Environmental and therapeutic modulators of neuropsychiatric disease

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DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute of higher education, except where due acknowledgement has been made in the text.

Bartosz Tomasz Adamcio Göttingen, 29 September 2009

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1. Introduction to the scientific project

The present cumulative thesis has been prepared in the framework of a large interdisciplinary effort in Göttingen (GRAS, Göttingen Research Association for Schizophrenia) to delineate the biological basis / biological subgroups of schizophrenia and to define novel treatment strategies. The thesis includes three original publications that investigate the role of stress in the development of psychiatric disorders and examine new treatment strategies. The first paper focuses on early psychosocial stress as an environmental cofactor that plays an important role in the development of psychiatric disorders, for example schizophrenia (Adamcio et al., 2009). The second study investigates the effects of erythropoietin (EPO) and its mechanisms of action on cognitive improvement in healthy mice (Adamcio et al., 2008). The third paper focuses on endogenous EPO production by stabilization of hypoxia-inducible factor 1 alpha subunit (HIF-1α) and describes the effects of HIF-1α stabilizer on cognitive performance in healthy mice (Adamcio et al., submitted).

Schizophrenia is a devastating psychiatric disorder that affects approximately 1 percent of the population across the world and cultures. The onset of the disorder occurs in young adulthood and is rare before adolescence or after middle age (Andreasen, 1995; Hafner, 2000; Freedman, 2003). Furthermore, the onset interacts with sex, such that men are likely to become ill earlier in life than women (MacDonald and Schulz, 2009).

People diagnosed with schizophrenia suffer from a variety of symptoms that can be divided into three main categories: (1) psychotic or ‘positive’ symptoms, (2) deficit or ‘negative’ symptoms, and (3) cognitive impairments (Wong and Van Tol, 2003). The positive symptoms are the symptoms in which normal functions are distorted or exaggerated. They include hallucinations, delusions, disorganization of thought and bizarre behavior (Andreasen, 2000; Thaker and Carpenter, 2001). The negative symptoms refer to the lack of important abilities and include loss of

motivation, lack of emotion, reduced ability to experience pleasure (anhedonia), poor or nonexistent social functioning, and alogia (Andreasen, 2000; Thaker and Carpenter, 2001; Wong and Van Tol, 2003). Cognitive impairments in schizophrenic patients are very severe and can reach 2 standard deviations below the mean of the healthy subjects (Harvey et al., 2004). They include deficits in working memory, attention, gating, executive function, abstraction and language (Reichenberg and Harvey, 2007; Keefe, 2008). The cognitive impairments and negative symptoms are more persistent and chronic, while the positive symptoms have an episodic pattern (Wong and Van Tol, 2003).

Since the identification of schizophrenia, various speculations have been made regarding its etiology. Detailed genetic epidemiological investigation of schizophrenia has been conducted for many years and the results of various family studies showed that the risk of developing the disorder is associated with the degree of biological relatedness to an affected individual. For example, in second degree relatives or in half siblings the risk increases to about 2-6 % (Gottesman, 1999). Furthermore, twin studies revealed that the degree of concordance for schizophrenia in monozygotic (share 100 % of their genes) and dizygotic (share 50 % of their genes) twins is typically about 48 and 17 % respectively (Gottesman, 1999). The observed rates in twins and other relatives suggest that genetic factors play an important role in the etiology of schizophrenia, though they are not adequate to explain all the observations (Gottesman, 1999). It appears that what is inherited is not necessarily the actual disease associated with a particular genotype, but rather the predisposition or potential to acquire the disease (Pesold et al., 2004), and that there must be other, non genetic factors that contribute to the development of schizophrenia.

Over the last 5 decades, many studies have been conducted in order to identify specific environmental factors that would increase the risk for schizophrenia (Oh and Petronis, 2008). The list of potential environmental risk factors is long and includes adverse live events, especially those occurring at early age (Read et al.,

2005), maternal nutrition (Brown et al., 1996), cannabis consumption (Moore et al., 2007), obstetric complication (Cannon et al., 2002), migration (Cantor-Graae and Selten, 2005), urbanicity (McGrath et al., 2004) and viral infection (Wright and Murray, 1993).
A decade ago, Bayer and colleges (1999) proposed a so called “Two hit hypothesis”, which integrated both, genetic and non-genetic risk factors. The suggested hypothesis postulates that only both hits together, genetic vulnerability and environmental hazards can trigger the disease process.

The treatment for schizophrenia is mainly based on antipsychotics. Discovery of first-generation antipsychotics (known as typical antipsychotics) in the 1950s led to a dramatic improvement in treatment of positive symptoms of schizophrenia (Wong and Van Tol, 2003). The antipsychotic action of these medications is linked to blockade of dopamine D2 receptor (Seeman and Lee, 1975; Seeman, 1992). Although the treatment with antipsychotics significantly decreases psychotic symptoms, it is also associated with a number of side effects, including extrapyramidal movement disorder. The introduction of clozapine in 1988 (Kane et al.) led to the establishment of a new group of antipsychotics, known as atypical antipsychotics. To this group of medication belong, for example, olanzapine, risperidone and quetiapine. These drugs, compared to typical antipsychotics, have a similar blocking effect on D_2 receptors but additionally they partially block serotonin receptors (particularly $5HT_{2A}$ receptors). They are less likely to cause movement disorders, but have still numerous side effects, including weight gain, prolactin and glucose elevation, and sedation (Snyder and Murphy, 2008). Additionally, and perhaps even more important, these medications, despite consistent improvements in positive symptoms, have not led to improvements in real-world functioning, perhaps due to the modest impact on cognitive functioning (Green, 1996; Green et al., 2000a; Harvey et al., 2004).

In 2001, based on the evidence that continuous neurodegenerative processes are involved in the pathogenesis of the disorder, a new concept of treatment for

schizophrenia has been proposed. Ehrenreich and Sirén suggested erythropoietin (EPO) as a neuroprotective add-on therapy in schizophrenia (Ehrenreich and Siren, 2001).

EPO is a 165 amino acid (~30 kDa) glycoprotein that is a member of the type I cytokine superfamily. Originally, it was believed that the only role of EPO was to regulate red blood cell differentiation by inhibiting apoptosis of erythroid precursors (Brines and Cerami, 2005). The production of EPO in humans, just as in other species, switches from liver to kidneys, with liver being active during human gestation and kidneys producing EPO in adults (Zanjani et al., 1977; Dame et al., 1998). Epo binds to its specific receptor (EPOR) that belongs to the type I cytokine receptor family. EPOR is a homodimer consisting of two peptide chains that in the absence of EPO do not interact with each other. Binding of EPO to the homodimer leads to a conformational change that brings two domains together and allows autophosphorylation of EPOR-associated Janus tyrosine kinase 2 (JAK2) and activation of several downstream signaling pathways, including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and activator of transcription 5 (STAT5) pathways (Siren et al., 2001; Brines and Cerami, 2005).

The finding that EPO and its receptor (EPOR) are expressed in the brain (Masuda et al., 1993; Masuda et al., 1994) led to the notion that EPO exerts direct, hematopoiesis-independent effects on the nervous system. Although the expression of EPO and EPO receptor in normal adult brain tissue is low, it gets upregulated as a response to hypoxia or metabolic distress of neurons even in adults, which could be interpreted as an endogenous neuroprotective system. Interestingly, also in schizophrenic patients the expression of EPOR is dramatically increased compared to healthy controls (Ehrenreich et al., 2004b; Ehrenreich et al., 2004d; Ehrenreich et al., 2004a; Ehrenreich et al., 2008; Ehrenreich, 2008; Siren et al., 2009).

Indeed, the list of neuroprotective properties of EPO is very long. EPO has been shown to be antiapoptotic (Siren et al., 2001), antioxidative (Chattopadhyay et al., 2000; Genc et al., 2002), anti-inflammatory (Agnello et al., 2002; Villa et al., 2003), glutamate-inhibitory (Kawakami et al., 2001), neurotrophic (Campana and Myers, 2001), angiogenetic (Sasaki et al., 2001). Moreover, EPO strongly influences stem cell differentiation (Jelkmann, 2000; Shingo et al., 2001). All these properties make EPO an ideal compound for neuroprotective strategies in neurological disorders.

2. Focus of the present work

The thesis includes 3 original publications that investigate mechanisms contributing to neuropsychiatric disorders and present novel treatment strategies for these diseases, in particular schizophrenia.

2.1 Aims of project I

The first original publication addresses stress as a potential (second) hit to the brain, contributing to the development of neuropsychiatric diseases, especially schizophrenia. The study has been designed in order to investigate late consequences of chronic psychosocial stress on pre-pulse inhibition of startle response (PPI) in a mouse model, and to explore a potential modifying influence of social deprivation versus social support.

We hypothesized that psychosocial stress in the absence of social support would lead to PPI disturbances in adulthood. Using the so called resident-intruder paradigm, 28-day old C57BL/6NCrl mice, housed either individually or in groups (5 mice per cage), were subjected daily for 3 weeks to social defeat. Basic behavior and PPI were analyzed 10 weeks later.

2.2 Aims of project II

The second project aimed at opening novel therapeutic strategies to address cognition in schizophrenia. This study investigated the mechanism of EPO action on cognitive performance in healthy mice. Young (28 days old) C57Bl6 mice, primary hippocampal neuronal cultures and autaptic hippocampal cultures were used.

In the first step, we aimed to develop a reliable, robust model for improvement of cognition by EPO in healthy mice. We focused on healthy mice in order to further study the mechanism of EPO action in brains independently of disease associated conditions. For that, young mice were injected for 3 weeks (every other day) with EPO and hippocampus dependent memory was measured in fear conditioning test 1, 3 and 4 weeks after the termination of treatment.

In the next step we aimed to investigate the mechanism of EPO action on cognitive performance. Using hippocampal slices and sections from EPO-treated mice as well as primary hippocampal cultures and autaptic hippocampal neurons we examined the influence of EPO on various readouts of synaptic plasticity.

2.3 Aims of project III

The purpose of the project III was to test whether stimulation of endogenous EPO production by stabilization of hypoxia-inducible factor 1 alpha subunit (HIF-1α) would lead to similar enhancement in hippocampus dependent memory. To test this, young mice were injected with prolyl hydroxylase inhibitor (FG-4497) for 3 weeks (every other day) and, similarly to the EPO study (project II), hippocampus dependent memory was measured in fear conditioning test 1, 3 and 4 weeks after the termination of treatment.

3. Effects of chronic psychosocial stress on pre-pulse inhibition of startle response (PPI) in a mouse model

3.1 Overview of project I

Psychotrauma (stressful life events) is an environmental co-factor that is believed to play an important role in the development of many psychiatric disorders. Adverse life events, especially those occurring at early age, have been associated with different psychopathologies e.g. schizophrenia (Geddes et al., 2000; Janssen et al., 2004; Scheller-Gilkey et al., 2004; Read et al., 2005), anxiety disorders including posttraumatic stress disorder (Phillips et al., 2005; Schaal and Elbert, 2006; Zlotnick et al., 2008) and depressive disorder (Kessler and Magee, 1993; Heim et al., 2004). One of the factors contributing to the prevention of these diseases is social support, a term that has been used to refer to the mechanism by which interpersonal relationships presumably protect people from the deleterious effects of stress (Kessler et al., 1985).
For many years various animal models have been used to investigate the role of stress in development of psychopathologies. However, in these models, very often aversive physical stimuli such as electric foot shock, restraint, forced swim and cold are employed that bear little resemblance to the natural conditions under which stress-related diseases develop (Tamashiro et al., 2005).

One stressor that is more ethologically relevant is social stress. This can be modelled in so called resident-intruder paradigm, which generates stress by using social conflict between members of the same species (Rygula et al., 2005).
In this paradigm an *intruder* animal is subjected to social stress by exposure to an unfamiliar, aggressive conspecific (*resident*). The social stress induced in this paradigm has been shown to cause alterations in several behavioral and physiological parameters (Tornatzky and Miczek, 1994; Meerlo et al., 1996; Koolhaas et al., 1997; Blanchard et al., 2001; Buwalda et al., 2001; Buwalda et al., 2005; Rygula et al., 2005; Rygula et al., 2008).

The development of psychiatric disorders is essentially influenced by the late effects of stressful interpersonal experiences occurring in childhood and adolescence (Sterlemann et al., 2008; Weber et al., 2008; Zlotnick et al., 2008). While the majority of animal studies have focused on the immediate effects of stress there is a lack of experiments investigating the crucial factor of the pathogenesis of psychiatric disorders, the long-term effects of stress experience at early age (Sterlemann et al., 2008). Therefore, in the present study we examined the late effects of three weeks of social stress exposure on pre-pulse inhibition (PPI) of startle response. This is a measure of sensorimotor gating abnormalities that are associated with several psychiatric and neurological disorders, including schizophrenia (Braff et al., 1978; Grillon et al., 1992; Green et al., 2000b; Weike et al., 2000), schizotypal personality disorder (Cadenhead et al., 1993) and post-traumatic stress disorder (Grillon et al., 1996). Even though stress, social support and PPI are strongly related to psychiatric disorders, their association was never examined in animal studies. Our objective was to investigate group and individually housed mice in order to determine to which extent social support vs. social deprivation influences the effects of chronic social stress on pre-pulse inhibition.

We found that chronic social stress occurring at very young age leads to a decrease of pre-pulse inhibition in adulthood only in individually housed mice but not in group housed mice. Additionally, we showed that the deleterious effects of chronic social stress are counteracted by rewarding interactions within a familiar social group.

3.2 Original publication

Adamcio B, Havemann-Reinecke U and Ehrenreich H. Chronic psychosocial stress in the absence of social support induces pathological pre-pulse inhibition in mice. ***Behav Brain Res. 2009 Dec 1;204(1):246-9.***

Personal contribution:

I was involved in the design of the studies, interpretation of results and preparation of the manuscript. I have performed all of the experimental work.

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Short communication

Chronic psychosocial stress in the absence of social support induces pathological pre-pulse inhibition in mice

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ABSTRACT

Chronic psychosocial stress has been suggested as "second hit" in the etiology of neuropsychiatric disease, but experimental evidence is scarce. We employed repetitive social defeat stress in juvenile mice, housed individually or in groups, and measured sensorimotor gating by pre-pulse inhibition (PPI), a marker of neuronal network function. Using the resident-intruder paradigm, 28-day old C57BL/6NCrl mice were subjected daily for 3 weeks to social defeat. PPI and basic behaviour were analyzed 10 weeks later. Whereas stress increased the level of anxiety in all animals, persistent PPI deficits were found only in individually housed mice. Thus, social support in situations of severe psychosocial stress may prevent lasting impairment in basic information processing.

Psychotrauma, caused by stressful life events that cannot be adequately compensated for, acts as environmental co-factor ("second hit") in the development of many psychiatric disorders. Adverse life events, especially those occurring at early age, have been associated with different psychopathologies, including schizophrenia [for review see: [16]]. Among the factors mitigating the deleterious effects of stress may be social support [11].

Animal studies, investigating the role of stress in the development of psychopathologies, mainly used aversive physical stimuli such as electric foot shock, restraint, forced swim and cold that poorly resemble natural conditions [20]. An etiologically more relevant stressor is psychosocial stress, modelled e.g. in the 'repeated change in group composition' [18] or the 'resident-intruder paradigm'. In this latter paradigm, an *intruder* animal is repeatedly subjected to social stress by exposure to an unfamiliar, aggressive conspecific (*resident*). The so caused psychosocial stress induces immediate alterations in several behavioural and physiological parameters [for review see: [2,3,12,15]], whereas experiments investigating long-term effects of chronic stress at early age, more relevant for the development of psychiatric disorders, are still limited.

Pre-pulse inhibition of the startle response (PPI) is a measure for sensorimotor gating and pre-attentive information processing that can be easily translated from man to mouse and vice versa [5]. Several psychiatric and neurological disorders are associated with PPI abnormalities, seen as a simple readout of neuronal network disturbances, including schizophrenia [e.g. [4]], schizotypal personality disorder [7] and post-traumatic stress disorder [9].

Even though severe psychosocial stress at early age, social support and PPI are all strongly related to the etiology of psychiatric disorders, their association has never been examined in animal studies. We hypothesized that stress in the absence of social support would lead to PPI disturbances. The present study has therefore been designed to investigate late consequences of chronic psychosocial stress on PPI in a mouse model, and to explore a potential modifying influence of social deprivation versus social support.

Immediately upon arrival, 21 days old C57BL/6NCrl male mice (Charles River Laboratories, Sulzfeld, Germany) were housed either individually or in groups (5 mice per cage). From each housing condition, mice were assigned to either control treatment or psychosocial stress. This resulted in four experimental groups: control/group housing, stress/group housing, control/individual housing and stress/individual housing ($n = 15$ each, Fig. 1A).

Three months old FVB male mice served as residents. Prior to the stress procedure they were housed for 6 weeks individually in a room separate from the experimental mice.

