (even at the cost of a difficult recruitment process with a ratio of screened versus included patients amounting to 4:1). Additionally, patients had to be free of other severe diseases, any suspicion of organic psychosis, alcohol or drug abuse, myeloproliferative disorder (including hematocrit $\geq 50\%$), allergies against or antibodies to EPO. Patients were treated with a weekly short intravenous infusion (15min) of 40000IU EPO (recombinant human erythropoietin-beta, Roche, Grenzach, Whylen, Germany) or placebo for 12 weeks. Cognitive readouts for the present study were assessed at baseline and at 12

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weeks of study drug treatment using the RBANS¹⁶. Psychopathology rating included the positive, negative and general psychopathological symptoms of schizophrenia as evaluated by the PANSS scales¹⁷. Blood workup was conducted weekly. MRI was performed at baseline and repeated after 12 weeks of study drug treatment (repetition in the same MR-scanner).

Data acquisition and preprocessing

For analysis of cognitive test results, raw data were subjected to ANCOVA with age as covariate. For determining the composite score out of selected significant RBANS subtests (figure recall, digit span and coding), raw data were z-transformed. Calculation of the z-scores was based on the means and standard deviations of the respective measurements at baseline. MRI scans were conducted on a Siemens Magnetom Sonata (Siemens Medical Systems, Erlangen, Germany) or a Philips Medical Systems MR-scanner (Gyrosan Intera Archieva, Hamburg, Germany), all operating at 1.5 Tesla. A T1-weighted 3-dimensional structural MRI of each subject was acquired (pulse sequence type: ultra fast gradient echo (Siemens: MPRAGE, Philips: 3D TFE), time to repetition (TR): 30ms; echo time (TE): 4.6ms; flip angle: 30°; matrix size: 256×256×150; voxel size of 1×1×1.5mm³). At the time of data processing, the investigator was neither aware of treatment assignment (EPO or placebo group) nor of any hypothesis regarding EPO effects (increase or decrease of GMD), or of brain regions typically affected in schizophrenia and likely to respond to neuroprotection. In other words, this

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investigator, despite performing essentially all imaging/statistical analysis, was initially completely 'blinded'.

Before preprocessing (*a priori*), all structural images were checked for too low signal-to-noise ratio (SNR), usually caused by motion artifacts or wideband and narrowband radiofrequency-noise, respectively. To this end, we calculated slice-wise SNR profiles of each image. The criteria applied were based on the investigations of Wetzel et al¹⁸. They reported for the T1-weighted gradient echo sequences, also used in our study, an SNR of 23.1 ± 5.2 for gray matter and of 27.8 ± 5.0 for white matter. Thus, we chose an SNR threshold of 20 for ALL slices containing parts of the brain to keep only those images with sufficient quality for further processing. Already a single slice with an SNR below 20 within the slices containing brain tissue was considered a sufficient exclusion criterion for the whole MRI scan (Supplementary Figure 1)^{18, 19}. Finally, images from only 32 (of originally 39) patients (16 EPO, 16 placebo) were included in data analysis. The image origin was placed on the anterior commissure, and images were reoriented manually to approximate the anterior and posterior commissure to the horizontal plane.

The Statistical Parametric Mapping 5 (SPM5) Software package (<http://www.fil.ion.ucl.ac.uk/spm/>) was used for the preprocessing and statistical analysis of the imaging data. The preprocessing of the imaging data was performed corresponding to an established voxel-based morphometry (VBM) protocol²⁰. This method minimizes the probability of misclassifications within the tissue segmentation by the introduction of additional preprocessing steps prior to

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normalization and subsequent segmentation. Briefly, this method comprises the following steps:

(i) Initially, a group-specific template was generated by normalizing each MR image to the template provided by the Montreal Neurological Institute, using linear (ordinary least square, 12 parameter affine transformation) and nonlinear (modeled by the linear combination of a set of harmonic basis functions, cut-off = 25mm) algorithms²¹. The normalized images were segmented into gray matter, white matter, and cerebrospinal fluid, smoothed with an isotropic Gaussian kernel of 10mm full width at half maximum (FWHM), and separately averaged for gray and white matter. Thus, 2 group- and tissue-specific templates were created with a voxel size of 1x1x1mm³.

