

Title:

Neural correlates of improved executive function following erythropoietin treatment in mood disorders

Running title: Effects of EPO on WM-related brain activity in mood disorder

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Abstract

Background

Cognitive dysfunction in depression and bipolar disorder (BD) is insufficiently targeted by available treatments. Erythropoietin (EPO) increases neuroplasticity and may improve cognition in mood disorders, but the neuronal mechanisms of these effects are unknown. This functional magnetic resonance imaging (fMRI) study investigated the effects of EPO on neural circuitry activity during working memory performance.

Methods

Patients with treatment-resistant major depression, who were moderately depressed, or with BD in partial remission, were randomized to 8 weekly infusions of EPO (40,000 IU) (N=30) or saline (N=26) in a double-blind, parallel-group design. Patients underwent fMRI, mood ratings and blood tests at baseline and week 14. During fMRI patients performed an n-back working memory (WM) task.

Results

EPO improved WM accuracy compared with saline ($P=0.045$). Whole-brain analyses revealed that EPO increased WM-load related activity in the right superior frontal gyrus (SFG) compared with saline ($P=0.01$). There was also enhanced WM-load related deactivation of the left hippocampus in EPO- compared to saline-treated patients ($P=0.03$). Across the entire sample, baseline to follow-up changes in WM performance correlated positively with changes in WM-related SFG activity and negatively with hippocampal response ($r=0.28-0.30$, $P<0.05$). The effects of EPO were not associated with changes in mood or red blood cells ($P\geq 0.08$).

Conclusions

The present findings associate changes in WM-load related activity in the right SFG and left hippocampus with improved executive function in EPO-treated patients. *Clinical trial registration: clinicaltrials.gov: NCT00916552.*

Introduction

Cognitive dysfunction in attention, memory and executive function is a core symptom of unipolar disorder (UD) and bipolar disorder (BD) that contributes to impaired workforce capacity (Depp *et al.* 2012; Jaeger *et al.* 2006). Executive dysfunction persists after remission and is seen in the domains of working memory and inhibitory control (Bora *et al.* 2013; Bourne *et al.* 2013).

Executive dysfunction across UD and BD is associated with WM-associated hypo-activity in the dorsolateral prefrontal cortex (dlPFC) during symptomatic phases (Fernandez-Corcuera *et al.* 2013; Garrett *et al.* 2011; Siegle *et al.* 2007; Townsend *et al.* 2010) and remission (Pomarol-Clotet *et al.* 2015; Townsend *et al.* 2010), although hyper-activity has also been observed (Harvey *et al.* 2005; Matsuo *et al.* 2007). This is accompanied by a failure to suppress activity in the default mode network (DMN) (Fernandez-Corcuera *et al.* 2013; Sheline *et al.* 2009), a network of medial brain regions that includes the hippocampus, medial PFC and inferior parietal cortex and is implicated in self-referential thoughts (Raichle *et al.* 2001).

There is no effective treatment for cognitive dysfunction in mood disorders. Current pharmacological treatments may in fact impair cognition because of anticholinergic, extrapyramidal, sedative, and/or blunting effects (Dias *et al.* 2012). Preliminary evidence indicates that psychological interventions like cognitive remediation (CR) may have beneficial effects on cognition in mood disorder (Demant *et al.* 2013; Porter *et al.* 2013). However, in a recent randomized, controlled study we found no overall cognitive benefits of 12 weeks CR in 44 remitted BD patients (Demant *et al.* 2015).

The discovery of effective treatments for cognitive dysfunction is hampered by the absence of rapid methods to screen novel candidate treatments. Identification of biomarker models for cognitive enhancement is therefore a priority for psychiatric research. Blood-oxygen-level dependent (BOLD)

functional magnetic resonance imaging (fMRI) is a sensitive biomarker of abnormal brain function (Nathan *et al.* 2014). Characterization of post-interventional change in task-related BOLD response within key neural circuits may therefore reveal neurobiological targets associated with cognitive enhancement (Nathan *et al.* 2014).

Erythropoietin (EPO) is a promising candidate treatment for cognitive dysfunction in mood disorders. EPO and its receptor are expressed in the brain and are important for neurodevelopment, neuroprotection and cognition (Kastner *et al.* 2012; Siren *et al.* 2009). Systemically administered EPO crosses the blood-brain barrier and enhances neuroplasticity and cognitive function in animal models of brain disease and in healthy animals (Miskowiak *et al.* 2012). In healthy volunteers, we showed that EPO modulates WM-associated fronto-parietal activation and improves executive function independent of red blood cell changes (Miskowiak *et al.* 2008). Further, 8 weeks EPO vs. saline treatment improved some aspects of cognition in treatment-resistant depression (TRD) and BD in recent studies from our group (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c). Prospective volumetric MRI assessments revealed a reversal of hippocampal cornu ammonis 1-3 and subiculum volume loss as a potential neural mechanism of patients' memory improvement (Miskowiak *et al.* 2014d).