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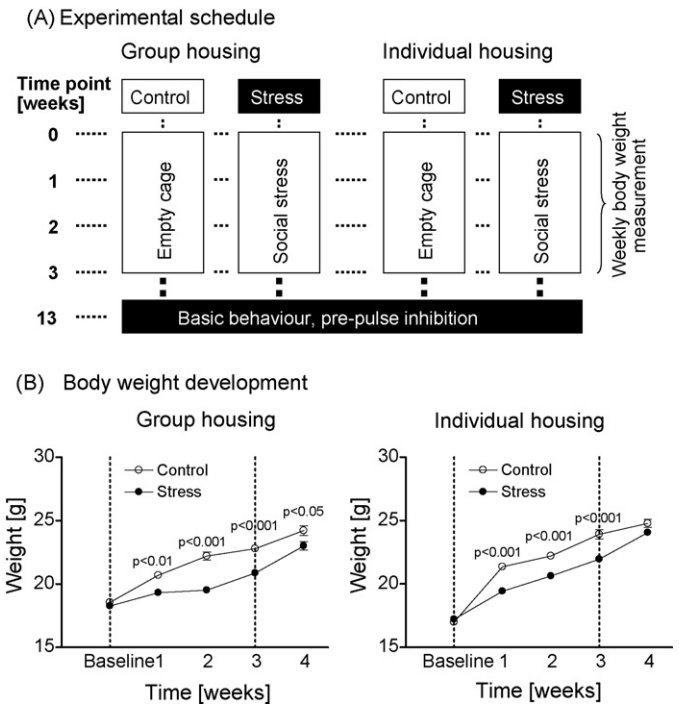

Fig. 1. Chronic psychosocial stress paradigm. (A) Experimental design of the study. Immediately upon arrival (21 days of age) mice were housed in groups ($n = 5$ per cage) or individually. At the age of 28 days, mice from each housing condition were exposed to either social stress in the resident-intruder paradigm or to the control treatment (placement into a novel, empty cage), daily for 3 weeks. Behavioural testing started 10 weeks after termination of the stress procedure. (B) Effects of psychosocial stress on body weight development. In group housed mice, two-way repeated measurement ANOVA yielded a significant effect of stress ($F_{1,112} = 20.93$, $p < 0.0001$) and a significant stress × time interaction ($F_{4,112} = 15.22$, $p < 0.0001$). Likewise, in individually housed mice, two-way repeated measurement ANOVA resulted in a significant effect of stress ($F_{1,108} = 15.8$, $p = 0.0005$) and a significant stress × time interaction ($F_{4,108} = 12.94$, $p < 0.0001$). Mean ± S.E.M presented; $n = 14$–15. Significance values after post-hoc Bonferroni test.

All mice were maintained temperature-controlled (21 ± 2 °C), 12 h light/dark cycle, food/water ad libitum. All experiments were approved by the local Animal Care and Use Committee.

After a habituation period (1 week) following arrival, experimental mice as *intruders* were subjected to social stress by daily exposure for 1 h to a *resident* mouse over 3 weeks. To prevent injuries, free interaction was immediately stopped when the first attack occurred (usually after 1 min, since only residents with attack latencies shorter than 2 min were used) and the *intruder* was covered with a grid cage within the *resident's* cage for the rest of the hour and afterwards placed back into its home cage. On each day, the intruder was confronted with a different resident in a Latin square design. Control mice were placed for 1 h in an empty novel cage, which in itself may be a stressor for the animal, and therefore serves as the adequate comparator for the experimental condition (psychosocial stress), composed of placement in a new cage, including handling, and exposure to the resident. Body weight was measured weekly. Behavioural testing started 10 weeks after termination of the daily stress procedure. Tests were selected to provide information on the most important basic behavioural readouts and were performed in the following order (number of days in brackets): Open field, observing spontaneous activity (day 1), hole-board, measuring exploratory activity (day 2), and rota-rod, testing motor function, balance and coordination (day 3). All tests were performed as described in detail previously [1]. Pre-pulse inhibition of startle response was measured after a resting period of 4 days, i.e. on day 8, in a 4-station testing system (TSE, Bad Homburg, Germany). An experimental session consisted of a 3 min habituation to the 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 90 s at background noise. After baseline recording, 6 pulse alone trials using the startle stimuli of 120 dB intensity and 60 ms duration were applied to decrease the influence of within-session habituation (data not included in analysis of PPI). For tests of PPI, the 120 dB/60ms startle pulse was applied either alone or preceded by a pre-pulse stimulus of 70, 75 and 80 dB intensity and 30 ms duration. An interval of 150 ms with background white noise was employed between each pre-pulse and pulse stimulus. All trials were presented in a pseudorandom order, with an average of 17 s separating trials. Amplitudes of the startle response were averaged for each individual animal, separately for both types of trials (stimulus alone, stimulus preceded by a pre-pulse). PPI was calculated as a percentage of the startle response using the formula: % pre-pulse inhibition = 100 − [(startle amplitude to pulse after pre-pulse)/(startle amplitude after pulse only) × 100].

Statistical significance was evaluated using two-way ANOVA and unpaired *t*-test including Bonferroni testing where applicable. Significance level was set to $p < 0.05$. Data are represented as mean ± S.E.M. in figures and text. The data was analyzed using Prism 4 (GraphPad Software, San Diego, CA, USA).

Stress transiently reduced body weight gain in group and individually housed mice. At the time of behavioural testing, i.e. 10 weeks after the stress period, group differences in body weight had become smaller (Fig. 1B).

At this late time point, neither stress nor type of housing had left any significant effects on spontaneous activity in the open field (all $p > 0.05$, data not shown). However, both stress and individual housing increased the basic anxiety level of mice, indicated by reduced time spent in the central zone of the open field (Fig. 2A). While there were no differences between stressed and control animals regarding behaviour in the hole-board test, individual housing significantly reduced exploration of holes (Fig. 2B). Rota-rod testing resulted in similar performance of all groups with respect to falling latency (all $p > 0.05$, data not shown).

At 75db, PPI showed a strong tendency of impairment in individually housed stressed mice (Fig. 2C; ANOVA: effect of stress: $F_{1,54} = 3.62$, $p = 0.062$), and at 80 db a significant main effect of stress (ANOVA: $F_{1,53} = 4.9$, $p = 0.031$) and stress × housing interaction ($F_{1,53} = 4.83$, $p = 0.032$). The subsequent *t*-test analysis revealed a significant difference between control and stressed animals only upon individual housing but not group housing (Fig. 2C).

The present study provides experimental evidence in mice for the induction of lasting disturbance in brain functioning by chronic psychosocial stress at young age. The deleterious stress effects, operationalized by measuring a decrease of PPI in adulthood, occurred only upon single but not upon group housing. Interestingly, in previous studies on rats, lasting physiological (e.g. endocrine) and behavioural (e.g. anxiety) effects of defeat are also observed upon single but not social housing [for review see: [6]].

In addition to this readout of disturbed sensorimotor gating, i.e. neuronal network dysfunction, persistent alterations in the anxiety level are induced by psychosocial stress and amplified by single housing. Similarly, anxiety-like behaviours in adulthood are provoked by a combined application of 3 different stressors (forced swim, elevated platform, and restraint stress) to juvenile rats. This consequence is prevented by enriched environment [10]. Thus, social interaction and enriched environment appear to play a comparable prophylactic role. These findings emphasize the significance of avoiding social or environmental deprivation for coping with stressful life events and prevention of psychiatric disease.

The hole-board test, used to assess exploratory behaviour/curiosity, is also believed to reflect anxiolytic/anxiogenic states in mice [19]. In the present study, individually housed mice showed a decrease in head-dipping behaviour compared to group housed

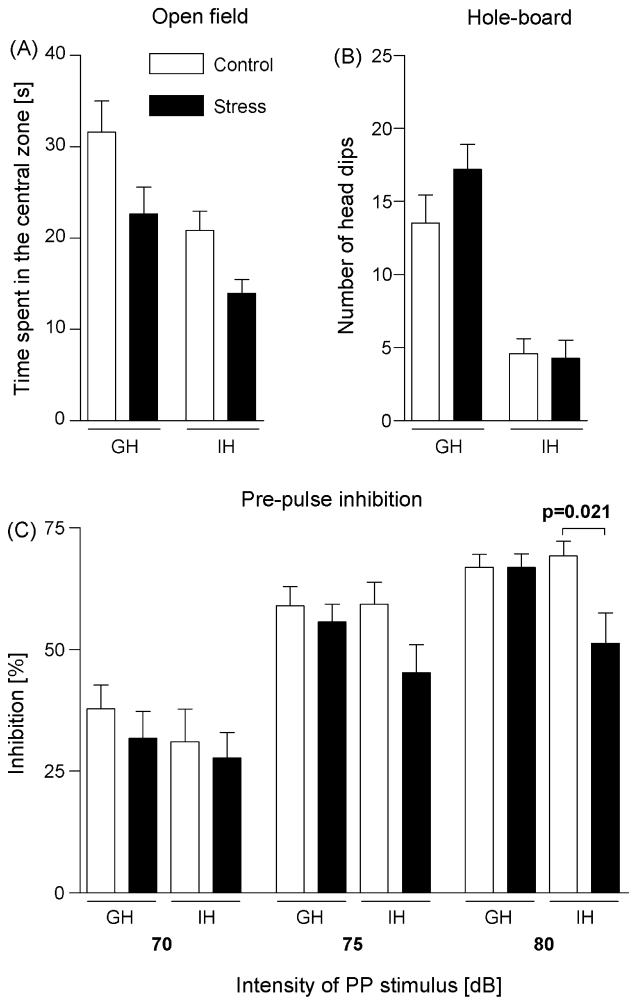

Fig. 2. Behavioural consequences of chronic psychosocial stress. (A) Two-way ANOVA showed a significant effect of stress ($F_{1,55} = 9.32$, $p = 0.004$) and a significant effect of housing ($F_{1,55} = 13.99$, $p = 0.0004$) on time spent in the central zone of the open field (anxiety readout). (B) Individually housed mice had less head dips in the hole-board test compared to mice that were housed in groups (two-way ANOVA: $F_{1,55} = 51.38$, $p < 0.0001$). (C) In individually but not group housed mice, stress decreased pre-pulse inhibition (PPI) at 80 dB (two-way ANOVA: main effect of stress $F_{1,53} = 4.9$, $p = 0.031$; and stress × housing interaction $F_{1,53} = 4.83$, $p = 0.032$). Significance value refers to post-hoc t-test. Mean ± S.E.M presented; $n = 13$–15. GH: group housing, IH: individual housing.

mice, consistent with previous reports of reduced head-dips after social isolation [23]. Thus, social deprivation, independently of psychosocial stress, can in itself be a stressor and alter exploratory behaviour and emotionality.

Several studies suggest that dopaminergic pathways in regions such as striatum (caudate/putamen) and nucleus accumbens play an important role in PPI regulation via activation of mainly dopamine D2 receptors [e.g. [22]]. A possible explanation for the observed deficits in PPI upon chronic psychosocial stress in association with single housing could thus be alterations in the dopaminergic system. Along these lines, social defeat stress in rats was found to increase extracellular dopamine levels in nucleus accumbens and prefrontal cortex [21]. Importantly, the mice in the present study were stressed during adolescence, a sensitive period of development, as indicated by rapid growth of the mesocorticolimbic dopamine system known to be activated by stressors [13].

Consistent with recent articles, we found psychosocial stress to lead to a temporary decrease in body weight gain [14,17]. In contrast to findings of others [8], social housing in our study did not prevent the stress-induced decrease in body weight gain, most likely due to the fact that the present stress procedure lasted for 3 weeks compared to single social defeat used by De Jong et al. [8].

In summary, the present study indicates (1) that there are additive effects of psychosocial stress and single housing (a stressor in itself) in the open field, (2) that there are effects of single housing in the hole-board test, with no additional effect caused by psychosocial stress, and (3) that only a combination of psychosocial stress and individual housing causes persistent impairment in basic information processing/neuronal network function in adulthood (PPI). These findings, when translated to man, underline the importance of immediate and continuous social support for preventing downstream damage after psychotrauma.

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4. Effect of EPO on hippocampus dependent memory

4.1 Overview of project II

EPO is a protein hormone that was originally described to be produced in the kidneys and to act as a hematopoietic growth factor. In recent years it evolved as a promising candidate for neuroprotection in human brain disease (Ehrenreich et al., 2004b). It was found that EPO and EPO receptor are abundantly expressed not only in neurons and glial cells in culture, but also in the embryonic rodent brain and spinal cord as well as in the developing human central nervous system. The expression of EPO and EPO receptor in postnatal and adult brain tissue is low under normal condition, but it is observed to be upregulated upon hypoxia, ischemia, inflammation or neurodegeneration (Ehrenreich et al., 2004b; Ehrenreich et al., 2004c; Ehrenreich et al., 2004a; Ehrenreich, 2008; Siren et al., 2009).

Recent literature showed that EPO has an effect on cognition in various models of brain pathology in rodents, including ischemia/hypoxia and traumatic brain injury (Catania et al., 2002; Mogensen et al., 2004; Siren et al., 2006). There is also one study that was performed on healthy animals. Hengemihle et al. (1996) have shown that 19 weeks of treatment with EPO improved water maze performance in healthy mice. The beneficial action of EPO on cognitive functioning was also shown in humans. In 2007, Ehrenreich and colleagues published the results of a double-blind, placebo-controlled, proof-of-principle (phase II) study, showing a significant and lasting improvement of cognitive performance in chronic schizophrenic patients after EPO treatment (Ehrenreich et al., 2007b). Furthermore, improvement in motor and cognitive function upon EPO treatment was noted in chronic progressive multiple sclerosis (Ehrenreich et al., 2007a).

These data indicate that EPO has a direct effect on the nervous system of both, rodents and men. However, there still is little known about the mechanism of

action of EPO in improving cognitive performance which is essential for further development of novel treatment strategies in neuropsychiatric disorders. Therefore in the project II we aimed to investigate the mechanism of EPO action in healthy mice where interference of disease-related effects can be excluded.

First we defined the experimental condition under which cognitive improvement can be observed in healthy mice. Young, 28 days old B57Bl6 mice were injected every other day for three weeks with EPO (5000 IU/kg) or Placebo (dilutant for EPO) and tested in fear conditioning test, either 1, 3 or 4 weeks after termination of treatment. EPO-treated mice have shown significant improvement of contextual memory 1 week after the cessation of treatment, when tested 72 hours after the training in the same context. This effect was still measurable 3 weeks after the last EPO injection but had disappeared after 4 weeks. Moreover, there was no difference in hematocrit level between EPO- and Placebo-treated mice 3 weeks after the termination of treatment indicating that cognitive improvement and hematopoietic effects of EPO are not directly related. We have also found that EPO treatment had no effect on anxiety, spontaneous activity, exploratory behavior, and motor performance.

One possible explanation of contextual memory enhancement after EPO treatment could be a direct effect of EPO on synaptic plasticity in hippocampus. In order to examine this possibility we performed electrophysiological measurements at Schaffer collateral CA1 pathway in hippocampal slices obtained from mice at 1 week after the cessation of treatment. To evoke field excitatory postsynaptic potentials (fEPSPs), the stimulation electrode was placed in stratum radiatum at CA3/CA1 junction and the recording electrode was placed in the stratum radiatum of the CA1 region. In hippocampal slices obtained from EPO treated mice we observed enhanced paired-pulse facilitation, long term potentiation (LTP), short term potentiation (STP) and short term depression (STD).

Next, in order to study cellular mechanisms of EPO action, we performed whole-cell patch-clamp recordings on CA1 pyramidal neurons in acute hippocampal slices from mice at 1 week after the termination of treatment. We have found that EPO treatment led to decrease of both amplitude and frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and to increase of frequency of spontenous inhibitory postsynaptic currents (sIPSCs).

Because the peritoneal application of EPO could cause indirect effects on the neurons, in the next approach we studied the effects of EPO on spontaneous neuronal network activity of primary hippocampal cultures. To do this, we obtained hippocampal neurons from mice at E17 and let them grown on multi-electrode array (MEA) dishes. We found that chronic EPO treatment, extending from an advanced developmental stage (day 5 in culture) to over 3 weeks leads to lower overall spiking activity but enhanced bursting in discrete neuronal assemblies.

Additionally, we performed whole-cell patch-clamp recordings in autaptic hippocampal neurons (neurons forming synapses on themselves) in order to directly measure the EPO effect on presynaptic transmitter vesicle exocytosis and postsynaptic receptor responses. Using this approach we were able to show that treatment with EPO at day 7 in culture leads to significant reduction in evoked excitatory postsynaptic current (EPSC) amplitude and readily-releasable pool size when measured 2-7 days after the EPO application.

Furthermore, in order to see if EPO effects on synaptic function could be explained by affecting the number of synapses, we performed counting of synapses on hippocampal sections and in autaptic neuron cultures. This revealed no differences between EPO and Placebo treatment indicating that changes in synaptic function might be rather due to the shifting of the balance between excitatory and inhibitory transmission and not due to altered total synapse number.

4.2 Original publication

Adamcio B*, Sargin D*, Stradomska A*, Medrihan L, Gertler C, Theis F, Zhang M, Müller M, Hassouna I, Hannke K, Sperling S, Radyushkin K, el-Kordi A, Schulze L, Ronnenberg A, Wolf F, Brose N, Rhee JS, Zhang W, Ehrenreich H. Erythropoietin enhances hippocampal long-term potentiation and memory. ***BMC Biol. 2008 Sep 9;6:37.***

* Indicates equal contribution for the publications

Personal contribution:

I was involved in the design of the study, interpretation of results and preparation of the manuscript. I have performed all behavioral experiments and was also involved in brain dissection as well as in performing western blot and qRT-PCR. Electrophysiological analyses were performed by our collaborators.