(ii) MR images (in native space) were segmented into tissue classes, followed by a series of additional automated morphological operations to remove unconnected non-brain voxels from the segments. The gray and white matter images were normalized to the group-specific templates as described in (i).

(iii) The calculated normalization parameters were then reapplied to the structural images and these images were re-sliced to a voxel size of 1x1x1mm³. This approach led to improved normalization for the full brain images. It also guaranteed an optimal final segmentation because of the probability maps used as Bayesian priors being located in stereotactic space.

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(iv) The normalized structural images were segmented again. Because of local volume effects (growing or shrinking) in nonlinear spatial normalization, a voxel-wise multiplication with the Jacobian determinant (derived from normalization parameters) was performed to preserve the signal within a particular brain region.

(v) Finally, the tissue segments were smoothed with a 10mm FWHM Gaussian kernel²¹.

For the following statistical analyses we only utilized those smoothed gray matter tissue segments.

Data analyses

In search of structural treatment effects and their relation to clinical readouts the following analyses were carried out: (1) Due to the factorial character of the presented study, we performed a voxel-wise analysis of covariance (ANCOVA) to identify brain regions showing significant interaction between study medication and evolution of GMD over time of treatment. (2) To verify the regional findings of ANCOVA, we conducted a voxel-wise linear discriminant analysis (vwLDA). (3) Subsequently, regions with joint effects were outlined computing the voxel-wise logical AND-relation between ANCOVA and vwLDA. (4) A region of interest based linear discriminant analysis (rbLDA) was employed to test the association between GMD changes within joint regions and clinical readouts. Details of these approaches are explained in the following paragraphs.

Regional gray matter density analysis

(1) A voxel-wise 2-factorial analysis of covariance for repeated measurements (ANCOVA, between subject factor: TREATMENT, within subject factor: TIME, covariates: age, MR-scanner, study site) was applied to identify gray matter regions which show an interaction of TREATMENT*TIME. These regions were anatomically and, if possible, probabilistically cytoarchitectonically identified using the Anatomy Toolbox as implemented in SPM5²². To test for the direction of changes in GMD, we calculated post-hoc t-tests for the individual GMD values at the peak voxel positions. For this purpose, we adjusted the data for the group specific median of GMD in the first MRI.

(2) To verify the results of the ANCOVA, a voxel-wise linear discriminant analysis (vwLDA) using the pre- and post-treatment GMD (corrected for age, MR-scanner and study site) was calculated as implemented in the statistics toolbox²³ of MATLAB Version 7.4 (Mathworks, Inc., Natick, MA). The estimated discriminant function separates the data into 2 subsets. Goodness of classification (GoC), the corresponding χ^2 value and the error probability for false positive findings were calculated.

(3) Although potentially important brain regions might have been identified by only one statistical approach (ANCOVA or vwLDA, see Supplementary Tables 1 and 2), we decided to go for a more conservative procedure, particularly in light of the small sample size potentially increasing the instability of results. Anatomical

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regions with a treatment effect detectable by only one of the before mentioned statistical analyses were not considered. In fact, to yield more robust results with respect to regional differences, we calculated the spatial intersection of both statistical maps. For this calculation, only voxels which passed a significance threshold of $p < 0.005$ (uncorrected for multiple comparisons) and a minimal cluster size of 100mm^3 were used.

A mean anatomical image was calculated from the normalized T1-weighted images of all 32 patients. The 3 statistical maps calculated (ANCOVA: Interaction TREATMENT*TIME; vwLDA; Intersection Interaction \cap vwLDA) were overlaid on this mean anatomical image using MRICron software (<http://www.sph.sc.edu/comd/rorden/mricron/>).