Here we report the results of a concurrent prospective fMRI investigation in 56 patients from the above trials (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c), which aimed to elucidate the changes in BOLD fMRI underlying the EPO-associated improvement in executive function. Given WM-related hypo-activity of dlPFC and deficient task-related suppression of DMN activity across BD and UD and the increase in WM-related right dlPFC activation after a single EPO administration in healthy volunteers, we hypothesized that EPO would (i) reinforce task-related activation of right dlPFC and (ii) suppress hippocampal activity during a spatial WM task.

Materials and methods

Study design and participants

This report is based on our double-blind, placebo-controlled efficacy studies (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c). Patients, 18-65 years of age, were screened with Schedules for Clinical Assessment in Neuropsychiatry to confirm ICD-10 diagnosis. Eligible patients had unipolar TRD with moderate depression (Hamilton Depression Rating Scale 17-items [HDRS-17] score ≥ 17) or BD in partial remission (HDRS-17 and Young Mania Rating Scale [YMRS] scores ≤ 14) with subjective cognitive difficulties. Patients' medication remained stable from ≥ 2 weeks before inclusion and throughout the study. For a detailed description of the screening, exclusion criteria and safety precautions, see (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c). The study was approved by the local ethics committee, Danish Medicines Agency, and Danish Data Agency, and registered at clinicaltrials.gov (no. NCT00916552). After complete description of the study, written informed consent was obtained from all participants.

Randomization and masking

Block randomization was stratified for age ($<$ or ≥ 35 years) and gender. Outcome assessors were blinded to patients' treatment allocation throughout the study period and data analysis (see (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c)).

Procedures

Patients received 8 weekly intravenous infusions of EPO (Eprex; 40,000 IU; Janssen-Cilag) or saline (NaCl 0.9%). **The** dose and treatment schedule were chosen because under conditions of an intact blood-brain-barrier, around 1% of peripherally injected EPO **enters** the brain **and leads** to concentrations in brain tissue comparable to the optimal concentrations **for** neuroprotection in

neuron cultures (Brines *et al.* 2000; Ehrenreich *et al.* 2004). Whole-brain fMRI was performed at baseline and week 14, six weeks after treatment completion at which time point the hematological parameters were expected to have normalized in the EPO group (Miskowiak *et al.* 2014d). Blood tests and blood pressure were taken weekly during the treatment period and in week 14 with the examiners being blind to the results. Mood symptoms were assessed with the HRDS-17, Beck Depression Inventory (BDI) and YMRS at weeks 1, 5, 9, and 14.

Spatial n-back working memory task

N-back WM tasks are widely used to probe memory aspects of executive function (Wager and Smith, 2003). Here we used a spatial n-back WM task during fMRI (Figure in supplementary material). The task consisted of blocks of three types of conditions that differed in terms of WM load (0-back, 1-back and 2-back). In each condition, a yellow circle appeared in a sequence of 14 appearances at random locations in a 5 by 5 grid. The circle was displayed for 300 ms followed by an empty grid for 1200 ms. In 1-back and 2-back conditions, patients indicated with a button press when the circle appeared at the same location as one trial and two trials back, respectively. During the 0-back condition, patients pressed a button when the circle appeared in one of the four grid corners. The blocks had an average of three target trials and were presented successively five times (15 blocks in total) interleaved with 8 s fixation crosses. The total task length was 7 min 35 s.

Control task

To investigate whether WM-related neural responses were confounded by global effects of EPO on cerebral blood flow, neural activation in the occipital cortex was assessed with a control visual stimulation paradigm: a flashing checkerboard (8 Hz) presented in blocks of 14 s alternating with

14 s of a fixation cross for a total of 6 cycles (duration 3 min 8 s). Patients were instructed to lie with their eyes open during this time.

Other fMRI paradigms

We also investigated the effects of EPO on picture encoding and emotional face processing in the same scan session using fMRI paradigms from our previous studies (Miskowiak *et al.* 2007; Miskowiak *et al.* 2014b). For clarity reasons, the results of these tasks will be reported elsewhere.

Magnetic resonance imaging

Whole-brain MRI data were collected at the Danish Research Centre for Magnetic Resonance with a 3 Tesla Siemens Trio MR scanner using an eight-channel head array coil. BOLD-sensitive fMRI used a T2*-weighted echo-planar imaging (EPI) sequence with an echo time (TE) of 30 ms, repetition time (TR) of 2.49 ms and a low flip angle of 20° to minimize physiological noise (Gonzalez-Castillo *et al.* 2011). For the WM task, a total of 184 brain volumes were acquired in a single fMRI session, each consisting of 42 slices with a slice thickness of 3 mm and a field of view (FOV) of 192x192 mm using a 64x64 grid. The control task employed an identical MR sequence as the WM task with 68 brain volumes acquired. High-resolution 3D structural T1-weighted spin echo images were obtained after the first session of BOLD fMRI (TI=800, TE=3.93, TR=1540 ms, flip angle 9°; 256x256 FOV; 192 slices).

fMRI data analysis

Our analysis approach was to investigate our a priori hypotheses that EPO would (i) increase WM-related right dlPFC activity and (ii) suppress hippocampal activity by comparing the task-related mean percent signal change within predefined Regions of Interest (ROIs) between groups (details

below). We explored additional effects of EPO versus saline on changes in neural response with whole-brain exploratory analysis.