BMC Biology

Research article

Erythropoietin enhances hippocampal long-term potentiation and memory

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Abstract

Background: Erythropoietin (EPO) improves cognition of human subjects in the clinical setting by as yet unknown mechanisms. We developed a mouse model of robust cognitive improvement by EPO to obtain the first clues of how EPO influences cognition, and how it may act on hippocampal neurons to modulate plasticity.

Results: We show here that a 3-week treatment of young mice with EPO enhances long-term potentiation (LTP), a cellular correlate of learning processes in the CA1 region of the hippocampus. This treatment concomitantly alters short-term synaptic plasticity and synaptic transmission, shifting the balance of excitatory and inhibitory activity. These effects are accompanied by an improvement of hippocampus dependent memory, persisting for 3 weeks after termination of EPO injections, and are independent of changes in hematocrit. Networks of EPO-treated primary hippocampal neurons develop lower overall spiking activity but enhanced bursting in discrete neuronal assemblies. At the level of developing single neurons, EPO treatment reduces the typical increase in excitatory synaptic transmission without changing the number of synaptic boutons, consistent with prolonged functional silencing of synapses.

Conclusion: We conclude that EPO improves hippocampus dependent memory by modulating plasticity, synaptic connectivity and activity of memory-related neuronal networks. These mechanisms of action of EPO have to be further exploited for treating neuropsychiatric diseases.

Background

The hematopoietic growth factor erythropoietin (EPO) has long been observed to exert beneficial effects on cognition. Upon introduction of recombinant human EPO into the clinic, cognitive improvement of patients with chronic renal failure was noted during EPO treatment, but attributed to its hematopoietic effects (for review see [1-4]). Indeed, anemia after isovolemic hemodilution, induced in healthy volunteers, impairs cognitive performance, which is completely restored by subsequent autotransfusion [5].

However, the finding that EPO and its receptor (EPOR) are expressed in the brain [6,7] (for review see also [1,3,8-11]) led to the notion that EPO exerts direct, hematopoiesis-independent effects on the nervous system. The manufacturing of EPO analogues with no hematopoietic but potent neuroprotective properties, e.g. CEPO (carbamoylated EPO) [12], delivered proof-of-principle that brain effects of EPO are not necessarily mediated by its hematopoietic actions.

Beneficial effects of EPO on cognitive functioning have been shown in different animal models of neuropsychiatric diseases, e.g. on place navigation after global ischemia or neurotrauma [13-17]. In a recent double-blind, placebo-controlled, proof-of-concept study in chronic schizophrenic patients, we showed that EPO improved schizophrenia-relevant cognitive performance independently of its hematopoietic effects. In fact, EPO turned out to be the first compound to exert a selective and lasting beneficial effect on cognition in schizophrenia [18]. Similarly, an increase in cognitive performance upon EPO in patients with chronic progressive multiple sclerosis occurred independently of changes in hemoglobin levels, and persisted for months after termination of EPO treatment [4,19].

Recently, the application of a single high intravenous dose of EPO in healthy human volunteers was reported to enhance the functional MRI-detectable hippocampus response during memory retrieval 1 week later, before any effect on hemoglobin was measured [20]. However, data on hippocampus dependent memory in healthy human subjects upon EPO are still missing. Altogether, little is known about potential cognitive effects of EPO in healthy individuals. Hengemihle et al. [21] reported that 19 weeks of low-dose EPO treatment increased spatial memory performance, and a conditioned learning task, taste aversion, was enhanced by a single high-dose injection of EPO in healthy mice [22].

In summary, the currently available data clearly indicate that EPO can improve cognitive function of both rodents and man by directly acting on the nervous system. To be able to fully exploit this beneficial cognitive effect of EPO for treatment of neuropsychiatric diseases, it is essential to understand the cellular mechanisms of EPO action in healthy brain, where interference of disease-related effects can be excluded. Here, we systematically addressed this problem. We developed a reliable, robust model for improvement of cognition by EPO in healthy mice and examined correlated effects of EPO on hippocampal synaptic transmission and learning/memory-relevant synaptic plasticity. Further, we analysed effects of EPO on cultured hippocampal neurons at network and single cell levels. Our data indicate that EPO improves memory by modulating synaptic connectivity of memory-related neuronal networks within the hippocampus.

Results

EPO improves hippocampus dependent memory in healthy young mice

First goal of this study was to define an experimental condition to test potential abilities of EPO to improve cognitive functions. We used young (28 day old) male mice. In our experimental set-up with 11 intra-peritoneal EPO versus placebo injections (5000 IU/kg) every other day for 3 weeks (Figure 1), EPO-treated mice showed significant improvement of contextual memory in fear conditioning 1 week after the last injection, when tested 72 h after training in the same context (Figure 1, Exp. 1, Figure 2a). This effect was still measurable 3 weeks after cessation of EPO treatment but had disappeared after 4 weeks (Figure 1, Exp. 2 and Exp. 3; Figure 2b, c). In contrast, EPO had no effect on cued memory (Figure 2a–c; all $P > 0.05$). Whereas at 1 week after termination of treatment, hematocrit was still increased in EPO-treated mice (control mice: 36.5 ± 0.84%, $N = 8$; EPO mice: 53.3 ± 1.34%, $N = 10$; $P < 0.0001$), there was no difference anymore between groups at 3 weeks (control mice: 39.4 ± 1.19%, $N = 14$; EPO mice: 40.8 ± 0.92%, $N = 13$; $P = 0.338$), indicating that cognitive improvement and hematopoietic effects of EPO are not directly related.

In two additional experiments, EPO was given only three times either at the beginning or at the end of the 3-week treatment period while the respective other eight injections consisted of placebo. In this setting, no improvement in cognitive performance was obtained (data not shown), suggesting that a certain amount of EPO treatment is required for improving cognition.

The effect of EPO on hippocampus dependent (contextual) memory was selective. There was no EPO effect on anxiety, spontaneous activity, exploratory behavior, and motor performance (Figure 2d–g; all $P > 0.05$). Time spent in open arms of elevated plus maze (Figure 2d) and time spent in the three different zones of open field was similar in both groups (Figure 2e). Total distance traveled in open

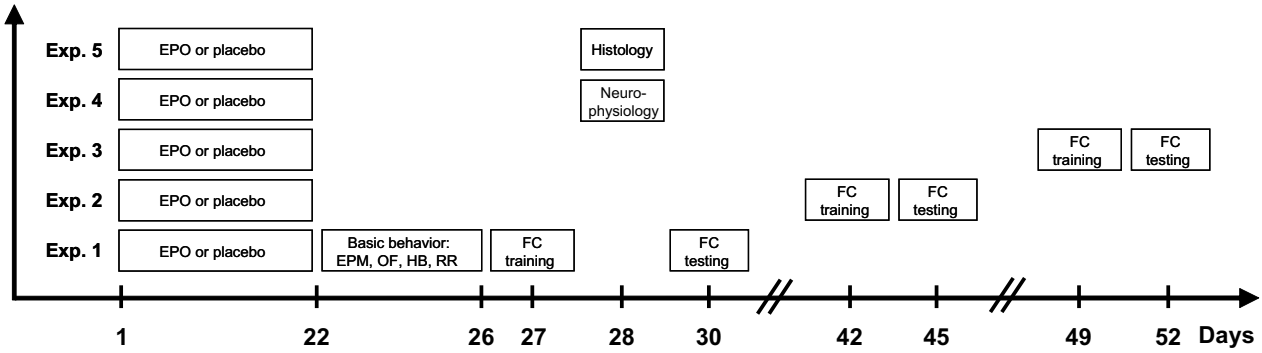

Figure 1
Experimental design of the *in vivo* studies. The time line of behavioral testing and brain dissection is presented. EPO or placebo was injected every other day for 3 weeks (11 injections in total). Tests performed were elevated plus maze (EPM), open field (OF), hole board (HB), rota-rod (RR), and fear conditioning (FC), including training and testing 72 h later.

field did not differ between groups nor did exploratory activity in hole board test (Figure 2e, f). Over two days of rota-rod testing, both groups did not differ in falling latency (Figure 2g), indicating that motor performance and motor learning were comparable. Taken together, EPO treatment over 3 weeks leads to selective and long-

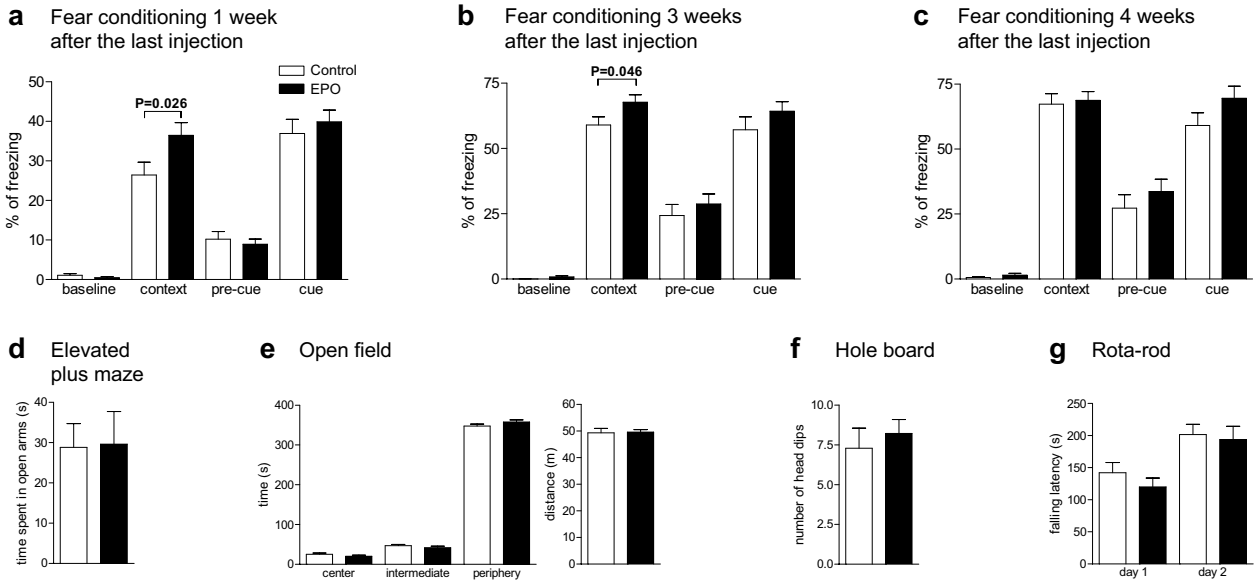

Figure 2
Effects of EPO on hippocampus dependent memory. Percentage of freezing as a readout of memory function in fear conditioning shows significant effects upon EPO treatment in the contextual memory (context) task at 1 week **(a)** and 3 weeks **(b)**, but no longer at 4 weeks **(c)** after the last EPO injection. Percentage of freezing measured during training (baseline), exposition to the new context (pre-cue), and testing for cued memory (cue) is not different between the groups. No differences are seen in EPM **(d)**, OF **(e)**, HB **(f)**, and RR **(g)**. Mean ± S.E.M. *N* = 28 for experiment in **(a)** and *N* = 14 for all other experiments **(b-g)**.

lasting improvement of hippocampus dependent (but not of global) memory in healthy mice, independent of hematopoietic effects.

Synaptic plasticity is significantly increased at Schaffer collateral CA1 synapses in EPO-treated mice

One likely explanation for the selective improvement of contextual memory would be a direct influence of EPO on synaptic plasticity in the hippocampus. We therefore investigated the effect of EPO in acute hippocampal slices from mice at 1 week after the last injection (Figure 1, Exp. 4). We first performed extra-cellular recordings of field excitatory postsynaptic potentials (fEPSPs). Input-output curves were obtained by evoking responses from stratum radiatum of the CA1 region after stimulation of Schaffer collaterals with increasing stimulation strengths (Figure 3a, b). Average of fEPSP slopes (Figure 3b) between stimulus intensities of 110–150 μA from all slices yielded no difference between control and EPO groups. Half-maximal stimulation strength was also comparable (Figure 3b, inset). Thus, EPO treatment for 3 weeks, followed by a treatment-free week, does not alter basal excitability.

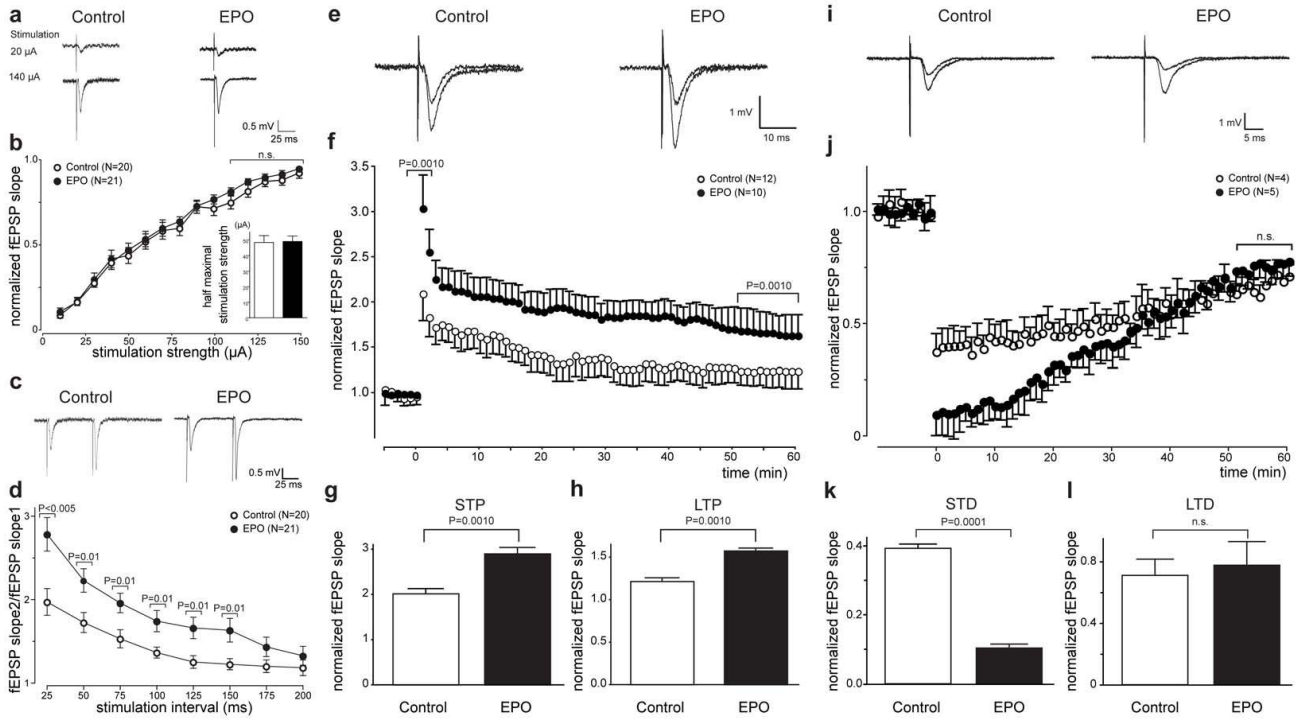

Figure 3

Neurophysiology of acute hippocampal slices: *Extracellular recordings*. (a-b) Input-output relation is not altered at Schaffer collateral-CA1 synapses in EPO-treated mice. **(a)** Sample recordings at 50% of maximal response (average of four traces) are shown for control and EPO-treated mice. **(b)** Input-output curve as a measure of baseline excitatory synaptic transmission: fEPSP slope, plotted against the stimulation strength, is not altered in EPO-treated mice compared to control (P = 0.3094). Inset: Half maximal stimulation strengths are not significantly different. **(c-d)** Paired-pulse facilitation is enhanced in EPO-treated mice. **(c)** Sample traces are presented. **(d)** Paired-pulse ratio (fEPSP slope for the second stimulus/fEPSP slope for the first stimulus) at inter-stimulus intervals of 25–150 ms is significantly greater in EPO-treated mice. **(e-h)** Increased LTP at Schaffer collateral CA1 synapses in EPO-treated mice. **(e)** Sample traces of responses are shown before and after high frequency stimulation (HFS; 3 × 100 Hz for 1 s each, 20 s interval). **(f)** Long-term potentiation elicited by HFS: Slopes of fEPSP are normalized to baseline and plotted against time. Time-point 0 represents application of HFS. **(g)** Magnitude of STP, determined as maximal responses within 1 min after HFS, is significantly greater in EPO-treated mice. **(h)** Magnitude of LTP, determined as responses between 50 and 60 min after HFS, is significantly greater in EPO-treated mice. **(i-l)** Increased STD at Schaffer collateral-CA1 synapses in EPO-treated mice. **(i)** Sample traces of responses are shown before and after low frequency stimulation (LFS; 1 Hz for 900 stimulations). **(j)** Long-term depression elicited by LFS: Slopes of fEPSP are normalized to baseline and plotted against time. Time 0 represents application of LFS. **(k)** Magnitude of STD, determined as maximal responses within 1 min after LFS, is significantly greater in EPO-treated mice. **(l)** Magnitude of LTD, determined as responses between 50 and 60 min after LFS, is not significantly changed in EPO-treated mice (P = 0.0869).