Analysis of associations between joint regions of GMD change and clinical readouts

A region of interest based linear discriminant analysis (rLDA) was performed in order to demonstrate interactions between changes of GMD (corrected for age, MR-scanner and study site) over time [$z(\Delta\text{GMD})$] and cognition (corrected for age), determined as sum of z-scaled longitudinal differences for selected RBANS subscales, i.e. cognitive sum score of figure recall, digit span and coding [$\sum z(\Delta\text{CSS})$]. Likewise, possible interactions between [$z(\Delta\text{GMD})$] and psychopathology (PANSS subscales) were evaluated. Moreover, potential effects

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of hemoglobin or hematocrit changes over time (ΔHb or ΔHct week 12 minus baseline) on cognition [$\Sigma z(\Delta\text{CSS})$] were controlled for by employing ANCOVA (covariates age and ΔHb or ΔHct).

Results

Chronic schizophrenic men were randomly assigned to receive weekly intravenous infusions of high-dose (40000IU) EPO (N=16) versus placebo (N=16) over 12 weeks in a double-blind setting. Patient characteristics were found well matched in both groups (Table 1). Only age tended to be different, with EPO patients being slightly younger. Therefore, all calculations include age correction together with correction for MR-scanner and study site (see above).

EPO prevents progressive gray matter loss in discrete brain areas typically affected in schizophrenia

VBM results revealed a recognizable neuroprotective effect of EPO in schizophrenic patients after a treatment period of only 12 weeks (Table 2, Figure 1), extending to a total of 4470mm³ of brain volume (sum of all clusters listed in Supplementary Table 1, referring to ANCOVA). In the **voxel-wise 2-factorial analysis of covariance for repeated measurements (ANCOVA)**, the effect of EPO treatment became apparent in the interaction TREATMENT*TIME,

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demonstrating clearly reduced evolution of GMD loss upon EPO compared to placebo. Such interaction was noted in several frontal and temporal cortical and subcortical regions known for cortical thinning in schizophrenia. The most pronounced and extensive effect ($\sim 860\text{mm}^3$) of EPO was found in the right ventral frontal cortex including ventral striatum with nucleus accumbens and rectal gyrus. In the left nucleus accumbens, a smaller effect ($\sim 120\text{mm}^3$) was noted. In frontal regions, effects were found in the ventral part of the right anterior cingulate gyrus ($\sim 460\text{mm}^3$), the right middle frontal gyrus ($\sim 140\text{mm}^3$) as well as in the left insula ($\sim 200\text{mm}^3$). Several perisylvian cortical regions of the left hemisphere, like inferior frontal gyrus, including Broca's area ($\sim 680\text{mm}^3$), inferior parietal cortex ($\sim 660\text{mm}^3$), including the parietal operculum and the supramarginal gyrus, as well as 2 clusters of about 290mm^3 and 150mm^3 volume in the middle temporal gyrus exhibited a delay in cortical atrophy upon EPO. In the right temporal cortex, the GMD in the temporal pole was influenced by EPO treatment ($\sim 330\text{mm}^3$). Effects of treatment were further detectable in the left entorhinal cortex ($\sim 340\text{mm}^3$), specifically the fusiform gyrus, several parts of the hippocampus (cornu ammonis, subicular complex, fascia dentata) and a small part of the superficial section and laterobasal nucleus of the amygdala ($\sim 250\text{mm}^3$) (Supplementary Table 1).

Compared to ANCOVA, the **voxel-wise linear discriminant analysis (vwLDA) for morphological data** provided more spatially extended results ($\sim 17470\text{mm}^3$) (Table 2, Figure 1, and Supplementary Table 2). GMD data from the 2 experimental groups, corrected for age, MR-scanner and study site, arranged in a