FMRI data processing was performed with the FMRI Expert Analysis Tool (FEAT; version 6.00) part of FMRIB's Software Library (FSL; www.fmrib.ox.ac.uk/fsl). Pre-processing included image realignment, non-brain removal, spatial normalization to an MNI (Montreal Neurologic Institute) template and spatially smoothing (Gaussian kernel, 5 mm full-width-half-maximum). The time series in each session were high pass-filtered (to max 0.008 Hz). Three conditions, '1-back', '2-back' and '0-back' were modeled as blocks convolved with a canonical hemodynamic response function.

To investigate hypothesis (i), we defined a spherical ROI with a 10 mm radius around the peak right dlPFC region ($x=40, y=34, z=29$) involved in active spatial WM processes as reported in a meta-analysis (Wager and Smith, 2003). The ROI was constructed on the Montreal Neurological Institute template using the WFU PickAtlas toolbox (Maldjian *et al.* 2003). Given the absence of meta-analyses of WM-associated DMN deactivation, we tested hypothesis (ii) by extracting mean percent signal change *at baseline* in a functional mask in the left and right hippocampus (i.e. clusters showing negative linear relation with increasing load obtained by using the hippocampal structural masks for small volume correction in FSL (thresholded at $Z>2.0$ and $P<0.05$, corrected for multiple comparisons at a cluster level). We extracted the mean percent BOLD signal change in the dlPFC and hippocampal ROIs during 1-, 2- and 0-back and computed the differential signal change for the 2-back>0-back and 2-back>1-back contrasts to assess the effects of EPO on neural response during **high-load WM and high-load *specific* WM**, respectively.

For analysis of our *a priori* ROIs, we conducted repeated-measures analysis of variance (ANOVA) with time and group to examine *differential change* in WM-related activity between groups from baseline to follow-up. Because of high within-subject variation in BOLD fMRI

signal change across repeated measurements (Zandbelt *et al.* 2008), we also conducted exploratory between-group comparisons *at the post-treatment session* given the randomized controlled trial design with well-matched groups. Activity in these ROIs was also compared between groups at baseline. We used residual plots to check the model assumptions and excluded any outliers with standardised residual values ≥ 5 . Signal change analyses were performed in Statistical Package for Social Sciences (SPSS; version 22; IBM Corporation, Armonk, New York, United States).

For the whole-brain exploratory analysis, a fixed-effects analysis was conducted for each participant to identify regions of activity increase and decrease from baseline to follow-up. These ‘difference images’ were entered into group level analyses, which employed a full mixed-effects approach (Woolrich *et al.* 2004). Z (Gaussianised T/F) statistic images were thresholded using clusters determined by an extent threshold of $Z > 2.0$ and a (corrected) cluster significance threshold of $P \leq 0.05$ (Worsley, 2001). We used the high-load WM measure (2-back > 0-back) and the high-load specific WM measure (2-back > 1-back) in the ROI analyses. A standard anatomical atlas (Talairach and Tournoux, 1988) was used to localize peak cluster activation. For clusters showing either a group x time interaction or change in the EPO group over time, we extracted mean percent BOLD signal change for each participant and examined this with analysis of variance (ANOVA) to identify what was driving the interaction/ activity change.

For the control paradigm, we calculated the mean percent BOLD signal change at baseline and follow-up within an ROI within the occipital (calcarine) cortex for each participant, consistent with the approach in our previous studies (Miskowiak *et al.* 2007; Miskowiak *et al.* 2008; Miskowiak *et al.* 2009). Repeated-measures ANOVA with group and time was conducted to examine differential change between groups in neural response to photic stimuli in this ROI (Maldjian *et al.* 2003).

Statistical analyses of behavioral and mood data

The effect of EPO on n-back WM performance was examined with repeated-measures ANOVA with time (weeks 1, 14) and WM load (1, 2) as within-subject factors and group as the between subject factor (all tests were two-tailed). Significant interactions were followed up by simple main effect analyses. We corrected for non-sphericity when appropriate using the Greenhouse-Geisser correction. Signal detection theory was applied to obtain a measure of WM accuracy corrected for participants' response tendency (d') (Grier, 1971). Changes between groups in mood symptoms were analysed in repeated-measures ANOVA. Associations between changes in BOLD response and WM accuracy, and the correlations between these variables and changes in mood, hemoglobin and blood pressure, were examined with Pearson's correlations. Medication status in EPO vs. saline groups was examined with Pearson's Chi Square and Fisher's Exact tests. Hemoglobin and blood pressure at follow-up were examined with simple main effect analyses (t-tests). Statistical analyses were performed in SPSS. Significance level was set at $P \leq 0.05$.

Results

Patient flow and characteristics

Table 1 and the CONSORT chart in the supplementary material display patient characteristics and flow, respectively. Of the 84 patients included in the trials, one patient withdrew at baseline, MRI data collection was incomplete for 14 and behavioral data was missing for 10 because of technical difficulties with the response box. Of the remaining 59 patients, 3 outliers with WM accuracy of ≥ 2 SD below the mean were excluded. Data was thus analyzed for N=56 patients (EPO: N=30; Saline: N=26). Groups were well-matched on baseline characteristics and medication ($P > 0.05$; see Table 1).