We then measured paired-pulse facilitation (PPF), the shortest form of plasticity at many synapses [31], at different inter-stimulus intervals (25–200 ms) in the Schaffer collateral CA1 pathway as ratio of the second fEPSP slope to the first fEPSP slope. Slices from EPO mice showed significantly enhanced paired-pulse facilitation at inter-stimulus intervals 25–150 ms (Figure 3c, d). Next, the effect of EPO on short-term potentiation (STP) and long-term potentiation (LTP) at the Schaffer collateral CA1 pathway was examined (Figure 3e–h). The magnitude of STP was defined as the maximal responses within the first minutes after induction by a train of 100 Hz stimuli. STP was significantly enhanced in slices of EPO mice compared to control (Figure 3f, g). Furthermore, the magnitude of LTP, determined as the average of responses between 50 and 60 min after induction by a train of 100 Hz stimuli, was also enhanced in slices of EPO mice compared to control (Figure 3f, h).

Another form of synaptic plasticity is long-term depression (LTD). We determined the effect of EPO treatment on short-term depression (STD) and LTD at Schaffer collateral CA1 pathway (Figure 3i–l). Magnitude of STD was defined as maximal responses within the first minutes after induction by a train of 900 stimuli (1 Hz). We found that STD was significantly enhanced in slices of EPO mice compared to control (Figure 3j, k). On the other hand, the magnitude of LTD, determined as average of responses between 50 and 60 min after induction by a train of 900 stimuli (1 Hz), was not significantly different in slices of EPO mice compared to control (Figure 3j, l). Collectively, these data show that EPO modulates synaptic plasticity and LTP in the hippocampus, but has no significant effect on LTD.

EPO differentially influences inhibitory and excitatory synaptic transmission in the Schaffer collateral CA1 pathway

To study cellular mechanisms of EPO action, we performed whole-cell patch-clamp recordings on CA1 pyramidal neurons in acute hippocampal slices from mice at 1 week after the last injection (Figure 1, Exp. 4; Figure 4a–f). Compared to control mice, the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in CA1 pyramidal neurons of EPO mice was increased, while the amplitude of sIPSCs was unchanged (Figure 4b, c). In con-

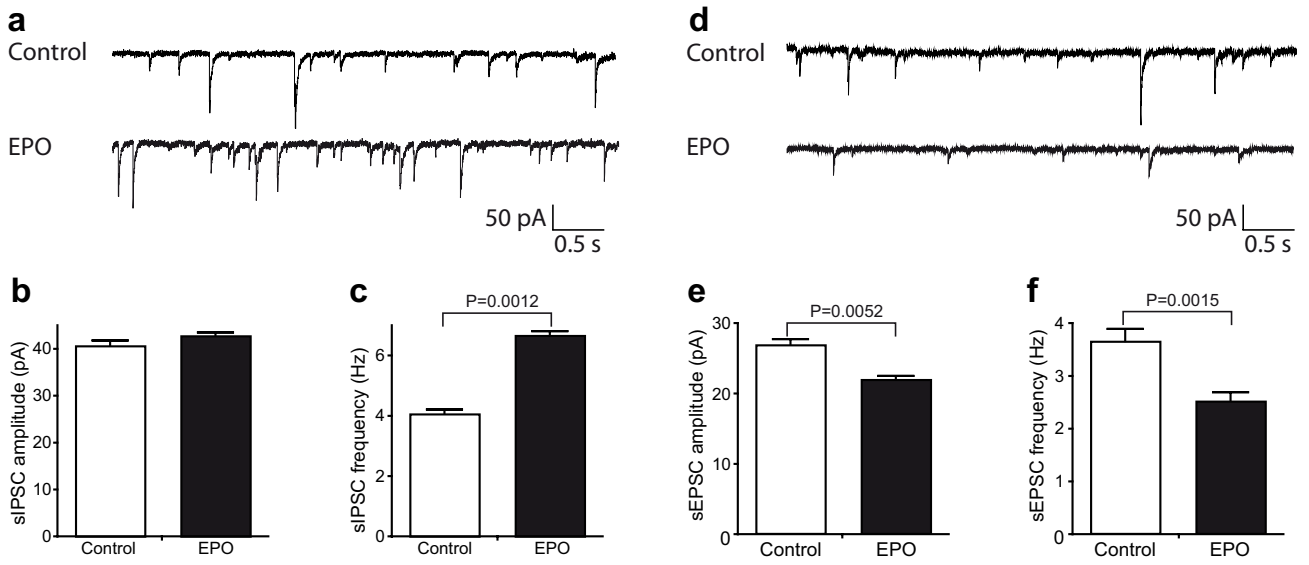

Figure 4

Neurophysiology of acute hippocampal slices: *Intracellular recordings*. **(a-c)** EPO enhances inhibitory transmission. **(a)** Representative recordings of spontaneous, pharmacologically isolated inhibitory postsynaptic currents (sIPSCs) from CA1 neurons. **(b)** Averaged amplitude of sIPSCs is not significantly altered in EPO-treated mice (*N* = 6 neurons/5 mice) compared to control (*N* = 4 neurons/4 mice; *P* = 0.0869). **(c)** Averaged frequency of sIPSCs is significantly enhanced in EPO-treated mice (*N* = 6 neurons/5 mice) compared to control (*N* = 4 neurons/4 mice). **(d-f)** EPO decreases excitatory transmission. **(d)** Representative recordings of spontaneous, pharmacologically isolated excitatory postsynaptic currents (sEPSCs) from CA1 neurons. **(e)** Averaged amplitude of sEPSCs is significantly decreased in EPO-treated mice (*N* = 4 neurons/4 mice) compared to control (*N* = 4 neurons/3 mice). **(f)** averaged frequency of sEPSCs is significantly decreased in EPO-treated mice (*N* = 4 neurons/4 mice) compared to control (*N* = 4 neurons/3 mice).

trast, EPO led to a significant decrease of both amplitude and frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in CA1 pyramidal neurons (Figure 4e, f). Importantly, there were no significant differences in input resistance or basic noise level between neurons of control and EPO mice (data not shown). Thus, EPO modulates inhibitory and excitatory synaptic transmission inversely.

We wondered whether the neurophysiological changes found in hippocampal slices upon EPO treatment would be due to alterations in total volume or synapse counts in the involved areas, CA1 and CA3. Neither volume of CA1 (control: 3.97 ± 0.11 mm^3, $N = 9$; EPO: 4.02 ± 0.16 mm^3, $N = 10$; $P = 0.81$) nor CA3 (control: 3.29 ± 0.21 mm^3, $N = 8$; EPO: 3.56 ± 0.25 mm^3, $N = 10$; $P = 0.42$), nor total hippocampal volume (control: 9.54 ± 0.34 mm^3, $N = 8$, versus EPO: 9.74 ± 0.39 mm^3, $N = 10$; $P = 0.71$) were significantly different. Moreover, density of synaptic boutons in CA1 (control: 1.28 ± 0.08 boutons/μm^2, $N = 7$; EPO: 1.32 ± 0.11 boutons/μm^2, $N = 9$; $P = 0.75$) and CA3 (control: 0.71 ± 0.13 boutons/μm^2, $N = 7$; EPO: 0.78 ± 0.08 boutons/μm^2, $N = 9$; $P = 0.61$) was not changed. Quantitative RT PCR and/or Western blotting using extracts of whole hippocampus did not reveal differences in expression of synaptic proteins (synapsin1, synaptophysin), postsynaptic receptor proteins ($GABA_A$1,2,3,4; NMDAR1, R2A, R2B) or BDNF, as potential mediating neurotrophic factor [20,32] (data not shown).

EPO modulates spontaneous electrical network activity in primary hippocampal neurons as determined by multi-electrode measurements

Above data demonstrated distinct and long-lasting effects of temporary high-dose EPO treatment on hippocampus dependent memory and synaptic plasticity in hippocampal slice preparations. As the peritoneal applications of EPO might have caused indirect effects on nerve cells, we next studied primary hippocampal cultures. We tested whether chronic EPO treatment, extending from an advanced developmental stage (day 5 in culture) to over 3 weeks leads to alterations in spontaneous neuronal network activity, and whether such changes would persist upon cessation of EPO treatment.

First, our long-term cultures were characterized regarding morphological appearance (Figure 5a), total cell numbers (day 10: control: 202.4 ± 11.03, $N = 6$; EPO: 191.0 ± 8.834, $N = 6$; $P = 0.436$; day 30: control: 147.9 ± 26.26, $N = 6$; EPO: 152.8 ± 27.87, $N = 6$; $P = 0.902$), and relative contribution of different cell types (Figure 5b). In none of these parameters were differences upon EPO found at days 10 or 30 in culture. Also, quantitative RT PCR and protein expression, determined by Western blotting, failed to uncover differences in synapsin1 or synaptophysin gene expression at any of the time points tested (days 8, 14, and 30). Thus, EPO treatment did not cause changes in morphology under our culture conditions.

Figure 5c illustrates primary hippocampal neurons grown on multi-electrode array (MEA) dishes. Group statistics for spontaneous electrical activity in the MEAs are presented in Figures 5d and 5e, contrasting silencing (number of channels with < 5 spikes per 2 min) and bursting behavior (percentage of strongly bursting channels of all active channels, with strongly bursting channels defined as channels with a coefficient of variation > 2.6). With increasing age and maturation of culture, the number of silent channels decreased in control MEAs, as expected (Figure 5d). This was not the case in EPO cultures. Whereas during the treatment phase itself, cultures behaved largely similarly (weeks 3 and 4 with $P = 0.41$ and $P = 0.18$, respectively), differences became obvious at later time points (weeks 5 through 7 with $P = 0.047$, $P = 0.0043$ and $P = 0.0043$, respectively). This indicates that temporary EPO treatment causes a significant number of channels to remain silent for an extended period after cessation of EPO addition to cultures.

The bursting channel analysis, presented in Figure 5e, showed that EPO provoked a consistently higher number of bursts in hippocampal cultures, obvious only at late time points, i.e. 2–3 weeks after termination of EPO treatment. This effect (expressed as percentage of all active channels in order to exclude the influence of silencing) was less pronounced as compared to the silencing effect of EPO. Whereas medians at week 6 were not yet significantly different ($P = 0.10$), difference reached significance at week 7 ($P = 0.019$). Together, the trend of weeks 6 and 7, when compared with the almost equal-bursting situation at week 5 ($P = 0.70$), confirms that bursting tends to increase as a late consequence of transient EPO treatment, in parallel with the persistently high percentage of silent channels.

Reduction of synaptic vesicle priming and transmitter release in the EPO pre-treated neurons

The finding of long-lasting EPO-induced dampening of spontaneous electrical activity in our primary hippocampal cultures together with a selective increase in bursting activity following EPO treatment prompted us to test individual neurons. We examined the effect of EPO in hippocampal autaptic cultures [30], to directly assess the EPO effect on presynaptic transmitter vesicle exocytosis and postsynaptic receptor responses. Autaptic neurons are neurons forming synapses on themselves, making electrophysiological stimulation and respective effect determination (recording) simple. Cultures were treated with EPO (0.3 IU/ml = 10^{-10}M) or the respective buffer solution only once at day 7 and then measured from days 9 to 14. There were no morphological differences detectable upon

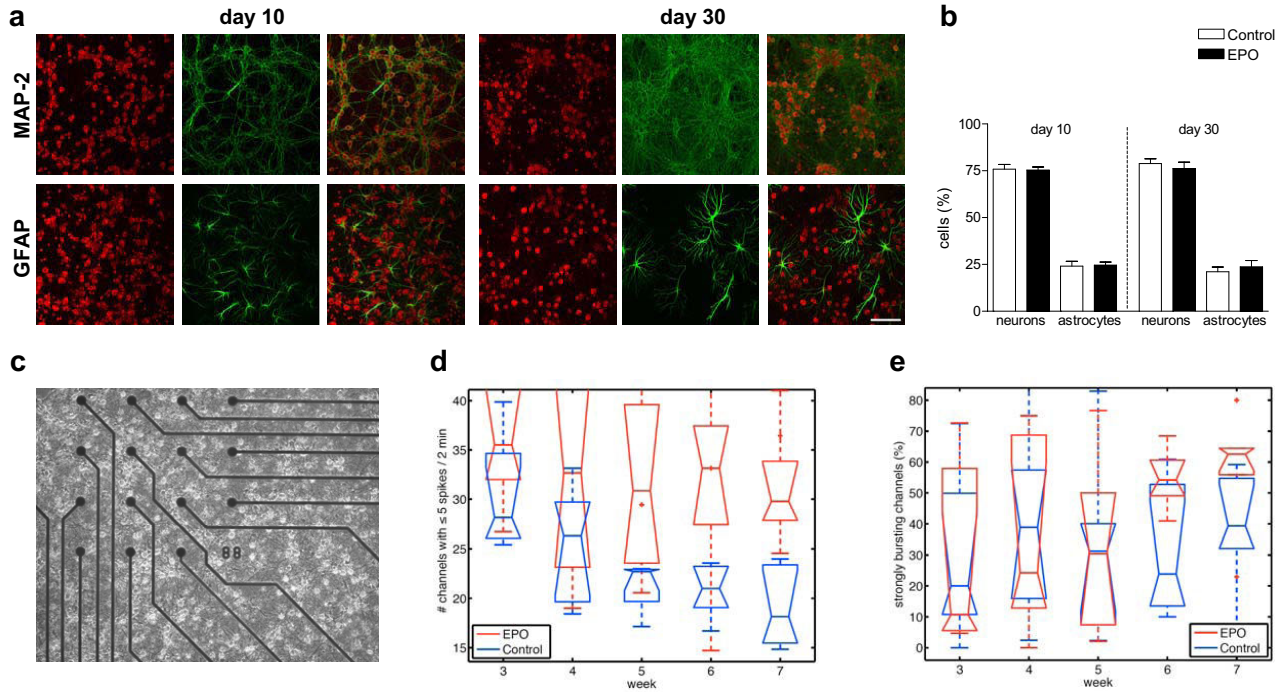

Figure 5

Multi-electrode array studies of primary hippocampal neurons. **(a-b)** Characterization of the cultures. **(a)** Immunocytochemical staining demonstrates maturation of cellular networks from day 10 to day 30. Propidium iodide staining of all nuclei (red), visualization of cell types by MAP-2 (mature neurons) or GFAP (astrocytes) staining (green), as well as merged pictures are presented (scale bar = 100 μm). **(b)** Cellular composition of networks remains stable over time and is not altered by EPO treatment (0.3 IU/ml every other day) from day 5 through 25 in culture (Mean ± S.E.M. of *N* = 3 independent cultures per time point). **(c)** Demonstration of primary hippocampal neurons grown on multi-electrode array dishes, containing 60 electrodes/dish. **(d-e)** Spontaneous electrical activity of primary hippocampal neuronal networks in culture is measured daily from week 3 through week 7. Group statistics of the multi-electrode array recordings over each week show significant dissociation over time of EPO versus control cultures. **(d)** Silencing group statistics reveal a global decrease of channels with low activity in control cultures that cannot be observed in EPO-treated cultures. **(e)** Bursting group statistics show that the percentage of strongly bursting channels increases in the EPO group after termination of treatment. Medians ± S.E.M. presented of *N* = 7 independent cultures. *P* values are given in the text.

treatment, and sizes of somata as estimated by measurement of whole cell capacitance were comparable between EPO-treated and control neurons (control neurons: 49.61 ± 2.75pF, *N* = 54; EPO neurons: 46.0 ± 2.73pF, *N* = 49; *P* = 0.355).