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2-dimensional scatter plot, can be separated using a linear discriminant function. For discrete brain regions, data allow to predict with an accuracy of $\geq 75\%$ whether they are derived from EPO or placebo treated individuals. Similar to ANCOVA, clusters with significant classification accuracy are located mainly in frontal and temporal regions. The largest cluster with significant differences in GMD evolution between EPO and placebo ($\sim 2470\text{mm}^3$) and 2 additional distinct regions of about 440mm^3 and 430mm^3 volumes were found in the medial frontal cortex associated with primary and supplementary motor function. In the area of the left medial wall, 2 clusters in middle cingulate structures ($\sim 240\text{mm}^3$ and $\sim 210\text{mm}^3$) also show treatment effects. Further discriminant effects in the left frontal lobe were found in the inferior frontal gyrus ($\sim 1840\text{mm}^3$, Brodmann areas 44 and 45), superior frontal gyrus ($\sim 740\text{mm}^3$) and precentral gyrus ($\sim 220\text{mm}^3$). Effects detected with a peak location in the right ventral frontal cortex including nucleus accumbens, head of caudate nucleus and anterior part of putamen were comparable to those found in the ANCOVA ($\sim 400\text{mm}^3$). In the left hemisphere, effects were noted in insula ($\sim 260\text{mm}^3$) and more extended in superior ($\sim 1130\text{mm}^3$), middle ($\sim 390\text{mm}^3$) and inferior temporal gyrus ($\sim 420\text{mm}^3$). Also, a part of the transverse temporal gyrus ($\sim 190\text{mm}^3$, Heschl) was affected. In ventromedial occipital and temporal regions, we found effects in left fusiform gyrus and several left hippocampal regions (subiculum, entorhinal cortex) ($\sim 730\text{mm}^3$). Slightly weaker effects were observed for other hippocampal (cornu ammonis, subiculum, fascia dentata) and amygdala clusters (laterobasal nucleus, superficial section) in left temporal regions ($\sim 570\text{mm}^3$) as well as for the superior temporal gyrus ($\sim 230\text{mm}^3$). More

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pronounced effects were noted in homologous regions of the right hemisphere (~1540mm³). In the parietal lobe, 2 clusters are located in the left postcentral gyrus (~930mm³) and parietal regions containing the left angular gyrus (~1050mm³) and parts of superior and inferior parietal lobules (~330mm³). Even though weaker as in the left hemisphere, significant treatment effects were detected also in the right postcentral gyrus (~340mm³). Occipital regions, comprising superior (~260mm³) and middle gyrus (~370mm³), as well as the left cuneus (~1200mm³), calcarine sulcus and lingual gyrus (~760mm³) showed significant EPO effects (Supplementary Table 2).

To further increase the probability of detecting true regional changes in GMD upon treatment, particularly in light of the small patient sample size, we chose a more conservative procedure: We calculated the **spatial intersection of the 2 statistical maps, ANCOVA and vwLDA** (Table 2, Figure 1). This intersection uncovers the regions with methodologically independent findings for the treatment effect and extends over an area of about 1400mm³. Thereby, ANCOVA and vwLDA show a mean overlap of about 19% of their volume (Supplementary Table 3). Joint effects were found in left medial ventral temporal lobe: The first cluster (~290mm³) covers entorhinal cortex and hippocampal subiculum. The second cluster (~200mm³) contains hippocampal subregions cornu ammonis, subiculum, fascia dentata as well as a superficial section and the laterobasal nucleus of amygdala. Great overlap (~290mm³) between ANCOVA and vwLDA results was found in inferior frontal cortex within Broca's area (BA45). Also in frontal lobe about

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240mm³ of right ventral striatal areas with nucleus accumbens belong to the spatial intersection. Further overlap (~240mm³) was detected in left sided inferior parietal cortex comprising supramarginal gyrus and a part of parietal operculum (OP1). Finally, a focal joint effect was found in the posterior part of the left middle temporal gyrus (~140mm³).

EPO induced reduction of gray matter loss in specific brain areas is associated with cognitive but not psychopathological improvement

In order to further elucidate the clinical significance of these VBM findings, we explored a potential relationship between protection of GMD and increase in cognitive performance. **ANCOVA with age as covariate** had revealed a highly significant improvement of EPO as compared to placebo patients in attention/memory functions from baseline to the 12-week treatment time point, even though an improvement was also measurable in the placebo group¹⁵. In further exploratory analyses on the MRI subgroup of patients (N=32), the sum score of the neuropsychological tests figure recall, digit span and coding showed the strongest age-corrected interaction TREATMENT*TIME ($p=0.003$) which was essentially unaffected by introducing ΔHb or ΔHct as further covariates (interaction TREATMENT*TIME $p=0.018$ and $p=0.016$, respectively). Therefore, we computed an additional **region of interest based LDA (rbLDA)** to investigate the relationship between this cognitive sum score (CSS) and changes in GMD. For this purpose, GMD difference in all 6 voxels with the maximal joint effect between

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ANCOVA and vwLDA were z-scaled for each patient [$z(\Delta\text{GMD})$] (Table 2). In addition, the sum score of the z-scaled longitudinal differences in the 3 neuropsychological tests was calculated [$\Sigma z(\Delta\text{CSS})$] (Table 2). The rbLDA, based on $z(\Delta\text{GMD})$ and $\Sigma z(\Delta\text{CSS})$, yielded highly significant results in goodness of classification for all 6 clusters (Table 2, Figure 1).