Safety of EPO treatment

EPO treatment was well-tolerated (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c). Seven of the 41 (17%) EPO-treated patients had to discontinue treatment after weeks 5-7 because of increased thrombocytes but completed all assessments. Six patients (EPO: N=5 [12%]; Saline: N=1 [2%]) reached the criteria for bloodletting (hematocrit levels >50% and >48% for men and women, respectively, on two consecutive measurements) in weeks 3-7; three were bled once, two were bled twice and one was bled three times. All hematological parameters were normalized 2-6 weeks after treatment completion (for further details see (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c)).

Behavioral results

The WM accuracy (d') scores data showed a negative skew due to near-ceiling accuracy rates. As d' scores are in the range 0-1, we arcsine transformed the data to approach normal distribution prior to statistical analysis (McDonald, 2014). At baseline, there was no difference in WM accuracy between groups ($P \geq 0.31$). EPO improved WM accuracy from baseline to follow-up compared with saline ($F(1,54)=4.23$, $P=0.045$; post-hoc t-tests non-significant) (see Table 2). Speed of WM was not influenced by EPO ($P \geq 0.70$) (see Table 2). For more details, see Table 2.

fMRI results

Dorsolateral prefrontal cortex

In line with previous studies (Wager and Smith, 2003), the right dlPFC was significantly activated by **high-load** WM (2-back>0-back) ($t=12.40$, $df=55$, $P<0.001$) and showed greater response **specifically** under high vs. low loads (2-back>1-back) ($t=-4.79$, $df=55$, $P<0.001$). Baseline dlPFC response showed no differences between groups during **high-load WM or high-load specific WM**

($P \geq 0.50$). One outlier (saline) was excluded because of a standardized residual value ≥ 5 for WM-related dlPFC response at follow-up and data was thus analysed for $N=55$ (EPO: $N=30$, Saline: $N=25$). **There was no differential change in WM-related dlPFC activity between groups over time ($P \geq 0.36$). However, exploratory post-treatment comparisons revealed greater right dlPFC response in EPO- vs. saline groups during 2-back>1-back WM ($t=2.21$, $df=53$, $p=0.032$) (see Figure 1) in the absence of differences during 2-back>0-back WM ($P \geq 0.12$).**

Functional cluster in the hippocampus

WM was associated with deactivation in regions of the DMN including the hippocampus, medial frontal and inferior parietal regions (see Figure 2). There were no differences between groups at baseline in WM-associated deactivation of the hippocampus ROIs ($P \geq 0.46$). **EPO enhanced left hippocampal deactivation during 2-back>0-back WM from baseline to follow-up compared with saline ($F(1,54)=5.24$, $P=0.026$), which was driven by greater deactivation in EPO- vs. saline groups at follow-up ($t=2.28$, $df=54$, $P=0.027$) (see Figure 2). There was also a greater hippocampal deactivation in EPO vs. saline groups over time during 2-back>1-back WM ($F(1,54)=5.02$, $P=0.029$), which reflected reversal of greater baseline activity in EPO group (baseline: $t=2.44$, $df=54$, $P=0.018$; follow-up: $P \geq 0.65$). In contrast, there were no WM-related activity differences between groups in the right hippocampal ROI ($P \geq 0.38$).**

Whole-brain exploratory analyses

High-load (2-back>0-back) and high-load specific (2-back>1-back) WM activated a broad network of overlapping regions including the dlPFC, SFG, cerebellum, temporo-parietal and occipital regions consistent with previous findings (Wager and Smith, 2003) (for peak cluster activations, see Table 3). Exploratory whole-brain analysis revealed no differences between groups

in neural activity changes during **high-load WM or high-load specific WM** over time. However, in the EPO group there was an **activity** increase over time in **the right caudal** SFG **specifically under high vs. low WM loads** (at $Z > 2.0$, cluster corrected $P = 0.05$) (see Figure 1A; for peak cluster activation see Table 3). Extraction and between-group comparison of mean percent signal change showed that this effect was driven by increased high-load specific activity in the EPO vs. saline groups at follow-up (see Figure 1B). In contrast, the SFG showed no activity changes **during 2-back > 0-back WM** in EPO vs. saline groups ($P \geq 0.25$).

Visual stimulation control experiment

Mean percent BOLD signal change to visual stimulation within the occipital (calcarine) cortex ROI showed no baseline differences between groups or any effects of EPO vs. saline ($P \geq 0.39$).

Associations between BOLD fMRI and WM performance

To investigate the functional relevance of the EPO-associated effects on neural activity, we correlated *baseline* WM accuracy with baseline WM-related BOLD response in the right dlPFC, the left hippocampal functional mask and the SFG cluster identified in the whole-brain analysis. We further correlated the *change* in WM accuracy with the change in WM-related activity in these regions across the entire sample. At baseline, patients displayed a significant correlation between WM accuracy and (i) **high-load** WM-related right dlPFC activity ($r(54) = 0.27$, $p = 0.044$) and (ii) high-load *specific* activity in the SFG ($r(54) = 0.35$, $p = 0.009$), as well as (iii) a trend towards correlation between WM accuracy and hippocampal deactivation during **high-load WM** ($r(54) = -0.25$, $p = 0.066$). Again using the entire sample, there was a correlation between WM improvement and increase in (i) **high-load** WM-related right dlPFC response ($r(54) = 0.29$, $P = 0.030$), (ii) increase

in high-load **specific** SFG activity ($r(54)=0.28$, $P=0.035$), and (iii) greater **high-load** WM-related left hippocampal deactivation ($r(54)=-0.30$, $P=0.026$) (see Figures 1C and 2C). For exploratory purposes we also performed these correlation analyses for EPO-treated patients only but found no significant correlations ($P\geq 0.36$), possibly because of reduced statistical power due to the reduction in **numbers**.