Evoked excitatory postsynaptic current (EPSC) amplitudes in EPO-treated neurons were reduced to about 60% of control (Figure 6a), confirming the data obtained in acute slices (Figure 4e). This EPSC reduction was due to a parallel reduction in pool size of fusion-competent and primed (readily releasable) vesicles, whose release can be triggered by hypertonic solution containing 0.5 M sucrose [33]. EPO neurons showed a drastic reduction in readily releasable pool size to 60% of control (Figure 6b). Vesicular release probability, calculated by dividing the charge transfer during a single EPSC by the charge transfer measured during readily releasable pool release, was not different between control and EPO neurons (*P* = 0.4116; Figure 6c). To test whether the reduction of neurotransmitter release in EPO neurons can be attributed to a reduction in quantal size, we analysed miniature EPSCs (mEPSC). mEPSC frequency in EPO neurons was reduced to about 50% of control, without changes in mEPSC amplitudes (*P* = 0.5817; Figure 6d, e). The lack of a difference in NMDA/AMPA ratio indicates a comparable maturation state of cultures (Figure 6f). Using trains of action potentials we estimated the efficiency of Ca^{2+} triggered release. In general, vesicular release probability closely correlates with depression and steady-state level of EPSC amplitude sizes during high frequency stimulation. We therefore monitored EPSC amplitudes during 50 consecutive action

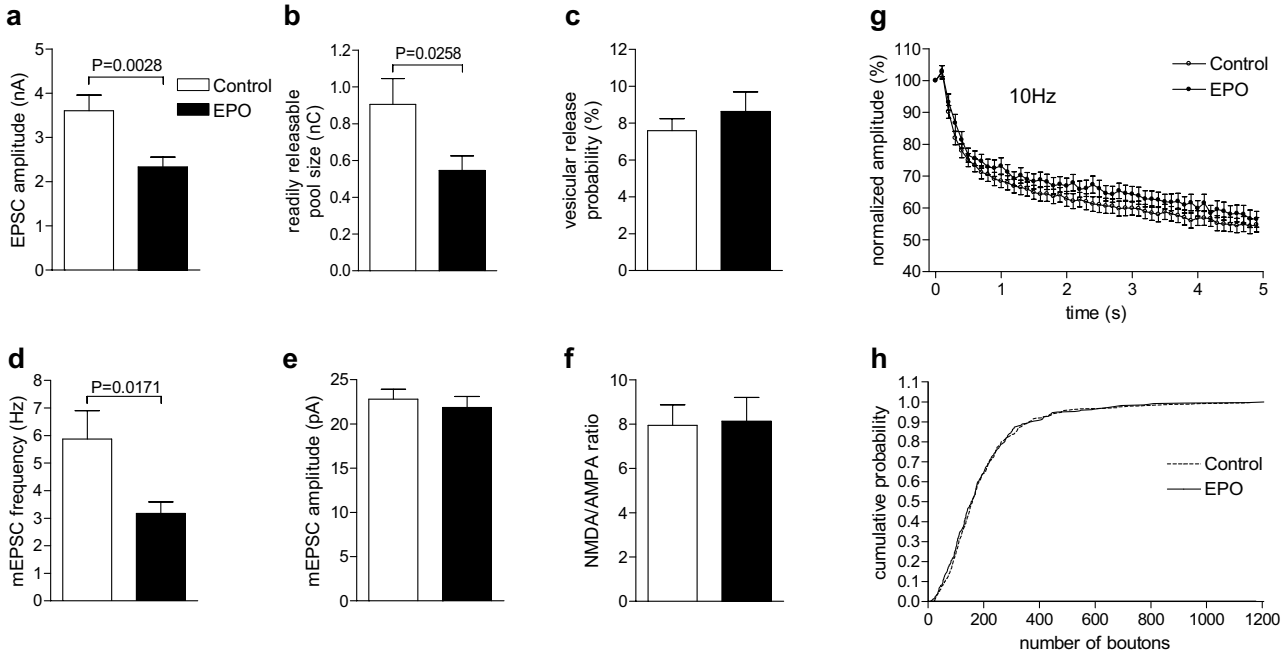

Figure 6

Autaptic hippocampal neuronal cultures. **(a-g)** Whole-cell electrophysiological recordings from single hippocampal neurons treated with either EPO (0.3 IU/ml) or control (diluent only) on day 7 and measured from day 9–14. Results indicate a reduction in the amount of primed vesicles without altering efficiency of vesicle fusion and vesicle dynamics. Mean ± S.E.M. presented. *N* = 40–60. **(h)** Analysing the number of synaptic boutons upon immunocytochemical staining for synapsin1 revealed an almost identical increase of boutons over time in EPO-treated and control neurons. Performed at 40x. Cumulative distribution over days 9–14. *N* = 100–120.

potentials applied at a frequency of 10 Hz. EPO and control neurons showed regular moderate depression of EPSC amplitudes (control: about 38%, *N* = 60; EPO: depression at the end of train about 35%, *N* = 60, Figure 6g). Stability of EPSC amplitudes during short-term plasticity, which is due to the quantitative balance between priming of synaptic vesicles and number of vesicles released, was identical in presynaptic terminals of each group.

Thus, EPO treatment of autaptic neurons leads to a reduction in the amount of primed vesicles or number of synapses without altering efficiency of vesicle fusion and vesicle dynamics. Counting of synaptic boutons per neuron revealed a considerable increase from day 9 to day 14 in culture, which, however, was not changed by EPO (Figure 6h). Therefore, EPO is likely to reduce the number of active synapses without altering total synapse number.

Discussion

We show that young mice, systemically treated with EPO for 3 weeks, exhibit improved hippocampus-associated memory. This selective improvement was maintained for an EPO treatment-free period of another 3 weeks, and was unrelated to increases in hematocrit, indicating a hematopoiesis-independent effect of EPO on neuroplasticity. The long-lasting effect of EPO on neuroplasticity was confirmed by analyses of paired-pulse facilitation, STP, LTP and STD, as well as of spontaneous synaptic activity in acute hippocampal slices, obtained from EPO-treated mice at the time point of EPO-induced enhancement of memory. MEA recordings of neuronal assemblies *in vitro* and the analysis of individual autaptic hippocampal neurons did not only confirm direct effects of EPO on neural cells, but also reveal potential mechanisms of action: EPO leads to a reduction in the amount of primed vesicles without altering number of synapses or efficiency of vesicle fusion and vesicle dynamics. Thus, most likely via increasing the proportion of silent synapses, EPO reduces overall spiking activity of neurons and enhances bursting efficiency of selected neuronal networks. Most of these data are consistent with EPO shifting the balance between excitatory and inhibitory transmission (i.e. functionally silencing a subset of excitatory presynaptic sites and increasing activity of inhibitory neurons), although other mechanisms cannot be entirely excluded at this point.

In humans, improvement of cognitive function upon treatment with EPO has only been demonstrated in dis-

ease states [18,19,34], i.e. in conditions of reduced/disturbed baseline performance. Exploring healthy individuals has therefore been a risky endeavour, although, if successful, promised to deliver a cleaner picture of mechanisms of EPO action, lacking interference with potential disease variables. Similar to what is observed with endurance and muscular performance during doping [35], where healthy individuals show dramatic improvement, we found significant memory effects in healthy mice. EPO-treated compared to placebo-treated mice had a significantly longer duration of freezing, as readout of memory function, during a contextual memory test that is known to be critically dependent on the hippocampus [36,37]. This finding implies that in healthy individuals the potential cognitive capacity is not fully exhausted. Although results were obtained in mice, the work of Miskowiak and colleagues [20] may indicate that respective effects can be expected in healthy humans.

Similar to the findings of Miskowiak et al. [20], the effect of EPO on hippocampal functions was measurable at 1 week after injection. In our setting, treatment for 3 weeks (11 injections) was necessary to obtain positive results on cognitive performance. Reduced to only three injections, no measurable effect on the behavioral readout of hippocampal functions was obtained. Healthy humans showed increased hippocampal response (perfusion equivalent) in functional magnetic resonance imaging upon memory retrieval already 1 week after a single EPO dose. However, effects on memory function were also not detectable after this single dose [20]. In other words, for cognitive improvement (and not only for increase in perfusion), more than a single injection is needed also in humans. In both studies, the hematocrit seems irrelevant. In the human study, a single dose of EPO had not changed the hematocrit after 1 week [20]. In our study, the hematocrit was already back to control levels when we still observed a significant effect on cognition, and direct effects of EPO on synaptic plasticity were found in hippocampal cultures.

The persistent effect of EPO on cognition, lasting for over 3 weeks after cessation of treatment, indicates alterations in neuroplasticity induced by EPO that do not require its continuous presence. Interestingly, our studies in MS patients showed beneficial effects of EPO on motor function, which lasted for up to 6 months after termination of a 6-months treatment [19]. In search for a mechanism explaining the lasting influence of EPO on hippocampus-associated memory, we detected pronounced EPO effects on short-term and long-term plasticity, as well as on excitatory and inhibitory synaptic transmission in the Schaffer collateral CA1 pathway. These electrophysiological parameters of plasticity have been associated with learning and memory [38-40].

Further exploring mechanisms of action of EPO, we employed multi-electrode arrays to study network activity in primary hippocampal cultures. We found that chronic application of EPO in a fashion similar to our *in vivo* approach resulted in persistence of a large population of silent channels but enhanced bursting efficiency of discrete neuronal circuits. In acute hippocampal slices as well as autaptic hippocampal cultures, excitatory synaptic transmission was decreased upon EPO treatment, whereas inhibitory synaptic transmission was increased. In line with these data, EPO-mediated inhibition of glutamate release has been reported for cerebellar granule cells [41].

Together, these findings may point to an enhanced lateral inhibition within the hippocampal neuronal network by EPO, leading to amplification of active synaptic connections. A concurrent suppression of surrounding synapses by EPO, consistent with lasting functional silencing, may ultimately achieve segregation/refinement of neuronal networks (for review see [42]). Interestingly, signal transduction pathways known to be activated in hippocampal neurons by EPO, include PI3K-PKB/Akt1 and RAS-MAPK [43,44]. Both, the MAPK-mediated pathway [45-47] and PI3K have been linked to LTP [48,49].

Conclusion

Although not providing complete mechanistic insight at this point, our data indicate that the selective enhancing effect of EPO on hippocampus dependent memory is mediated via profound changes in neuroplasticity. These plastic changes, in turn, may be based on a more efficient bursting activity of selected synapses together with persistent silencing of other synapses.

Methods

Animals

All experiments were approved by and conducted in accordance with the regulations of the local Animal Care and Use Committee. For all experiments, young (28 days old) C57/Bl6 male mice were used. They were housed in groups of five in standard plastic cages and maintained in a temperature-controlled environment (21 ± 2°C) on a 12 h light/dark cycle with food and water available ad libitum.

Drug treatment

For experiments 1–5, mice were injected intra-peritoneal with EPO (Epoetin-alpha, Janssen-Cilag, Neuss, Germany, 5 IU/g in 0.01 ml) or placebo (diluent for EPO, 0.01 ml/g) every other day for 3 weeks (11 injections in total). Two additional groups of mice received only three injections of EPO or placebo either at the beginning or at the end of the 3-week treatment period. The remaining eight injections were all placebo. Before each injection, the body weight was measured. The experimenter, who

administered the injections and performed the tests, was blinded concerning group assignment.

Experimental design of mouse studies

The experimental design including behavioral tests, neurophysiology, and brain tissue analyses is presented in Figure 1.

Experiment 1

EPO effects on basic behavior and cognition of young healthy mice after termination of EPO treatment were assessed. Mice were tested, starting on the day after the last injection, for anxiety (EPM, elevated plus maze), spontaneous loco-motor activity (OF, open field), exploratory activity (HB, hole board), motor functioning (RR, rota-rod) and memory (FC, fear conditioning).

Experiments 2 and 3

In these experiments, mice were tested in FC either 3 or 4 weeks after the last EPO injection to explore the duration of EPO effects on cognition. Hematocrit was determined immediately after FC.

Experiments 4 and 5

These experiments were set up to obtain brain tissue of mice for neurophysiology and histology at the time point with the most prominent effect of EPO on hippocampus dependent memory.

Behavioral testing

Group size in all behavioral experiments amounted to *N* = 15–28. Exact numbers of individual experiments are given in the legend of Figure 2.

Elevated plus maze

The mouse was placed in the central platform, facing an open arm of the plus-maze. Behavior was recorded by an overhead video camera and a PC equipped with 'Viewer' software (Biobserve, Bonn, Germany) to calculate the time each animal spends in open or closed arms. The time spent in open arms was used for estimation of open arm aversion (fear equivalent).

Open field

Spontaneous activity in open field was tested in a grey Perspex arena (120 cm in diameter, 25 cm high). The mouse was placed in the center and allowed to explore the open field for 7 min. The behavior was recorded by a PC-linked overhead video camera. 'Viewer' software was used to calculate velocity, distance traveled and time spent in central, intermediate or peripheral zones of the open field.

Hole board

The hole board test measures exploratory activity. The apparatus consisted of a 21 cm × 21 cm × 36 cm transparent Perspex chamber with a non-transparent floor raised 5 cm above the bottom of the chamber with 12 equally spaced holes, 2 cm in diameter. Mice were allowed to explore the chamber for 3 min and the number of explored holes (head dips) was scored by a trained experimenter.

Rota-rod

Rota-rod is a test for motor function, balance and coordination and comprises a rotating drum (Ugo Basile, Comerio, Varese, Italy), which is accelerated from 4 to 40 revolutions per minute over the course of 5 min. Each mouse was placed individually on a drum and the latency of falling from the drum was recorded using a stop-watch. To assess motor learning, the rota-rod test was repeated 24 h later.

Cued and contextual fear conditioning

The fear conditioning test was performed as described in detail earlier [23]. Briefly, mice were trained within the same session for both contextual and cued fear conditioning. Training consisted of exposing mice for 120 s to the context to assess the baseline level of activity. This period was followed by a 10 s, 5 kHz, 85 dB tone (conditioned stimulus, CS). Immediately after the tone, a 2 s, 0.4 mA foot shock (unconditioned stimulus, US) was applied. This CS-US pairing was repeated 13 s later. All mice remained in the conditioning chambers for an additional 23 s following the second CS-US pairing. The contextual memory test was performed 72 h after this training. Mice were monitored over 2 min for freezing in the same context as used for training. The cued memory test was performed 76 h after training in a new chamber. First, mice were monitored for freezing over a 2 min pre-cue period with no tone to assess freezing in the new context. Next, a 2 min cue period followed during which the tone was presented. Duration of freezing behavior, defined as the absolute lack of movement (excluding respiratory movements), was recorded by a video camera and a PC equipped with 'Video Freeze' software (MED Associates, St. Albans, Vermont, USA).

Brain dissection and sections preparation

For RNA and protein analysis, mice were deeply anaesthetized and decapitated. Hippocampi were taken out, immediately frozen on dry ice and stored at -80°C. For histology, mice were perfused under deep anesthesia with 4% paraformaldehyde. Brains were dissected, postfixed overnight at 4°C and transferred into 30% sucrose/PBS solution. After having sunk, they were frozen in liquid nitrogen and stored at -80°C. Whole mouse brains were cut into 30 μm thick coronal sections on a cryostat (Leica, Wetzlar, Germany) and kept in a storage solution (25% ethyleneglycol and 25% glycerol in PBS). Every 10th section throughout the dorsal part of the hippocampal for-

mation was selected for staining, yielding five to six sections per brain, used for either volumetrical analysis or confocal microscopy.

Volumetric measurements using histological sections

The sections were mounted on Super Frost microscopic slides, washed in phosphate buffer, then immersed for 25 min in a dilute cresyl violet stain (0.01%) in acetate buffer (pH 4.5), dehydrated in serial dilutions of ethyl alcohol and finally coverslipped using DePeX (Serva, Heidelberg, Germany). Calculation of the volume of CA1, CA3 subregions and the total hippocampus was based on thickness of the sections and areas obtained by tracing contours around the regions of interest, using a light microscope (Olympus BX50) modified for stereology with a 10× objective, a computer-driven motorized stage, *Z*-axis position encoder (microcator), and a microfire video camera interfaced to a PC with the software Stereo Investigator 6.55 (MicroBrightfield, Inc., Williston, VT, USA). Volumetric determinations were performed on both sides of the hippocampus.

Confocal analysis

For counting of synaptic boutons, sections were washed in PBS, permeabilized and blocked in 5% blocking serum for 1 h at 4°C, and incubated at 4°C overnight with rabbit polyclonal synapsin1 antibody (1:4000; Synaptic Systems, Goettingen, Germany). After PBS washes, the sections were incubated with anti-rabbit AlexaFluor555-labeled secondary antibody (1:2000; Invitrogen, Karlsruhe, Germany). Following PBS washes, sections were mounted on Super Frost microscopic slides, air dried and coverslipped with fluorescence mounting medium (Vector, Burlingame, CA, USA) containing DAPI. Synapsin1 immunoreactive presynaptic boutons were analysed within stratum radiatum of area CA1 and stratum lucidum of area CA3 of hippocampus. Images were obtained at a zoom factor 4 using an inverted confocal laser scanning microscope (LSM 510; Zeiss, Goettingen, Germany) with a 63× oil-immersion objective. For intensity comparisons, gain and offset were held constant across images. Synapsin1 immunoreactive punctae were quantified using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Images were manually thresholded and particle analysis plugin was used to calculate the number of immunoreactive punctae.

Hippocampal slice preparation and solution

Acute hippocampal slices were prepared from 56 days old mice (Figure 1, Exp. 4). As in all experiments performed here, the experimenter was blinded regarding group assignment. Mice were deeply anesthetized with diethyl ether before decapitation. The brain was quickly removed and immersed for 2–3 min in ice-cold artificial cerebrospinal fluid (ACSF). The ACSF had the following composition (in mM): 130 NaCl, 3.5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 24 $NaHCO_3$, 1.25 NaH_2PO_4, 10 Glucose, with the pH adjusted to 7.4. Transverse slices of 400 μm thickness were cut with a vibroslicer (752 M, Campden Instruments, Loughborough, UK). The slices were then transferred to an interface recording chamber of the Oslo type and allowed to recover for at least 90 min. The recording chamber was continuously perfused with ACSF, aerated with 95% O_2 and 5% CO_2 (3–4 ml/min). The temperature was kept at 34°C.