Similar to cognitive readouts, all schizophrenic patients, independent of the treatment group, improved over the 12 weeks of study in psychopathology scores (PANSS). In contrast to cognition, however, showing a significantly better outcome upon EPO, PANSS scores improved in both placebo and EPO patients to a comparable degree¹⁵. Accordingly, there was no association of GMD in any of the brain regions of interest with positive, negative and general psychopathological symptoms of schizophrenia in either treatment group (data not shown). This is in distinction to the clear relation between GMD and cognition.

Discussion

Collectively, these data suggest that progressive loss of cortical GMD in discrete brain regions of a highly homogeneous cohort of chronic schizophrenic patients is significantly reduced during EPO treatment over a period as short as 12 weeks. Clear interactions between TREATMENT*TIME were found in cortical and subcortical regions, typically affected in schizophrenia²⁴. Protection of GMD in

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cortical areas was associated with better cognitive performance. Thus, provided that these results can be consolidated in a larger clinical trial, perhaps extending now to a patient population to be recruited with less strict eligibility criteria, EPO would be the first hopeful compound for prevention of cortical GMD loss and improvement of neurocognition.

Certain brain regions deserve particular attention in this study. These are the regions that displayed the most prominent overlap between ANCOVA and vwLDA, and where significant relationships between cognitive and GMD changes were detected by rbLDA. In mesolimbic structures, hippocampus and amygdala, known to undergo volume reduction²⁴⁻²⁷ and to be linked to attention and memory deficits in schizophrenia²⁸⁻³⁵, EPO prevented GMD loss as shown by ANCOVA and vwLDA. An association of GMD changes and cognition was documented by rbLDA, with EPO patients having a clearly better outcome. Similarly, the fusiform gyrus, found to be reduced in volume in chronic schizophrenia³⁶⁻³⁸ benefited from EPO treatment.

Also the inferior frontal gyrus, a brain area involved in semantic processing and integration^{39, 40}, has previously been found to undergo GMD loss in schizophrenia⁴¹, and showed protection by EPO in the present study. Another area with remarkable overlap of EPO effects in both ANCOVA and vwLDA was the left middle temporal gyrus. The GMD alterations found here were again correlated with cognition according to rbLDA. This brain area is engaged in auditory and language processing, visual perception and multimodal sensory integration^{36, 42-44}. Reduced

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GMD volumes in temporal lobe have also been associated with auditory hallucinations⁴⁵. Thus, EPO prevents GMD reductions in temporal areas consistently found associated with hallucinations and delusions in schizophrenia. To turn this EPO effect into clinical benefit in a chronic schizophrenic population with long-term antipsychotic pretreatment and low positive scores at study entry (as required inclusion criterion), however, may take more than 3 months. Along these lines, there was no difference in positive or negative symptom scoring nor in general psychopathology between EPO and placebo treated patients despite improvement in cognition upon EPO.

The inferior parietal cortex has been implicated in sensory integration, body image, concept of self, and executive function⁴⁶. Schizophrenic patients show reduced GMD in the left inferior parietal lobule⁴⁷. Again, this area was protected by EPO treatment and protection was associated with improved cognition. In the right nucleus accumbens, known to be associated with motivation/reward and to dysfunction in schizophrenia⁴⁸, GMD protection and cognitive amelioration were also interrelated in our patient population.

An interesting observation is the lateralization of the morphological effects: Whereas only the nucleus accumbens showed EPO-mediated prevention of GMD loss on the right hemisphere, all other regions with EPO effects, hippocampus, amygdala, fusiform gyrus, middle temporal gyrus, inferior frontal gyrus, and inferior parietal cortex exhibited a prominent effect on the left hemisphere. The unequal distribution of GMD loss is consistent with earlier reports on selective cortical

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thinning²⁴ including work comparing effects of antipsychotics^{4, 49}. The basis for the lateralized preference of EPO-mediated GMD protection is still unclear but likely indicates areas with most progressive neurodegeneration inherent to the disease process.