No influence of changes in hematocrit, blood pressure, mood or medication

EPO increased hemoglobin during the active treatment phase (for details see (Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c)), but this effect had tapered off before the follow-up fMRI scan at which time there was no difference between groups ($t=-0.39$, $df=54$, $P=0.70$). There was also no difference between groups in blood pressure ($P\geq 0.39$). EPO-treated patients showed no correlation between changes in hemoglobin and neural response within the identified ROIs or WM improvement ($P\geq 0.21$). There were no significant correlation between changes in blood pressure and neural response in these regions ($P\geq 0.08$). EPO had no significant effect on mood symptoms (**HDRS: $P\geq 0.13$; BDI: $P\geq 0.08$**) and there was no **significant** correlation between changes in **these** mood symptoms and **changes in (i)** WM accuracy in EPO- or saline groups ($P\geq 0.69$ and $P\geq 0.12$, respectively) or **(ii) high-load specific activity in the dlPFC or SFG (EPO: $P\geq 0.38$; saline: $P\geq 0.43$) or hippocampal ROIs (EPO: $P\geq 0.09$; saline: $P\geq 0.42$)**. Finally, there was no correlation between age and neural response or WM accuracy ($P\geq 0.18$). Post-hoc comparisons of patients with or without lithium, antipsychotic and antidepressant treatment, respectively, revealed no effects of these medications on post-interventional activity in the right dlPFC ($p\geq 0.5$) or hippocampus ROI ($p\geq 0.4$) or in SFG activity change ($p\geq 0.14$).

Discussion

This fMRI study provides a cerebral basis for the EPO-associated improvement of executive function in patients with mood disorders. **Our data show that 8 weeks of EPO treatment of patients with TRD or BD increased right caudal SFG response specifically during high WM loads and enhanced WM-related deactivation of the left hippocampus compared with saline. Post-treatment comparisons also revealed greater high-load specific activity in the a priori dlPFC ROI in EPO vs. saline groups, although notably dlPFC activity change over time did not differ between groups.** EPO treatment also improved patients' WM accuracy. Across the entire sample, WM improvement correlated with increase in WM-related dlPFC and SFG activity and hippocampal deactivation. The present effects of EPO were not associated with changes in mood, hemoglobin or blood pressure.

The effects of EPO on WM-related neural activity may counteract **hypo-activity in dorsal PFC regions** and failure to suppress DMN activity in the hippocampus across mood disorders (Fernandez-Corcuera *et al.* 2013; Garrett *et al.* 2011; Pomarol-Clotet *et al.* 2015; Sheline *et al.* 2009; Siegle *et al.* 2007). Greater task-related *suppression* of hippocampal activity in the EPO group may be an *indirect* marker of improved WM (increased *disengagement* from task-irrelevant thoughts), consistent with the observed correlation between hippocampal activity suppression and WM performance. The PFC shows low activity at low load, increased activity at high load, and decreased activity when the load exceeds WM capacity (Callicott *et al.* 1999). Given this, the greater **prefrontal** response **specifically** during high WM loads in EPO vs. saline treated patients at follow-up suggests that EPO may prevent break-down of WM capacity. These results are in accordance with **increased dorsal PFC activity during executive function tests after cognitive training** in multiple sclerosis (Filippi *et al.* 2012) and schizophrenia (Ramsay and MacDonald, III, 2015). The observed effects on WM-related neural circuit activity may therefore not be *specific* to EPO but also occur in response to *other* compounds with pro-cognitive effects. This would

highlight normalization of ***hypo-activity in dorsal PFC regions*** and DNM *hyper*-activity during executive control tasks as key neurobiological targets associated with pro-cognitive effects of both biological and psychological treatments across a range of neuropsychiatric disorders.

Several neurobiological mechanisms may underlie the observed effects of EPO. Although EPO increased hemoglobin during the active treatment phase, this was normalized at follow-up and there were no correlations between changes in hemoglobin and BOLD response within the identified ROIs. Blood pressure has been shown to correlate positively with BOLD response (Wang *et al.* 2006). However, we found no significant differences in blood pressure between groups or correlations between EPO-associated change in BOLD response and blood pressure. The visual control task also showed no difference between groups in occipital activation to photic stimuli, suggesting that the observed effects of EPO did not result from any global hemodynamic changes. Finally, there were no significant effects of EPO on mood symptoms across the UD and BD groups or correlations between changes in mood and neural response or WM accuracy. Taken together, this suggests that direct neurobiological actions mediate the observed effects of EPO. Impaired neuroplasticity may be a central cause of patients' executive dysfunction and aberrant prefrontal and hippocampal activity (Carlson *et al.* 2006; Marsden, 2013). The ability of EPO to increase neuroplasticity through activation of anti-inflammatory, anti-apoptotic and anti-oxidant signaling cascades (Siren *et al.* 2009) and inhibition of glycogen synthase kinase 3 beta (GSK3 β) (Ge *et al.* 2012) may hence be important for the observed effects of EPO on neural and behavioral measures of WM. These effects may be generalizable to other neuropsychiatric disorders, consistent with the findings of EPO-associated improvement of executive function in multiple sclerosis, schizophrenia (Ehrenreich *et al.* 2007a; Ehrenreich *et al.* 2007b) and in healthy individuals (Miskowiak *et al.* 2008).