Extracellular recordings of hippocampal slices

The recording electrodes were pulled from thin-walled borosilicate glass capillaries (GC150TF-10, Harvard Apparatus, Holliston, MA, USA) using a horizontal Flaming-Brown micropipette puller (P-80/PC, Sutter Instrument Co., Novato, CA, USA). They were filled with ACSF. Monopolar stimulation electrodes made from bare stainless steel microwire (50 μm diameter, AM-Systems) were used for stimulation. The stimuli were generated by photoelectric stimulus isolation units (Grass PSIU6) triggered by a stimulator (Grass S88). Extracellular field potential recordings were done using a custom-built DC amplifier. Data were digitized by a DigiData 1322A (Molecular Devices, Sunnyvale, CA, USA). Initial analysis of the data was done in Clampfit 9.0 (Molecular Devices, Sunnyvale, CA, USA). To evoke field excitatory postsynaptic potentials (fEPSPs), the stimulation electrode was placed in stratum radiatum at CA3/CA1 junction for the activation of Schaffer collaterals. The recording electrode was placed in the stratum radiatum of the CA1 region. The magnitude of fEPSPs was measured as amplitude (baseline to peak) and slope (20–80% level of the falling phase). For input-output relationship, fEPSPs were evoked with 0.1 ms stimuli at 0.25 Hz and an average of four consecutive responses was taken. fEPSP amplitudes and slopes were plotted against the stimulus intensity (10 to 150 μA). Paired-pulse facilitation (PPF) was measured at different interstimulus intervals (25, 50, 75, 100, 125, 150, 175 and 200 ms) as the ratio of the second fEPSP to the first fEPSP. Here also the paired stimuli were given at 0.25 Hz and an average of four consecutive responses was taken. To study long-term potentiation (LTP), baseline responses were evoked every 20 s for 5 min and LTP was induced by three trains separated by 20 s, each train consisting of 100 Hz stimulation for 1s. The post-train responses were then measured every 20 s for 60 min. The magnitude of LTP was measured as the average of responses between 50 and 60 min after induction. To study long-term depression (LTD), baseline responses were evoked every 15 s for 5 min and LTD was induced by 900 stimuli delivered at 1 Hz. The post-train responses were then measured every 15 s for 60 min. The magnitude of LTD was measured as the average of responses between 50 and 60 min after induction.

Whole-cell patch clamp-recordings

Acute transverse 300 μm hippocampal slices were prepared as described above. After preparation, slices were incubated for 30 min at 34°C, followed by room temperature incubation for more than 1 h. All recordings were performed in CA1 hippocampal pyramidal neurons. The extracellular solution in all experiments was the same as the one used in LTP experiments. The pipette solution for all experiments contained (in mM): 140 KCl, 1 $CaCl_2$, 10 EGTA, 2 $MgCl_2$, 4 Na_3ATP, 0.5 Na_3GTP, 10 HEPES at pH 7.3. Spontaneous inhibitory PSCs were recorded at a Cl-reversal potential of about 0 mV in 10 μM CNQX and 40 μM AP5. Spontaneous excitatory PSCs were recorded in the presence of 1 μM strychnine and 1 μM bicuculline. Signals with amplitudes of at least two times above the background noise were selected. Patches with a serial resistance of > 10 MΩ, a membrane resistance of < 0.2 GΩ, or leak currents of > 200 pA were excluded. The membrane currents were filtered by a four-pole Bessel filter at a corner frequency of 2 kHz, and digitized at a sampling rate of 5 kHz using the DigiData 1322A interface (Molecular Devices, Sunnyvale, CA). Data acquisition and analysis were done using commercially available software: pClamp 9.0 (Molecular Devices, Sunnyvale, CA), MiniAnalysis (SynaptoSoft, Decatur, GA) and Prism 4 (GraphPad Software, San Diego, CA).

Primary hippocampal neuronal culture

Mice at embryonic day 17 (E17) were used for preparation of hippocampal primary neuronal cell cultures [24,25] Briefly, after complete removal of meninges, hippocampi were dissected in warm HBSS solution (Invitrogen, Karlsruhe, Germany), supplemented with penicillin and streptomycin, and trypsinized. After mechanical trituration with fire polished Pasteur pipettes, cells were plated on poly-D-lysine- and laminin-coated 6-well plates (for Western blotting and quantitative RT PCR) or on poly-D-lysine- and laminin-coated MEA dishes (for multi-electrode array, MEA) or on poly-D-lysine- and laminin-coated glass cover slips in 6-well plates (for immunocytochemistry) at a density of 200000 cells per well. Neurons were cultured in MEM/B27 medium (Invitrogen, Karlsruhe, Germany) supplemented with sodium bicarbonate, sodium pyruvate, L-glutamine, penicillin, streptomycin and 0.6% glucose. Cultures were incubated at 37°C under 7.5% CO_2/92.5% air and 90% humidity. One-third of medium volume was exchanged every 5th day. Contamination with glial fibrillary acidic protein positive astrocytes on day 5 in culture was consistently less than 7%. For all MEA experiments, EPO or control treatment (0.3 IU/ml = 10^{-10} M) was started on day 5 and continued by addition of EPO every other day until day 25. Cell cultures were maintained until day 50 for MEA, until day 8, 14, or 30 for Western blotting and quantitative RT PCR, until day 10 and 30 for immunocytochemistry.

Immunostaining of cultured cells

After 10 or 30 days in culture, cells were washed in PBS, fixed with 4% paraformaldehyde in PBS, permeabilized and blocked in 0.2% Triton X-100/PBS with 10% blocking serum, and incubated at 4°C overnight with mouse monoclonal MAP-2 (1:500; Chemicon, Hampshire, UK) or mouse monoclonal GFAP (1:500; Novocastra, Newcastle Upon Tyne, UK) antibodies diluted in 1% blocking serum/PBS. After PBS washes, the cells were incubated with Cy2-labeled secondary antibody (1:250; Jackson ImmunoResearch, Newmarket, UK), washed in PBS, air dried and coverslipped with fluorescence mounting medium (Vector, Burlingame, CA, USA) containing propidium iodide.

Multi-electrode array recordings and analysis

For determination of spontaneous electrical network activity in primary mouse hippocampal neuronal cultures, we used multi-electrode arrays (MEA) of 60 titanium nitride electrodes with 30 μm diameter each and 200 μm inter-electrode distance (Multi Channel Systems, Germany). Raw data from the MEA electrodes were amplified by MEA 1060 filter amplifiers (bandwidth 3 Hz-10 kHz; gain × 1100). Sampling frequency amounted to 25 kHz. The experiments were performed at 37°C, using a TC01 temperature controller. Recording of spontaneous network activity was carried out daily in the morning for 2 min, starting on day 14 and ending on day 50. This gave us five weeks of daily recordings, from week 3 until week 7 (total of 37 days). The choice of morning hours for measurements did not affect the statistics, as confirmed by an additional evening experiment showing little daily differences. Seven independent "sister" cultures (i.e. cultures derived from the same brain preparation), treated with EPO or control were analysed. Spike extraction from the continuous data is commonly achieved by spike sorting [26,27]. Having to process 481 2 min recordings, manual interaction, often used to improve sorting behavior, was not feasible. Thus, automated spike extraction using MEATools, a MATLAB-based toolbox for comprehensive analysis of multi-neuronal data http://material.brainworks.uni-freiburg.de/research/meatools/ was employed. For each channel, principal components were calculated, and spikes were identified via thresholds in the principal component contributions. In order to identify multivariate features explaining potential modifications by EPO in the cell cultures, single sample analyses were performed first (see Additional file 1). Due to a relatively high background noise and a low overall number of spikes in the channels, standard statistics, such as spike rates and spike time interval distributions, did not capture significant differences in EPO versus control cultures (see Additional files 2 and 3). A direct quantification of the variations in bursting and silent channels was therefore necessary. Similar clustering effects have been previously studied in

oscillator networks on a theoretical level [28,29]. Here, two indices were calculated: (1) In order to measure silencing in the groups, we determined the number of channels $c_i(t)$ of dish i at time t with basically no spikes (less than five spikes per 2 min). We then took the mean of $c_i(t)$ over each week and compared the time evolution of this mean channel activity using a Wilcoxon rank sum test in each week. The test was performed on the samples after outlier removal, where an outlier was defined as a sample not lying within 1.5 times the interquartile range from the median. (2) In addition to silencing effects, we analysed bursting behavior. For this, we calculated the coefficient of variation (CV) of the spike-time interval distribution in each channel, i.e. the ratio of standard deviation and mean. This measure of dispersion is larger than 1 for hyper-exponential distributions and lower than 1 for lower-variance distributions. In the case of bursting channels, over-proportionally many small spike-time intervals were observed, so the spike-time intervals obeyed a hyper-exponential distribution, which could be identified by high CV-values of the corresponding channels. We defined bursting behavior if the CV-value was above 1, and strongly bursting behavior if it was above a threshold of 2.6 (see also Additional file 1). In order to quantify bursting over all channels, we counted the percentage $b_i(t)$ of strongly bursting channels of all active channels of dish i at time t. By calculating relative bursting with respect to active channels, we were able to study bursting independent of the number of silent channels. Again, we took the mean over each week, and tested for differing medians of the EPO and the control group using a rank sum test.

Autaptic neuron experiments

Cell culture

Microislands of astrocyte feeder cells were prepared two days before plating hippocampal neurons [30]. Islands of substrate (10 mM acetic acid, 0.1 mg/ml poly-D-lysine, and 0.2 mg/ml collagen) were applied to agarose-coated glass coverslips using a stamp containing regularly spaced squares (200 μm × 200 μm). To obtain astrocytes and hippocampal neurons, P0 mice were decapitated, and brains were removed and cleaned of meninges and vascular tissue. To obtain hippocampal neurons, hippocampi were removed in HBSS, digested in papain (25 IU/ml, Worthington Biomedical) in DMEM (supplemented with 1 mM $CaCl_2$, 0.5 mM EDTA, and 1.65 mM L-cysteine) for 45 min at 37°C, incubated for 15 min at 37°C in serum-free medium (Neurobasal medium A supplemented with 2.5 mg/ml Albumin and 2.5 mg/ml Trypsin inhibitor) and dissociated. To obtain astrocytes, the cortices of separate animals were removed in HBSS, similarly dissociated (digested for 15 min at 37°C in Trypsin/EDTA) and plated at a density of 2500 per cm^2 in DMEM containing 10% fetal calf serum, penicillin/streptomycin, and MITO (Becton Dickinson). Before plating the dissociated hippocampal neurons at a density of 300 per cm^2, the medium of the astrocyte feeder cells was replaced with Neurobasal medium A (supplemented with B27, Glutamax-I and penicillin/streptomycin). Neurons were allowed to mature until days 9, 11, or 14 to be used for electrophysiology or immunocytochemistry. Only islands containing single neurons were examined. EPO versus control (diluent solution) treatment was performed on day 7. If not otherwise indicated, cell culture reagents were obtained from GIBCO/Invitrogen.

Immunostaining

For estimating the number of synaptic boutons in autaptic neurons, cells were washed in PBS, fixed with 4% paraformaldehyde in PBS, permeabilized and blocked in 0.2% Triton X-100/PBS with 10% blocking serum, and incubated at 4°C overnight with mouse monoclonal synapsin1 antibody (1:1000 SynapticSystems, Goettingen, Germany) diluted in 1% blocking serum/PBS. After PBS washes, cells were incubated with Cy3-labeled secondary antibody (1:1000; Jackson ImmunoResearch, Newmarket, UK), washed in PBS and incubated at 4°C overnight with mouse monoclonal MAP-2 (1:500; Chemicon, Hampshire, USA) antibody. Following PBS washes, the cells were incubated with Cy2-labeled secondary antibody (1:250; Jackson ImmunoResearch, Newmarket, UK), washed in PBS, air dried and coverslipped with fluorescence mounting medium (Vector, Burlingame, CA, USA) containing DAPI. Images of individual neurons were captured using an upright epifluorescence Olympus BX61 microscope (Hamburg, Germany) with a 40× oil-immersion objective. Images were photomerged to reconstruct individual neurons using Adobe Photoshop CS3 software. The number of synapsin1 immunoreactive punctae of 18–20 neurons per coverslip (six coverslips per condition) were quantified using ImageJ software with manual thresholding and particle analysis plugin. Estimation of the percentage of excitatory and inhibitory neurons was performed by visual distinction between the degree of arborization, thickness of processes and shape of soma. Amount of inhibitory neurons among the total neuronal population was found to be 10–20% per culture.

Electrophysiology

Cells were whole-cell voltage clamped at -70 mV with pClamp10 amplifier. All analyses were performed using Axograph 4.9 (Molecular Devices, Sunnyvale, CA, USA). The size of the readily releasable pool (RRP) of synaptic vesicles was determined by a 6 s application of the external saline solution made hypertonic by the addition of 0.5 M sucrose. Recordings of mEPSCs were performed in the presence of 300 nM tetrodotoxin (TTX). EPSCs were evoked by depolarizing the cell from -70 to 0 mV for 2 ms. The effect of high-frequency stimulation on the amplitude

of EPSCs was measured by applying depolarisations at a frequency of 10 Hz for 50 stimuli. To measure NMDA/AMPA ratio, EPSCs were stimulated in the presence of 10 mM glycine, 2.5 mM Ca^{2+} (no Mg^{2+}) to activate the synaptic NMDA receptors in hippocampal autaptic culture. The evoked EPSCs had a fast AMPA component followed by a slow NMDA component. To examine the changes in synaptic NMDA/AMPA ratios in presence and absence of EPO, the NMDA components relative to the AMPA component were measured.

Patch-pipette solutions contained (mM): 146 potassium gluconate, 18 HEPES, 1 EGTA, 4.6 $MgCl_2$, 4 NaATP, 0.3 Na_2GTP, 15 creatine phosphate and 5 U/ml phosphocreatine kinase (315–320 mOsmol/l, pH 7.3). The extracellular saline solution contained (mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 $CaCl_2$ and 4 $MgCl_2$ (320 mOsmol/l, pH 7.3). All chemicals, except for TTX (Tocris Cookson) and calcimycin (Calbiochem) were purchased from Sigma. All solutions were applied using a fast-flow system (Warner Instruments, Hamden, CT, USA) with custom made flow pipes.

Protein extraction and immunoblot analysis

Tissue samples or cells were lysed with lysis buffer [50 mM Tris HCL (pH 8.3), 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 1% Igepal, 0.1% Natriumdesoxycholat, 0.1% SDS] containing 1 mM Phenylmethysulfonylfluoride, 10 μg/ml Aprotinin and 1 mg/ml Leupeptin. The lysates were freeze-thawed four times and homogenized by pulling through a 1 ml syringe 10 times, transferred into microcentrifuge tubes and centrifuged (1200 rpm) at 4°C for 45 min. The supernatant was mixed with three volumes of Laemmli buffer [250 mM Tris HCL (pH 8.3), 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.04% pyronin Y], boiled for 5 min at 95°C and frozen at -20°C until blotting. The protein samples were run on NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Karlsruhe, Germany) for 35 min at 200 V and transferred to a nitrocellulose membrane. The blots were blocked with 2% ECL Advance blocking agent (Amersham, Freiburg, Germany) in Tween 20-Tris-buffered saline (TTBS) at room temperature for 1 h and incubated at 4°C overnight with primary antibody for synapsin1 (1:10000; Synaptic Systems, Goettingen, Germany) or synaptophysin (1:500; Sigma, Germany) or α-tubulin as an internal control (1:5000; Sigma, Germany). Immunoreactive bands were visualized by using secondary antibodies coupled to horseradish peroxidase by enhanced chemoluminescence (Amersham, Freiburg, Germany). Densitometric analysis of the protein bands was performed by using ImageJ software.

RNA isolation and expression analysis by quantitative real-time RT-PCR

RNA was isolated from tissue samples or cells by using the RNeasyPlus kit (Qiagen, Hilden, Germany). First strand cDNA was generated from total RNA using N9 random and Oligo(dT)18 primers. The relative concentrations of mRNAs of interest in different cDNA samples were measured out of four replicates using the threshold cycle method (Ct) for each dilution and were normalized to levels of murine 18S RNA. Reactions were performed using SYBR green PCR master mix (ABgene, Foster City, CA, USA) according to the protocol of the manufacturer. Cycling was done for 2 min at 50°C, followed by denaturation at 95°C for 10 min. The amplification was carried out by 45 cycles of 95°C for 15 s and 60°C for 60 s. The specificity of each primer pair was controlled with a melting curve analysis. Quantitative RT-PCR was performed with primers listed below:

NM_007540.3 *Mus musculus brain derived neurotrophic factor (BDNF)*, mRNA

mouse *BDNF* fwd: GCA TCT GTT GGG GAG ACA AG

mouse *BDNF* rev: TGG TCA TCA CTC TTC TCA CCT G

NM_010149.2 *Mus musculus erythropoietin receptor (EPOR)*, mRNA

mouse *EPOR* fwd: CCT CAT CTC GTT GTT GCT GA

mouse *EPOR* rev: CAG GCC AGA TCT TCT GCT G

NM_009305.1 *Mus musculus synaptophysin (Syp)*, mRNA

mouse *synaptophysin* fwd: CAA GGC TAC GGC CAA CAG

mouse *synaptophysin* rev: GGT CTT CGT GGG CTT CAC T

NM_013680.3 *Mus musculus synapsin1 (Syn1)*, mRNA

mouse *synapsin1* fwd: GGA AGG GAT CAC ATT ATT GAG G

mouse *synapsin1* rev: TGC TTG TCT TCA TCC TGG TG

Statistical analysis

Statistical significance was evaluated using two-tailed unpaired Student's *t*-test, with or without Welch's correction, depending on the distribution of the data (tested with a Kolmogorov-Smirnov test). Significance level was set to $P < 0.05$. Numerical values are represented as mean

± S.E.M. in Figures and text. Plotting of the data as well as statistical analyses were done in Prism 4 (GraphPad Software, San Diego, CA, USA) and MATLAB 7 (The MathWorks, Natick, MA, USA).