Whereas it seems logical that acute loss of GMD might lead to negative consequences with respect to brain function and, accordingly, prevention of this loss should maintain or improve function, this association has still not been proven by the various studies investigating effects of antipsychotics on brain dimensions and functions^{e.g. 3, 4}. In fact, during the time of most progressive GMD loss in acute first episode schizophrenia, improvement of clinical readouts, independent of the type of antipsychotic applied, can be observed⁴. This seemingly contradictory finding may change upon chronicity of an illness. In an acute disease state, the cellular / molecular underpinnings of rapid brain matter reduction most likely differ from those contributing to the slowly progressing brain atrophy in a more chronic condition. In the present study, reduction of GMD loss and associated improvement of cognitive performance have been observed upon neuroprotective treatment with EPO in chronic schizophrenic patients. A likewise effectiveness of EPO treatment in acute disease states, e.g. first episode schizophrenia, still needs to be demonstrated.

Even though in the present study, prevention of GMD loss in discrete brain areas might explain the improved cognitive performance of EPO treated chronic schizophrenic subjects, the cellular mechanisms involved remain to be determined

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which, however, is impossible in humans. In rodents, EPO is not only neuroprotective^{9, 10}. EPO also improves neuroplasticity, synapse formation and neuronal process outgrowth^{13, 14, 50}, all mechanisms potentially important for higher cortical functions, but with unclear relationship to the halt of brain atrophy. Since an increase in the number of microglia in schizophrenic brains might add to the gradual loss of axons and synapses through synaptic stripping⁵¹, a neuroprotective effect of EPO via prevention of microgliosis and injury-related cortical tissue loss, as recently demonstrated in mice^{11, 52} could play a role in this context. A potential contribution of the EPO induced increase in hemoglobin / hematocrit (and thus increased tissue oxygenation) to cognitive improvement / protection of GMD cannot be entirely excluded at this point but seems unlikely in light of the following points: (1) No influence of blood parameters was found upon their addition as covariates to ANCOVA in the present study. (2) EPO is known to pass an intact blood-brain-barrier in schizophrenic patients and to act directly on neural cells that express EPO receptor^{8, 9}. (3) Non-hematopoietic EPO analogues show effects on the brain comparable to those of EPO⁵³.

Taken together, the observations that EPO is the first compound promising to prevent progression of brain atrophy and to improve cognitive performance in humans should encourage further basic and clinical research, including prevention strategies in first episode schizophrenia.

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Conflict of interest

HE holds a user patent for the treatment of schizophrenia with EPO.

Supplementary information

Supplementary information (3 Tables, 1 Figure) is available at *Molecular Psychiatry's* website.

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Table 1: Patient characteristics at baseline

	EPO N=16 M (±s.d.)	Placebo N=16 M (±s.d.)	2 Sample t-test	
			T ₍₃₀₎	p
Age (years)	38.8 (7.5)	43.8 (6.4)	2.0	0.052
Duration of disease	15.0 (8.1)	18.4 (5.6)	1.4	0.173
Years of education	12.3 (3.7)	13.0 (3.6)	0.5	0.596
MWT-B (raw data; scale range 0-37) <i>(premorbid intelligence estimate)</i>	26.3 (9.3)	29.5 (5.8)	1.2	0.254
PANSS Positive Scale (scale range 7-49)	14.4 (4.1)	15.5 (4.2)	0.7	0.469
PANSS Negative Scale (scale range 7-49)	29.6 (5.9)	27.4 (4.7)	-1.2	0.255
PANSS General Scale (scale range 16-112)	54.1 (10.0)	49.9 (4.8)	-1.5	0.146
Chlorpromazine equivalents	473.4 (346.0)	445.5 (455.2)	-0.2	0.846
	EPO N=16 n (%)	Placebo N=16 n (%)	χ^2 -test	
			$\chi^2_{(30)}$	p
Unemployed	7 (43.8%)	7 (43.8%)	0.0	1.000
Patients without partnership	16 (100%)	14 (87.5%)	2.1	0.144
Patients with children	3 (18.8%)	2 (12.5%)	0.2	0.626
Antipsychotic medication	1 st generation 2 nd generation combination	7 (43.8%) 6 (37.5%) 3 (18.8%)	3.1	0.216
Anticholinergic drugs	2 (12.5%)	4 (25.0%)	0.8	0.365
Patients with benzodiazepine treatment	4 (25.0%)	2 (12.5%)	0.8	0.365
Cigarette smoking	non-smoker smoker ex-smoker	2 (12.5%) 11 (68.8%) 3 (18.8%)	1.7	0.435
Right handed	14 (87.5%)	14 (87.5%)	0.0	1.00