A strength of the study was the relatively large sample size of N=56 patients with complete pre- and post-treatment data. In comparison, previous prospective fMRI investigations of treatments targeting cognition involved N=20-28 patients (Filippi *et al.* 2012; Meusel *et al.* 2013; Subramaniam *et al.* 2014). In addition, the randomized, placebo-controlled design could accommodate for any practice effects and effects of repeated scanning. It is a potential limitation that patients were medicated since this could have produced non-specific changes in global brain activation. However, such confounding effects were unlikely since EPO and saline groups showed no differences in medication status or baseline neural response. In addition, we observed no effects of lithium, antipsychotic or antidepressant treatment on neural activity in the identified ROIs. Indeed, it is noteworthy that the effects of EPO on WM-related neural activity occurred over and above any non-specific effects of patients' medication. Another limitation is that our cohort included both TRD and BD patient groups who may be characterized by distinct although overlapping pathogenic processes. Assessment of the effects of EPO for the TRD and BD groups separately was not possible due to insufficient statistical power with the small number of participants for such subgroup analyses. Nevertheless, executive dysfunction and aberrant WM-related neural responses in the dlPFC and DMN occur across both disorders, and EPO has been shown to improve cognition across several brain disorders (Ehrenreich *et al.* 2004; Ehrenreich *et al.* 2007b; Miskowiak *et al.* 2014a; Miskowiak *et al.* 2014c). Since the study did not include a healthy control arm, it cannot be determined if our patients displayed WM-related prefrontal *hypo*-activity at baseline or if the EPO-associated change in BOLD signal represents normalization.

While we found a significantly higher dlPFC response in the EPO group post-treatment compared with saline, the effect of treatment on *change* in dlPFC activity was not significant. Consequently, caution should be applied when interpreting the effects of EPO on WM-related dlPFC activity. Given the evidence for abnormal task-related prefrontal and DMN activity in mood

disorders and our demonstration of correlations between BOLD signal and WM performance, **we suggest that our findings indicate an** improved neural network functioning in EPO-treated patients. Notably, this post-interventional fMRI investigation of the effects of EPO on neural circuitry activity was *exploratory* in nature **according to** our original published trial protocol (Miskowiak *et al.* 2010). Bonferroni correction for the three employed fMRI paradigms was therefore not applied. From a clinical perspective, the therapeutic use of EPO in patients with mood disorders is limited by its hematological effects. Although EPO was well-tolerated in our studies, it is a labor-intensive treatment because of the necessity to perform careful screening and regular medical examinations and blood tests. A clinically interesting alternative is therefore non-hemopoietic EPO molecules such as carbamylated EPO that cross the blood–brain barrier and enhance neuroplasticity and cognition in preclinical models (Siren *et al.* 2009). If pharmacokinetic studies indicate that such EPO derivatives can be safely administered to humans, it would be a logical next step to investigate if they produce target engagement in the PFC and DMN.

In conclusion, **our data links EPO-related improvement of executive function in mood disorders with increased SFG response specifically during high-load WM, and WM-related deactivation of DMN activity.** This is consistent with preclinical evidence for the ability of EPO to enhance neuroplasticity and cognition and highlights EPO as a candidate treatment for executive dysfunction in mood disorders.

Conflicts of interest

KWM reports having received consultancy fees from Lundbeck. MV discloses consultancy fees from Eli Lilly, Lundbeck; Servier and Astra Zeneca. HE has submitted / holds user patents for EPO in stroke, schizophrenia, and MS. OBP is a member of the board of directors of the Elsass Foundation. CJH has received consultancy fees from P1vital ltd, Lundbeck, Servier, Eli-Lilly and is a company director of Oxford Psychologists ltd. CJH has also received grant income from GSK, UCB, Janssen Inc, Lundbeck, Servier and Astra Zeneca. GMK received within the last 3 years honoraria as field editor for Int J Neuropsychopharmacology and as scientific advisor for Lundbeck. HRS discloses honoraria as reviewing editor for Neuroimage, as speaker for Biogen Idec Denmark A/S, and scientific advisor for Lundbeck within the past 3 years. LVK reports having been a consultant for Lundbeck and AstraZeneca within the last 3 years. All other authors report no biomedical financial interests or potential conflicts of interest.

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Table 1. Patient characteristics.