Authors' contributions

BA carried out the behavioral experiments. DS performed the immunohistochemical analysis and synapse counting. BA and DS participated in writing the manuscript. AS carried out most of the electrophysiological analysis of slice cultures. CG and JSR were involved in preparation and electrophysiology of autaptic cultures. FT and FW performed statistical analysis of MEA cultures. LM, MZ, MM and LS were involved in electrophysiological experiments with slice cultures. IH performed immunohistochemistry. KH was involved in cell culture experiments and western blot analysis. SS carried out mouse brain preparations for immunohistochemistry. KR, AEK and AR were involved in behavioral experiments. NB participated in the design of the study and helped to draft the manuscript. WZ supervised electrophysiology of slices. HE supervised the whole project, designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Analysis of single-sample MEA recordings.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7007-6-37-S1.pdf]

Additional file 2

Mean conditional firing rates for EPO and control samples.
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Additional file 3

Spike-rate (1/s) for EPO-treated and control dishes.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7007-6-37-S3.pdf]

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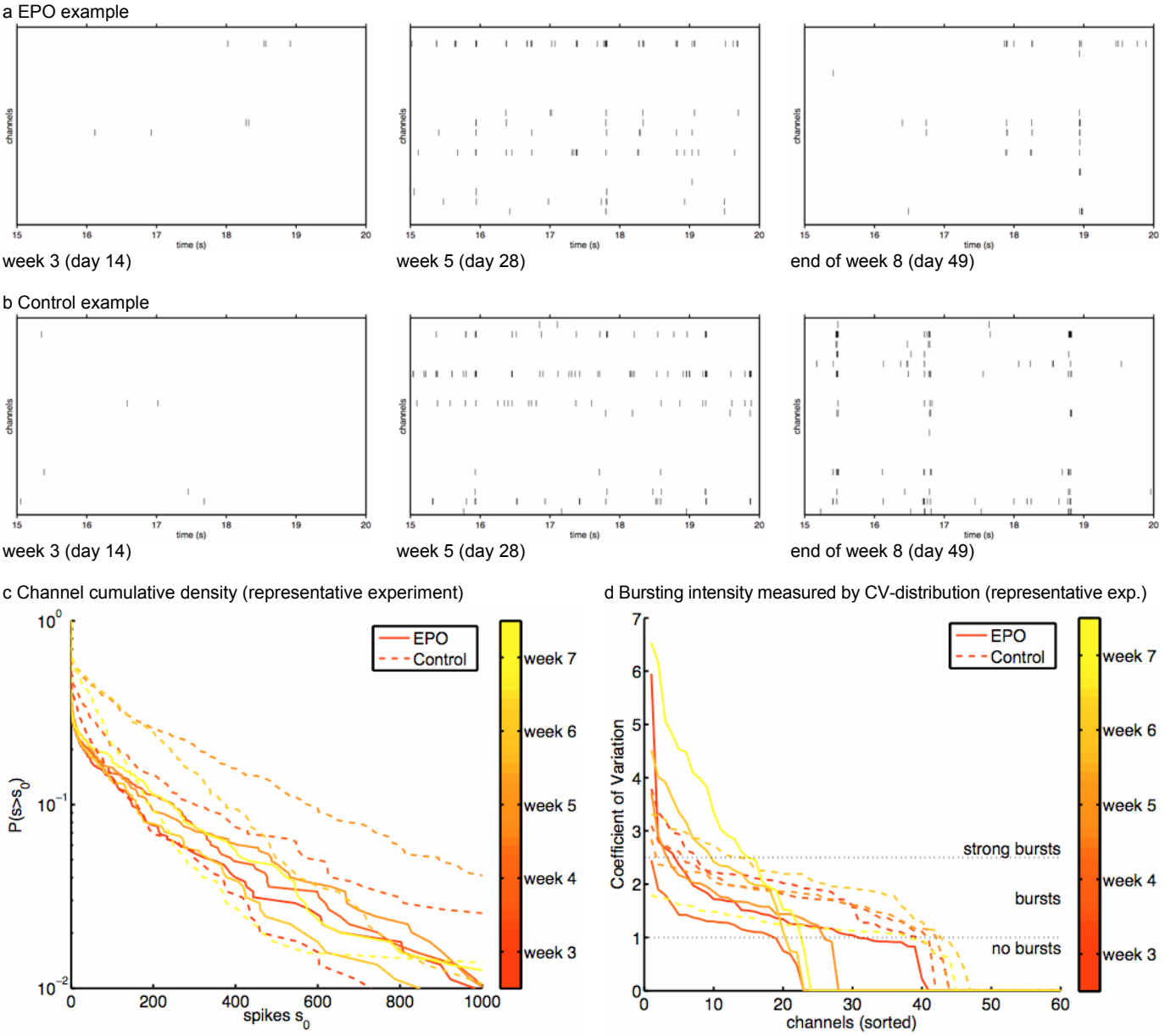
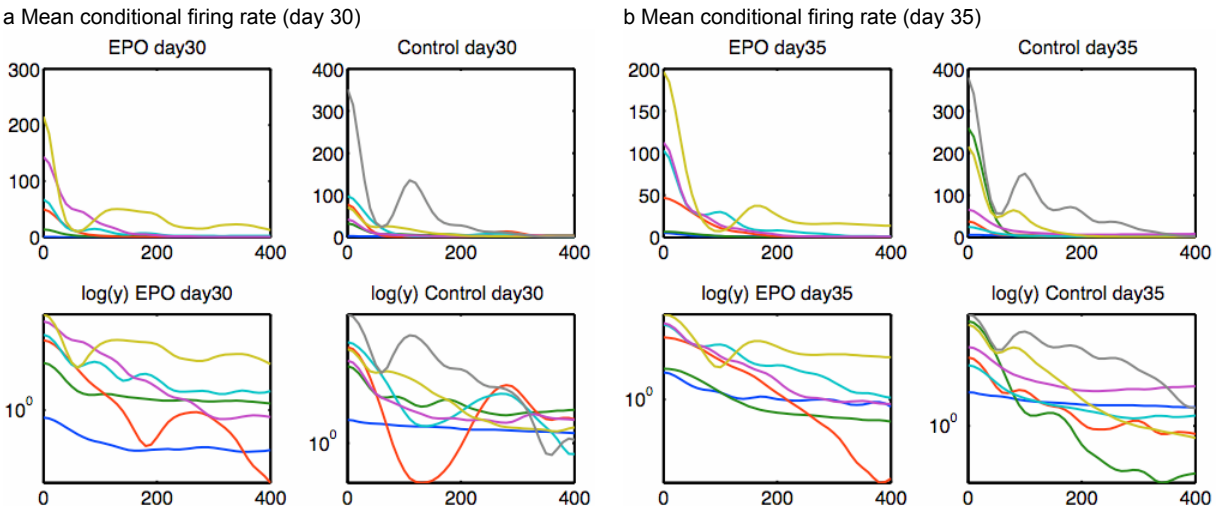
Additional file 1



Single-sample MEA recordings at different days are analysed. (a) shows an EPO sample at the beginning of treatment (week 3), directly after treatment (week 5) and at the end of the recording (week 8). Only the central 16 channels out of the total 60 channels are shown (electrodes are positioned on an 8x8 grid except for the 4 vertices, see Figure 4c). When compared with the control (b), channel activity is lower, with at the same time higher bursting behavior. This is illustrated for the same two samples in (c), where the channel cumulative density at the different 5 weeks is shown for both EPO and control; the lower onset of EPO can be numerically captured by counting the number of silent channels (compare Fig 4d). Similarly, considering bursting behavior in each channel, accumulated over each week, shows that EPO leads to more strongly bursting channels than the control at later weeks (d), which is confirmed by the group statistics (compare Fig 4e).

Additional file 2



Mean conditional firing rates between two different channels *i* and *j* after taking the mean over *i* and *j* at different lags 0 to 400ms; calculation was performed using a Gaussian kernel at each spike with standard deviation 10ms. We show the firing rates for the different samples (indicated by different colors) for both EPO and control at day 30 (a) and at day 35 (b). Using a logarithmic y-axis (lower subfigures), a tendency towards "bumpier" behavior in case of the control might indicate longer-range autocorrelations of the control versus EPO, however significant differences and time-shifts could not be identified.

Additional file 3

Spike-rate (1/s)

	week 3	week 4	week 5	week 6	week 7
EPO	42.5 ± 43.7	58.5 ± 49.1	65.3 ± 38.7	50.2 ± 17.5	49.9 ± 20.5
Control	43.3 ± 33.5	73.1 ± 19.8	83.8 ± 43.7	92.1 ± 44.2	81.9 ± 54.8

Spike-rate (1/s) for EPO-treated and control dishes when compared over a whole week. No significant differences were found.

5. Effect of hypoxia inducible factor stabilization on hippocampus dependent memory

5.1 Overview of project III

Hypoxia inducible factor (HIF) is a heterodimer of HIF-α and HIF-β subunits, belonging to the PAS (Per, Arnt, Sim) family of basic helix-loop-helix transcription factors. The expression of the HIF-β subunit is constitutive, whereas the expression and stability of the HIF-α subunit is precisely and negatively regulated by cellular oxygen levels that also determine the activity of HIF prolylhydroxylases, a family of iron and 2-oxoglutarate-dependent dioxygenases (for review see: Semenza, 2000; Semenza, 2004).

In normoxia (21% oxygen levels), HIF-α subunit is hydroxylated by prolylhydroxylases (PHD1, 2 and 3) in the presence of O^2, Fe^{2+}, 2-oxoglutarate (2-OG) and ascorbate. Hydroxylated HIF-α is recognised by Von Hippel-Lindau tumor suppressor protein (VHL), which, together with a multisubunit ubiquitin ligase complex, tags HIF-1α with polyubiquitin. This allows recognition by the proteasome and subsequent degradation (for review see: Semenza, 2000; Semenza, 2004; Poon et al., 2009). This process is extremely rapid. For example, in isolated, perfused, and ventilated lung preparation subjected to hypoxia and reoxygenation, the half-life of HIF-1α subunit was less than 1 min (Yu et al., 1998).

During hypoxia prolyl hydroxylation is inhibited and VHL is no longer able to bind and target HIF-α for proteasomal degradation, which leads to HIF-α accumulation and translocation to the nucleus. There HIF-α dimerises with HIF-1β and binds to hypoxia-response elements (HREs) within the promoters of target genes (Wang et al., 1995). There are over 70 genes known to be activated at the

transcriptional level by HIF, including EPO, vascular endothelial growth factor (VEGF), transferrin, and enolase (Semenza, 2004; Siddiq et al., 2005).

Currently, three different HIF-α subunits as well as three different HIF-β subunits are known. HIF-1α and HIF-1β mRNA are expressed in most, if not all, human and rodent tissues. In contrast, HIF-2α, HIF-3α, HIF-2β, and HIF-3β show a more cell-type specific expression (Semenza, 2000). Also the biological role of each of the subunits can be different. For example, during development, HIF-1α and HIF-2α were demonstrated to have nonoverlapping functions and various studies have shown that HIF-1α and HIF-2α can regulate both overlapping and distinct target genes (Poon et al., 2009).

In the project III we aimed to investigate whether stimulation of endogenous EPO production by stabilization of HIF-1α would lead to similar enhancement in hippocampus dependent memory that was observed upon exogenous EPO (project II). For stabilization of HIF-1α we used FG-4497, a HIF prolyl hydroxylase inhibitor that was synthesized at FibroGen (San Francisco, CA, USA) and has been recently shown *in vitro* to enhance proliferation, neurogenesis and dopaminergic differentiation of human mesencephalic neural progenitor cells (Milosevic et al., 2009) and *in vivo* to enhance endogenous EPO plasma levels in normal mice (Hsieh et al., 2007) as well as increase HIF dependent transcription in neonatal mouse brain (Schneider et al., 2009).
First, before starting the investigation of FG-4497 effects on cognitive performance, we intended to evaluate direct effects of FG-4497 on brain. For that, mice were injected with FG-4497 and 6h later hippocampi were taken out. We found that FG-4497 treatment was indeed proficient to stabilize HIF-1α subunit, which was indicated by an increase in HIF-1α protein concentration (Western blot). Moreover, quantitative RT-PCR of hippocampal extracts revealed elevated expression of EPO and VEGF mRNA upon FG-4497, genes that are known to be targeted by HIF. Additionally, in order to see whether observed changes in gene expression would be attributable to neurons, primary

hippocampal neuronal cultures were grown and upon maturation (day 8 in culture), also exposed for 6h to FG-4497. Similarly to the *in vivo* study, we found that cell culture FG-4497 treatment led to upregulation of EPO and VEGF mRNA.

Finally, after proving the ability of FG-4497 to stabilize HIF-1α subunit, we performed experiments that aimed to investigate the effects of FG-4497 on cognitive performance in healthy, young mice. For that we applied analogous experimental schedule like in project II but instead of EPO injections mice received FG-4497 (60 mg/kg). In the same way to EPO study, three independent groups of mice were examined in the fear conditioning test at either 1, 3 or 4 weeks after termination of FG-4497 treatment. Additionally, before fear conditioning training, tests for basic behavior were performed. They included: elevated plus maze (anxiety), open field (*s*pontaneous activity), hole-board (exploratory activity) and rota-rod (motor function, balance and coordination).

We have found that FG-4497 treated mice had improved contextual memory in fear conditioning test, when measured 3 weeks after the last injection, without a change in hematocrit. This effect was persisting for 4 weeks after the termination of treatment. No improvement in memory was detected at 1 week when the hematocrit was increased, indicating that cognitive improvement and hematocrit are not directly related. In addition, we observed slight sedative effects of FG-4497 indicated by reduced spontaneous activity in open field test and reduced exploratory activity in hole-board test (2 and 3 days after the last injections respectively). These effects were gone at later testing time points.

5.2 Original publication

Adamcio B, Sperling S, Hagemeyer N, Walkinshaw G, and Ehrenreich H. Hypoxia inducible factor stabilization leads to lasting improvement of hippocampal memory in healthy mice. ***(submitted)***

Personal contribution:

I was involved in the design of the study, interpretation of results and preparation of the manuscript. I have also performed all behavioral experiments. I was also involved in brain dissection as well as in performing western blot and qRT-PCR.

Hypoxia inducible factor stabilization leads to lasting improvement of hippocampal memory in healthy mice

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Abstract

Hypoxia-inducible factors (HIFs) are transcription factors involved in the cellular response to low oxygen, including upregulation of transcripts like vascular endothelial growth factor (VEGF) and erythropoietin (EPO). Under normal oxygen, prolylhydroxylases decrease HIF-α stability. This is banned by prolylhydroxylase inhibitors, which prevent oxygen dependent degradation and thus prolong HIF-α half-life. We investigated whether upregulation of endogenous EPO in brain by HIF stabilization would increase hippocampal memory similar to exogenous EPO. Healthy male 28 day-old mice received FG-4497, a HIF prolylhydroxylase inhibitor, or placebo intraperitoneally every other day for 3 weeks. Behavioral testing and hematocrit determinations were conducted in independent cohorts at 1, 3, or 4 weeks after treatment completion. Increased EPO and VEGF mRNA expression in hippocampus or primary hippocampal neurons 6h after application of FG-4497 confirmed its ability to stabilize HIF and upregulate HIF dependent transcription in brain. At 3 and 4 weeks after the last injection, respectively, FG-4497 treated mice compared to placebo mice had improved hippocampal memory in fear conditioning without a change in hematocrit. In contrast, no improvement in memory was detected at 1 week when the hematocrit was increased, indicating that cognitive improvement and hematocrit are not directly related. FG-4497 application for 3 weeks leads to delayed but lasting enhancement of hippocampal memory, making HIF stabilization an attractive target for pharmacological manipulation of cognition.

Key words: Fear conditioning test, erythropoietin, EPO, VEGF, FG-4497.

Introduction

The production of erythropoietin (EPO) is increased in low oxygen conditions not only in kidney and fetal liver but also in other tissues, particularly the nervous system. EPO and its receptor, EPOR, are strongly expressed in the developing brain where they appear – among others – to be involved in the regulation of physiological apoptosis and angiogenesis. In adult brain, EPO/EPOR expression is low but is rapidly upregulated upon metabolic distress, ranging from hypoxia/ischemia and inflammation to neurodegeneration, to provide an endogenous *"neuroprotective stand-by system"*. A certain baseline expression of EPO/EPOR in the postnatal brain, however, appears to contribute to cognitive functions even under physiological conditions [2,4,9,11,20].

High doses of peripherally applied EPO were not only found to improve cognition in human diseases like schizophrenia and multiple sclerosis [5,6], but also to lastingly enhance hippocampal longterm potentiation and memory [1] as well as discrete learning processes including stability of cognitive performance in healthy mice [7]. We wondered whether, analogous to peripheral application of high EPO doses, stimulation of endogenous EPO production in brain would improve cognition. To approach this question, we used a prolylhydroxylase inhibitor, FG-4497, which stabilizes hypoxia inducible factor (HIF), a transcriptional regulator of hypoxia-induced EPO expression [for review see: 17,18].

HIF is a heterodimer of HIF-α and HIF-β subunits, belonging to the PAS (Per, Arnt, Sim) family of basic helix-loop-helix transcription factors. The expression of the HIF-β subunit is constitutive, whereas the expression and stability of the HIF-α subunit is negatively regulated by oxygen levels that also determine the activity of HIF prolylhydroxylases, a family of iron and 2-oxoglutarate-dependent dioxygenases. The HIF-α and HIF-β heterodimer translocates to the nucleus where it binds to hypoxia-responsive element motifs and induces transcription of genes that counteract the effects of hypoxia, e.g. EPO, vascular endothelial growth factor (VEGF), transferrin, and enolase [17,19]. Currently, three different

HIF-α subunits as well as three different HIF-β subunits are known. The most widely expressed α-subunit in the mammalian organism is HIF1-α whereas the other HIF-α subunits appear to have more tissue specific functions [16].