Abbreviations: EPO - erythropoietin; M - mean; s.d. - standard deviation; MWT-B - Mehrfach-Wortschatz-Intelligenztest; PANSS - Positive and Negative Syndrome Scale; N - sample size; n - number of subjects

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Table 2: Statistical results demonstrating regional GMD and cognitive changes over 12 weeks of EPO versus placebo treatment in a cohort of chronic schizophrenic men

	Anatomical region Cytoarchitectonic probability (P_C)	Intersection cluster size [mm ³]	MNI-coor- dinates [mm] ANCOVA vwLDA			ANCOVA						vwLDA GMD (pre) versus GMD (post)			rbLDA $\Sigma z(\Delta CSS)$ versus $z(\Delta GMD)$				
						$F_{(1,59)}$		p		Post-hoc t-tests				GoC [%]	$\chi^2_{(2)}$	p	GoC [%]	$\chi^2_{(2)}$	p
										Paired Placebo (pre > post)		2 Sample post (Placebo > EPO)							
						x	y	z	T ₍₁₅₎	p	T ₍₃₀₎	p	GoC [%]	$\chi^2_{(2)}$	p	GoC [%]	$\chi^2_{(2)}$	p	
1	Fusiform gyrus (left) Hippocampus (left) EC: $P_C=0.5$ SUB: $P_C=0.3$	293	-32 -36	-16 -16	-32 -34	10.9	.002	1.6 -1.0	.142 .338	-2.4	.020	87	9.6	.008	66	8.7	.013		
2	Inferior frontal gyrus (left) BA45: $P_C=0.2$	286	-47 -45	+26 +23	-8 -9	13.6	.001	1.9 -1.3	.082 .229	-3.2	.004	87	13.5	.001	72	11.5	.003		
3	Inferior parietal cortex (left) PFop: $P_C=0.6$ PF: $P_C=0.3$ OP1: $P_C=0.3$	242	-60 -59	-31 -30	+22 +20	11.8	.001	2.0 -0.5	.064 .613	-2.1	.041	78	9.3	.010	66	8.7	.013		
4	Nucleus accumbens (right)	236	+9 +6	+10 +9	-11 -10	15.2	.001	2.3 -0.6	.038 .562	-3.5	.001	81	8.5	.014	72	9.3	.009		
5	Hippocampus (left) CA: $P_C=0.8$ FD: $P_C=0.7$ SUB: $P_C=0.4$ Amygdala (left) LB: $P_C=0.4$ SF: $P_C=0.2$	196	-25 -26	-13 -15	-23 -22	10.7	.002	1.5 -1.3	.144 .199	-2.3	.027	81	9.0	.011	72	11.6	.003		
6	Middle temporal gyrus (left)	139	-62 -62	-50 -54	+4 +5	10.2	.002	1.6 -1.1	.132 .294	-2.1	.045	78	8.9	.012	72	10.2	.006		

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Legend to Table 2:

Statistical results demonstrating GMD and cognitive changes over 12 weeks of EPO versus placebo treatment in a cohort of chronic schizophrenic men