	EPO (N = 30)	Saline (N = 26)	p-value
Diagnosis, no. BD/TRD	16/14	16/10	0.29
Age, Mean (SD)	39 (10)	40 (12)	0.61
Gender, No. Women (%)	20 (67)	15 (58)	0.06
Education, Mean (SD)	15 (4)	15 (3)	0.66
HDRS baseline, Mean (SD)	15 (7)	12 (7)	0.09
follow-up, Mean (SD)	11 (7)	9 (7)	0.36
BDI baseline, Mean (SD)	28 (11)	22 (11)	0.07
follow-up, Mean (SD)	20 (12)	17 (13)	0.30
YMRS baseline, Mean (SD)	2 (2)	2 (2)	0.92
follow-up, Mean (SD)	2 (2)	2 (3)	0.55
No. prior depressions, Mean (SD)	6 (5)	5 (3)	0.33
No. prior (hypo)manias, Mean (SD)	6 (7)	4 (3)	0.35
Bipolar subtype, No. of type II (%)	10 (33)	10 (38)	0.16
Current Medication			
<i>Lithium, no. (%)</i>	10 (18)	9 (16)	0.57
<i>Anticonvulsants, no. (%)</i>	17 (30)	13 (23)	0.64
<i>Antidepressants, no. (%)</i>	17 (30)	16 (29)	0.55
<i>Antipsychotics, no. (%)</i>	9 (16)	8 (14)	1.0
<i>Benzodiazepines, no. (%)</i>	9 (16)	8 (14)	0.77
<i>Melatonin, no. (%)</i>	6 (11)	2 (4)	0.26
<i>No medication, no. (%)</i>	1 (2)	1 (2)	1.0
<i>Number of medications, Mean (SD)</i>	2.4 (1.5)	2.3 (1.1)	0.93

Abbreviations: BD: bipolar, TRD: treatment-resistant depression, EPO: erythropoietin, HDRS: Hamilton Depression Rating Scale,

BDI: Beck Depression Inventory, YMRS: Young Mania Rating Scale, No.: number.

Table 2. N-back working memory (WM) performance at baseline (week 0) and follow-up (week 14) and effects of EPO vs. saline.

	Week 0 Baseline M (SD)	Week 14 Follow-up M (SD)	Time (weeks 0-14) Time by group P-value^a
N-back WM performance			
<i>Accuracy (arcsine transformed d' values)</i>			
<i>WM across 1- and 2-back</i>			
EPO (N=30)	1.07 (0.20)	1.16 (0.17)	0.12
Saline (N=26)	1.13 (0.20)	1.12 (0.20)	0.05* (DF=1,54)
<i>1-back WM</i>			
EPO (N=30)	1.21 (0.21)	1.28 (0.17)	0.48
Saline (N=26)	1.23 (0.20)	1.21 (0.20)	0.15 (DF=1,54)
<i>2-back WM</i>			
EPO (N=30)	0.93 (0.26)	1.05 (0.21)	0.06
Saline (N=26)	1.03 (0.23)	1.03 (0.25)	0.05* (DF=1,54)
<i>Number and percentage of hits 1-back WM</i>			
EPO (N=30)	14 (2) [93 (13)%]	14 (2) [93 (13)%]	0.66
Saline (N=26)	14 (2) [93 (13)%]	13 (2) [87 (13)%]	0.27 (DF=1,54)
<i>Number and percentage of hits 2-back WM</i>			
EPO (N=30)	10 (4) [67 (27)%]	12 (3) [80 (20)%]	0.070
Saline (N=26)	12 (3) [80 (13)%]	12 (3) [80 (13)%]	0.046* (DF=1,54)
<i>Number and percentage of false alarms 1-back WM</i>			
EPO (N=30)	2 (2) [13 (13)%]	1 (1) [7 (7)%]	0.11
Saline (N=26)	1 (2) [7(13)%]	1 (1) [7(7)%]	0.11(DF=1,54)
<i>Number and percentage of false alarms 2-back WM</i>			
EPO (N=30)	2 (1) [13(7)%]	1 (0) [7(0)%]	0.07
Saline (N=26)	2 (2) [13(13)%]	1 (1) [7(7)%]	0.81(DF=1,54)
<i>Response times (for correct detections)</i>			
<i>0-back control</i>			
EPO (N=30)	566 (94)	566 (98)	0.52
Saline (N=26)	544 (96)	558 (93)	0.51 (DF=1,54)
<i>1-back WM</i>			

EPO (N=30)	590 (135)	576 (143)	0.21
Saline (N=26)	603 (111)	580 (114)	0.75 (DF=1,53)
<i>2-back WM</i>			
EPO (N=30)	590 (135)	563 (142)	0.22
Saline (N=26)	603 (111)	570 (151)	0.70 (DF=1,53)

Abbreviations: M, mean; SD standard deviation; EPO, erythropoietin; WM, working memory. ^aDegrees of freedom (DF) are identical for time and time by group.

Table 3. Peak cluster activation in regions identified in whole-brain analyses (with $Z=2.0$, $P=0.05$, cluster-corrected) as showing (i) increased BOLD response during general n-back working memory (2-back>0-back), (ii) greater response during high WM load (2-back>1-back) and (iii) functional deactivation during working memory (2-back<1-back<0-back) across all participants at baseline, and (iv) regions showing activity *change* from baseline to follow-up in EPO-treated patients.

MNI coordinates (x, y, z) refer to the point of peak activation within each cluster identified using this threshold. BA: Brodmann area.

Task and Region	P- value	Cluster size (no. voxels)	Z- value	Coordinates X Y Z		
2-back versus 0-back						
Main effect of task at baseline						
Cerebellum	<0.0001	89,620	9.41	-36	-62	-28
Left inferior parietal gyrus (BA 40)			9.32	-36	-48	48
Right insula			9.03	34	24	0
Right inferior parietal gyrus (BA 40)			8.87	48	-44	48
Right superior parietal gyrus (BA 7)			8.75	42	-44	48
Right superior parietal gyrus (BA 7)			8.67	38	-50	52
2-back versus 1-back						
Main effect of task at baseline						
Cerebellum	<0.0001	23,343	6.89	-18	-66	60
Right medial frontal gyrus (BA 6)	<0.0001	19,104	6.38	24	2	50
Left medial frontal gyrus (BA 10)	0.0005	918	4.01	-42	48	-8
Left insula	0.003	724	4.23	-42	14	-6
Baseline < Follow-up, EPO						
Right superior frontal gyrus (BA 6)	0.047	476	3.91	26	8	64
Negative linear relation (default mode network)						
Main effect of task at baseline						
Right posterior cingulate cortex (BA 23)	<0.0001	7,602	6.61	2	-48	28
Left orbitofrontal gyrus (BA 11)	<0.0001	6,180	7.26	-2	44	-12
Left superior temporal gyrus (BA 41)	<0.0001	5,730	6.1	-40	-16	18
Right inferior temporal gyrus (BA 6)	<0.0001	2,767	6.53	56	0	8
Left medial frontral gyrus (BA 47)	0.0166	571	5.65	-42	32	-16
Left hippocampal functional mask	0.0012	274	4.85	-26	-24	-12
Right hippocampal functional mask	0.0015	260	4.53	28	-20	-14

Figure legend 1. (A) Neural network activated during high WM load (2-back>1-back) across all patients at baseline (green); Region in the superior frontal gyrus (SFG) (BA 6) showing increased response specifically during this high WM load in the erythropoietin (EPO) group in the exploratory whole-brain analysis (yellow); A priori ROI in the right dorsolateral prefrontal cortex (dlPFC) (red). Images are thresholded at $Z>2.0$ and $p<0.05$, corrected for multiple comparisons at a cluster level.

(B) Plot of change from baseline to follow-up in mean % blood oxygen level dependent (BOLD) signal change during high WM load (2-back>1-back) within SFG and dlPFC in EPO (red bars) and saline (blue bars) groups at baseline and follow-up. Bars show the mean; error bars show the SE. In the a priori ROI, dlPFC, between-group comparison of mean percent signal change **at the follow-up scan** with adjustment for baseline activity showed that EPO produced **greater** activity compared with saline during high WM loads ($P=0.03$). **Notably, this post-treatment group difference occurred in the absence of significant activity changes from baseline to follow-up within each group separately ($p\geq 0.40$).** EPO also increased high-load specific BOLD response in the SFG from baseline to follow-up compared with saline **(group by time interaction for extracted mean percent signal change: $F(2,54)=6.78$, $P=0.01$; t-test at follow-up: $t=2.34$, $df=54$, $P=0.02$).** Please note that **the ROIs were non-independent and that the effect sizes of the extracted mean percent signal change may therefore be exaggerated.**

(C) Linear relation across the entire sample between change in WM performance and change in BOLD response **in the SFG during high-load specific WM and in the dlPFC during high-load WM.** There was a positive correlation between change in WM accuracy and WM-related BOLD signal in these regions across the entire cohort ($r=0.30-0.35$, $P<0.05$).

Figure legend 2. (A) Default mode network (DMN) showing deactivation during WM (i.e., regions showing a negative linear relation with increasing WM load) across all participants at baseline (green); The (overlapping) functional cluster within the left hippocampus identified as showing WM-associated hippocampal deactivation (blue). Images are thresholded at $Z > 2.0$ and $P < 0.05$, corrected for multiple comparisons at a cluster level. (B) Plot of mean % blood oxygen level dependent (BOLD) signal change during **high-load** WM (2-back > 0-back) within the left hippocampal ROI in the erythropoietin (EPO) (red bars) and saline (blue bars) groups at follow-up. Bars show the mean; error bars show the SE. Erythropoietin produced greater WM-related deactivation compared with saline at follow-up ($P = 0.02$). Please note that the ROIs were *non-independent* and that the effect sizes of the extracted mean percent signal change may therefore be exaggerated. (C) Linear relation across the entire sample between change in WM performance and in WM-related BOLD response in the left hippocampal ROI. There was a negative correlation between change in WM accuracy and WM-associated hippocampal activity across the entire cohort, indicating increase in WM accuracy in patients with greater hippocampal deactivation ($r = -0.30$, $P = 0.03$).

Figure (I) for supplementary material. CONSORT flow chart.

Figure (II) for supplementary material. The implemented spatial n-back working memory task with 1-back, 2-back and 0-back (control) conditions.