Results of inference statistical tests within the intersection of brain regions showing a significant interaction TREATMENT*TIME for ANCOVA and a significant goodness of classifications (GoC) in the voxel-wise linear discriminant analysis (vwLDA) are presented. ANCOVA and vwLDA results include correction for age, MR-scanner and study site. The intersection contains all voxels which pass a statistical threshold $p < 0.005$ (uncorrected for multiple comparisons, minimum cluster size 100mm^3 , adjusted for non-isotropic smoothness). The coordinates of the voxel with the maximal joint effect within the cluster are given in millimeters according to the stereotactical reference system defined by the brain template from Montreal Neurological Institute (MNI). Notably, dependent on the maximum effects obtained with the respective statistical procedure, MNI coordinates differ slightly (compare Figure 1). If available, the cytoarchitectonic probability (P_C) for anatomical structures within the current cluster is specified. The results for the post-hoc paired t-tests of within group differences are displayed for the placebo group in regular letters, for the EPO group (underneath) in italic letters. The last 3 columns display the results for region of interest based LDA (rbLDA). Information on brain regions and cytoarchitectonic probability is derived from ²².

Abbreviations: EC – entorhinal cortex; SUB – subiculum; BA45 – Brodmann area 45; PFop – rostroventral part of inferior parietal cortex / supramarginal gyrus; PF – caudal part of inferior parietal cortex / supramarginal gyrus; OP1 – parietal operculum; CA – cornu ammonis; FD – fascia dentata; LB – laterobasal nucleus; SF – superficial section; GMD - gray matter density; CSS - cognitive sum score.

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Legend to Figure 1

Illustration of VBM and neuropsychological findings in a cohort of chronic schizophrenic men treated over 12 weeks with weekly high-dose EPO (N=16) versus placebo (N=16). Compare Table 2.

Panel A: Gray matter regions (1-6) with treatment effects: Significant effects ($p < 0.005$; uncorrected for multiple comparisons, minimum cluster size 100mm^3 , adjusted for non-isotropic smoothness) for the interaction TREATMENT*TIME (ANCOVA, brown), the voxel-wise linear discriminant analysis (vwLDA, blue) and the intersection of both maps ($\text{ANCOVA} \cap \text{vwLDA}$, yellow), overlaid to coronal sections of the mean T1-weighted MRI of the whole sample (N=32) are shown. As insets, respective transversal and sagittal sections are displayed. The crosshair highlights the cluster from which the data for panels B, C, and D were taken. L, left; R, right; A, anterior; P, posterior.

Panel B: Box plots display the group data for the peak effect obtained in ANCOVA within the corresponding cluster and results of post-hoc 2-sided t-tests (paired $T_{(15)}$ within group, 2 sample $T_{(30)}$ between groups). The horizontal lines of the boxes mark the lower quartile, median and upper quartile values. Whiskers extend from each end of the box to the values within 1.5 times the interquartile range from the ends of the box. Notches display the variability of the median between samples. The width of a notch is computed so that box plots whose notches do not overlap have different medians at the 5% significance level. In addition, single subject data are displayed as open squares (placebo group, gray)

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or filled circles (EPO group, black). Data were adjusted for the group specific median of GMD in the first MRI.

Panel C: Results for voxel-wise linear discriminant analysis (vwLDA) are shown as 2-dimensional scatter plots for the GMD changes over the study period. The pre-treatment GMD at the mentioned stereotactic position serves as x- and the post-treatment GMD as y-coordinate. The dashed black line denotes the stable state. Each deviation of the displayed open squares (placebo group, gray) or filled circles (EPO group, black) from this line indicates GMD decrease or increase, respectively. The linear discrimination function, showing the maximum separation between data from the placebo and the EPO group is displayed as solid black line.

Panel D: Results for region of interest based linear discriminant analysis (rbLDA) shown as 2-dimensional scatter plots for the relation between z-scaled differences in GMD [$z(\Delta\text{GMD})$] and sum of z-scaled differences for selected RBANS subscales, i.e. cognitive sum score of figure recall, digit span and coding [$\Sigma z(\Delta\text{CSS})$] over the entire study period. The $\Sigma z(\Delta\text{CSS})$ serves as x- and the $z(\Delta\text{GMD})$ at the mentioned stereotactic position as y-coordinate. The linear discrimination function, showing the maximum separation between data from the placebo and the EPO group is displayed as solid black line.

Wüstenberg et al. Figure 1 - part 1