The present study has been designed to explore the effect of longterm HIF stabilization on cognitive performance in mice. Thus, the HIF prolylhydroxylase inhibitor FG-4497 was employed, which has previously been shown *in vitro* to enhance proliferation, neurogenesis and dopaminergic differentiation of human mesencephalic neural progenitor cells [12] and *in vivo* to augment endogenous EPO plasma levels in normal mice [8] as well as increase HIF dependent transcription in neonatal mouse brain [15]. We show here that FG-4497 leads to lasting improvement in hippocampal memory.

Materials and Methods

Animals. All experiments had been approved by the local Animal Care and Use Committee. Young (28 day-old) C57BL/6NCrl male mice (Charles River Laboratories, Sulzfeld, Germany) were housed in groups of 5 in standard plastic cages and maintained in a temperature-controlled environment (21±2°C) on a 12h light/dark cycle with food and water available ad libitum.

Drug treatment. To test the effects of HIF stabilization on basic behavior and cognition, a total of 90 mice were injected intra-peritoneal with FG-4497 (60mg/kg, FibroGen, San Francisco, CA, USA) or placebo (diluent of FG-4497) every other day for 3 weeks (11 injections in total). An additional group of 20 mice was used to obtain brain tissue for extraction of RNA or protein at 6h after injection of either placebo or FG-4497.

Experimental design and behavioral testing. Three independent groups of mice were examined in the fear conditioning test at either 1, 3 or 4 weeks after termination of FG-4497 treatment. A comprehensive test description is given in

our previous paper [1]. In brief, mice were trained within the same session for both contextual and cued fear conditioning. After a 120s period, in which baseline freezing was assessed, mice received 2 paired presentations of a 10s, 5kHz, 85dB tone (conditioned stimulus, CS) and a 2s, 0.4mA foot shock (unconditioned stimulus, US). The contextual memory was assessed 72h after the training. Mice were monitored over 2min for freezing in the same conditioning chamber. Four hours later, mice were placed in a new chamber and re-exposed to the tone for cued memory assessment.

Basic behavior tests were always performed before fear conditioning training and included (always in the same order): elevated plus maze (anxiety, 1 day), open field (spontaneous activity, 1 day), hole-board (exploratory activity, 1 day) and rota-rod (motor function, balance and coordination, 1 day). All basic behavioral tests were also performed as described in detail previously [1]. Hematocrit was measured in all groups immediately after fear conditioning.

Brain dissection and hippocampal neuron cultures. To evaluate direct effects of FG-4497 on brain, mice were deeply anaesthetized and decapitated 6h after FG-4497 injection. Hippocampi were taken out, immediately frozen on dry ice and stored at -80˚C until analyzed. To see whether changes in gene expression, detectable in whole hippocampus, would be attributable to neuronal expression, primary hippocampal neuronal cultures were grown as described in detail previously [1], and upon maturation (day 8 in culture), also exposed for 6h to FG-4497 (3μM).

Protein extraction and immunoblot analysis. Tissue samples were lysed with lysis buffer [50mM Tris HCL (pH 8.3), 150mM NaCl, 40mM NaF, 5mM EDTA, 5mM EGTA, 1mM Na_3VO_4, 1% Igepal, 0.1% natriumdesoxycholate, 0.1% SDS], containing 1mM phenylmethysulfonylfluoride, 10μg/ml aprotinin and 1mg/ml leupeptin. The lysates were freeze-thawed 4 times and homogenized by pulling through a 1ml syringe 10 times, transferred into microcentrifuge tubes and

centrifuged (1200rpm) at 4˚C for 45min. The supernatant was mixed with 3 volumes of Laemmli buffer [250mM Tris HCL (pH 8.3), 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.04% pyronin Y], boiled for 5min at 95˚C and frozen at -80˚C until blotting. The protein samples were run on NuPAGE 3-8% Tris-Acetat Gel (Invitrogen, Karlsruhe, Germany) for 60min at 150V and transferred to a nitrocellulose membrane. The blots were blocked with 5% non fat dry milk in Tween 20-Tris-buffered saline (TTBS) at room temperature for 1h and incubated at 4ºC overnight with primary antibody for HIF1α (1:1000; Novus Biologicals, Littleton, USA). Immunoreactive bands were visualized by using secondary antibodies coupled to horseradish peroxidase by enhanced chemoluminescence (Amersham, Freiburg, Germany). Densitometric analysis of the protein bands was performed by using ImageJ software.

RNA isolation and expression analysis by quantitative real-time RT-PCR. RNA was isolated from whole hippocampus or hippocampal neuron culture samples by using the RNeasyPlus kit (Qiagen, Hilden, Germany). First strand cDNA was generated from total RNA using N9 random and Oligo(dT)18 primers. The relative concentrations of mRNAs of interest in different cDNA samples were measured out of 4 replicates using the threshold cycle method (Ct) for each dilution and were normalized to levels of murine 18S RNA. Reactions were performed using SYBR green PCR master mix (ABgene, Foster City, CA, USA) according to the protocol of the manufacturer. Cycling was done for 2min at 50°C, followed by denaturation at 95°C for 10min. The amplification was carried out by 45 cycles of 95°C for 15s and 60°C for 60s. The specificity of each primer pair was controlled with a melting curve analysis. Quantitative RT-PCR was performed with primers listed below:

HIF1 alpha, fwd: CAT GAT GGC TCC CTT TTT CA

HIF1 alpha, rev: GTC ACC TGG TTG CTG CAA TA

EPO, fwd: AAG GTC CCA GAC TGA GTG AAA ATA TTA C

EPO, rev: GGA CAG GCC TTG CCA AAC T

VEGF, fwd: AGC ACA GCA GAT GTG AAT GC

VEGF, rev: TTG ACC CTT TCC CTT TCC TC
GAPDH, fwd: CAA TGA ATA CGG CTA CAG CAA C
GAPDH, rev: TTA CTC CTT GGA GGC CAT GT
Beta-actin, fwd: CTT CCT CCC TGG AGA AGA GC
Beta-actin, rev: ATG CCA CAG GAT TCC ATA CC

Statistical analysis. Statistical significance was evaluated using 2-tailed unpaired t-test and two-way ANOVA. Significance level was set to $p<0.05$. Numerical values are presented as mean±S.E.M. in Figures and text. Plotting of the data and statistical analyses was done in Prism 4 (GraphPad Software, San Diego, CA, USA).

Results

FG-4497 increases HIF-1α protein concentration and elevates expression of EPO and VEGF genes in hippocampal tissue and in primary neurons

At 6 hours after injection of the HIF prolylhydroxylase inhibitor, FG-4497, or placebo, whole hippocampus was extracted to determine HIF-1α mRNA and protein concentrations. Whereas the former was (expectedly) unaltered, the latter showed a significant increase after FG-4497 treatment as compared to placebo (Figure 1A,B). Moreover, quantitative RT-PCR revealed elevated expression of EPO and VEGF mRNA upon FG-4497 both in hippocampus extracts and in primary hippocampal neurons (Figure 1C-F). In contrast, EPOR mRNA expression at 6h was unchanged by FG-4497 (placebo: 1.48±0.06, N=10; FG-4497: 1.40±0.08, N=9; p=0.46).

FG-4497 improves hippocampus dependent memory in healthy young mice

The intraperitoneal injections every other day over 3 weeks were well tolerated and did not lead to any obvious side effects. Body weight development, evaluated over 11 time points, in FG-4497 treated mice did not differ from placebo controls (two-way ANOVA: effect of treatment $F_{1,810}=1.45$, p=0.23; body

weight at the end of treatment in the placebo group: 21.9±0.2g, N=42; in the FG-4497 group: 21.4±0.2g, N=41; p=0.09). No effect on memory function in fear conditioning was detected at 1 week after cessation of treatment (Figure 2A). At 3 weeks after the last injection, however, FG-4497 treated mice showed clear improvement of contextual memory in fear conditioning (Figure 2B). This effect was still measurable 4 weeks after termination of FG-4497 applications (Figure 2C). Cued memory was not influenced by FG-4497 at any of the 3 time points tested (Figure 2A-C; all p>0.05).

Effects of FG-4497 on hematocrit

Whereas at 1 week after termination of injections, hematocrit was increased in FG-4497 versus placebo treated mice (Figure 2A), there was no difference anymore at 3 and 4 weeks (Figure 2B, 2C), indicating that cognitive improvement and hematopoietic effects of EPO are not directly related.

Effects of FG-4497 on basic behavioral readouts

There was no effect of FG-4497 on anxiety and motor performance (Figure 2A-C, all p>0.05). However, FG-4497 mice displayed significantly reduced spontaneous activity in the open field and diminished exploratory behavior in the hole-board test when measured within the first week, i.e. 2 and 3 days after termination of treatment, respectively (Figure 2A). These slight sedative effects of FG-4497 were no longer measurable at the 2 later testing time points, 3 and 4 weeks after treatment cessation (Figure 2B, 2C).

Discussion

Longterm treatment of healthy young mice with the HIF prolylhydroxylase inhibitor, FG-4497, led to selective improvement of contextual (hippocampal) memory in the fear conditioning test at 3 weeks after the last injection. As with EPO [1], there was no alteration of cued (hippocampus-independent) memory. The effect of FG-4497 on hippocampal memory occurred somewhat delayed

when compared to the effect of EPO, which was measurable at 1 week after termination of treatment [1]. However, the effect of FG-4497 seemed more pronounced than that of EPO and persisted for at least 4 weeks.

Regarding the time course of hematocrit changes, the effect of FG-4497 was identical to that of EPO, with an increase only measurable at 1 week after termination of treatment but no longer at 3 and 4 weeks. The magnitude of hematocrit increase, however, was less with FG-4497 treatment when compared to EPO (122.2±2.4%, N=15, after FG-4497 versus 146.0±3.7%, N=10, after EPO according to Adamcio and colleagues [1], with the respective mean control value expressed as 100%; $p<0.0001$). This perhaps indicates that lower plasma EPO levels are obtained upon HIF stabilization when compared to high-dose EPO injections.

Lower EPO levels may actually help explain the delay in cognitive enhancement seen with FG-4497 when compared to EPO [1], even though several other mechanisms may play a role. Recently, we reported that treatment with EPO enhances hippocampal longterm potentiation and memory in young mice by modulating neuronal plasticity, synaptic connectivity and activity of memory-related neuronal networks [1]. It is therefore reasonable to assume that the effects of FG-4497 on cognition may be mediated, at least in part, by stimulation of endogenous EPO production. Indeed, upregulation of EPO gene expression by FG-4497 was observed in hippocampus extracts as well as in hippocampal neurons, proving neuronal EPO stimulation. However, in parallel, FG-4497 augmented VEGF expression (and potentially other HIF dependent gene transcripts not tested) which may have contributed to the cognitive effects of HIF prolylhydroxylase inhibition. VEGF in hippocampus seems to be involved in cognitive performance [3,13] and plasticity [10], and transgenic mice with neuronal overexpression of human VEGF show faster spatial learning, better memory, and increased numbers of neuronal precursor cells [14]. Thus, it is well possible that endogenous EPO and VEGF (possibly together with other gene

products induced by HIF stabilization) all interacted to mediate the powerful effect of FG-4497 on hippocampal memory.

The temporarily reduced spontaneous activity in open field and hole-board (2-3 days after the last injection) observed in FG-4497 treated mice is still unexplained and points to a slight and transient sedative effect of the HIF prolylhydroxylase inhibitor. One possible mechanism to explain this phenomenon could be a transitory imbalance in striatal dopamine turnover. In fact, expression of tyrosine hydroxylase, a key enzyme of dopamine synthesis, is stimulated by HIF. Treatment of rats with another prolylhydroxylase inhibitor, FG-0041, increased extracellular dopamine levels in striatum, resulting in enhanced locomotor activity upon cocaine challenge [21]. If a similar increase in dopamine production, causing short-lived hyperactivity, had occurred in our mice upon FG-4497 injections, the observed hypoactivity at 2 and 3 days after treatment cessation could be interpreted as a rebound effect.

Even though a number of questions still remain unanswered, in particular the relative contribution of the various different gene transcripts stimulated upon HIF stabilization, we conclude that HIF prolylhydroxylase inhibition may be an important new target for pharmacological manipulation of cognition, leading to even more pronounced and longer lasting effects as compared to EPO.

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Figure legends

Figure 1: Effect of prolylhydroxylase inhibition induced HIF stabilization on hippocampal gene expression. Application of the HIF prolylhydroxylase inhibitor, FG-4497 (60mg/kg body weight), to healthy young mice did not change HIF-1α mRNA expression **(A)** but led to pronounced increase in HIF-1α protein in hippocampal extracts **(B)** at 6h after intraperitoneal injection. Both EPO **(C)** and VEGF-A **(D)** gene transcripts were significantly elevated upon FG-4497 in hippocampus. The same gene transcripts were found elevated in primary hippocampal neurons in culture 6h after addition of FG-4497 **(E,F).** Mean±SEM presented. N=6-10 per condition.

Figure 2: Effect of the HIF prolylhydroxylase inhibitor, FG-4497, on behavior and hematocrit of healthy young mice. Mice were tested at 1, 2, or 3 weeks after a 3-week treatment with FG-4497 (60mg/kg every other day intraperitoneal). While at 1 week **(A)**, fear conditioning results did not differ among experimental groups, there was a significant reduction in activity both in open field and hole-board, and an increase in hematocrit of mice treated with FG-4497 as compared to placebo. Rotarod as readout of motor performance was unaltered. At 3 and 4 weeks **(B,C),** respectively, an improvement in hippocampal memory (context in fear conditioning) remained the only significant difference between groups. Mean±SEM presented. N=14-15 per condition.

Figure 1

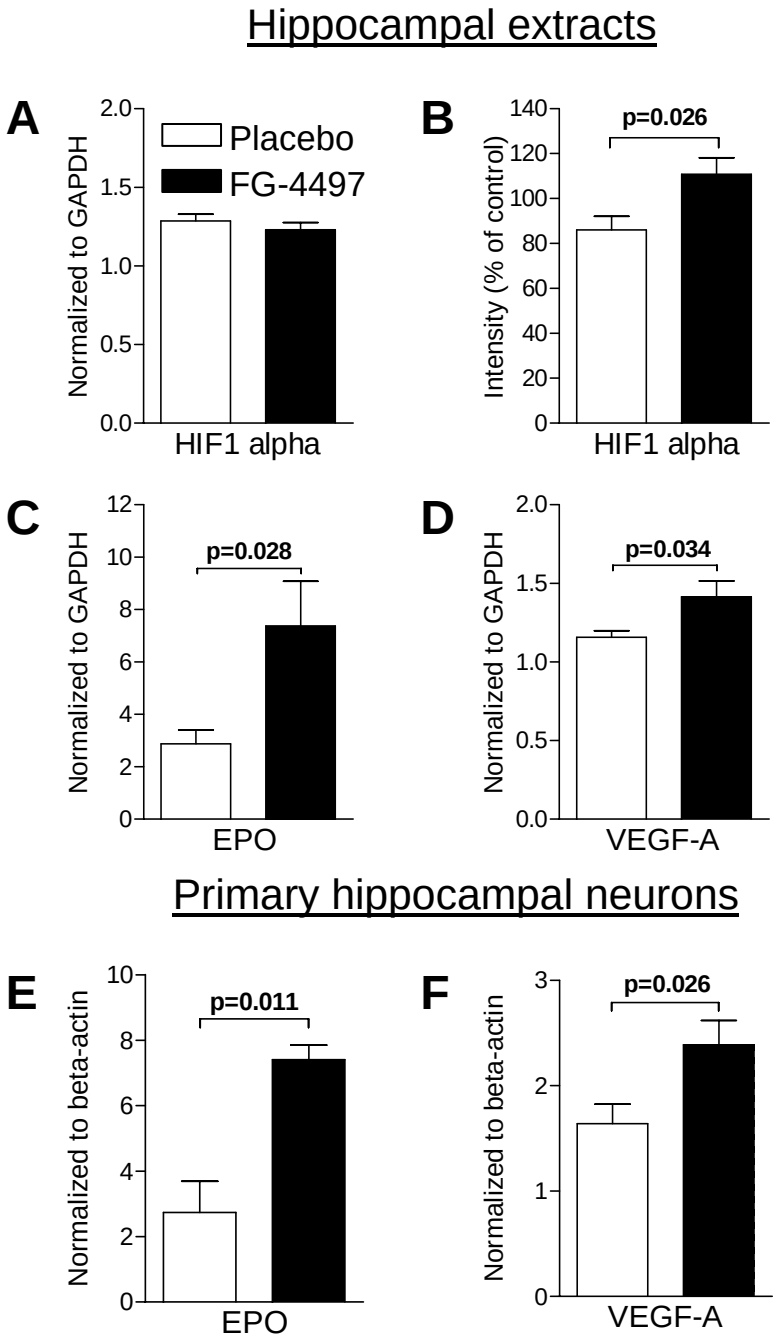
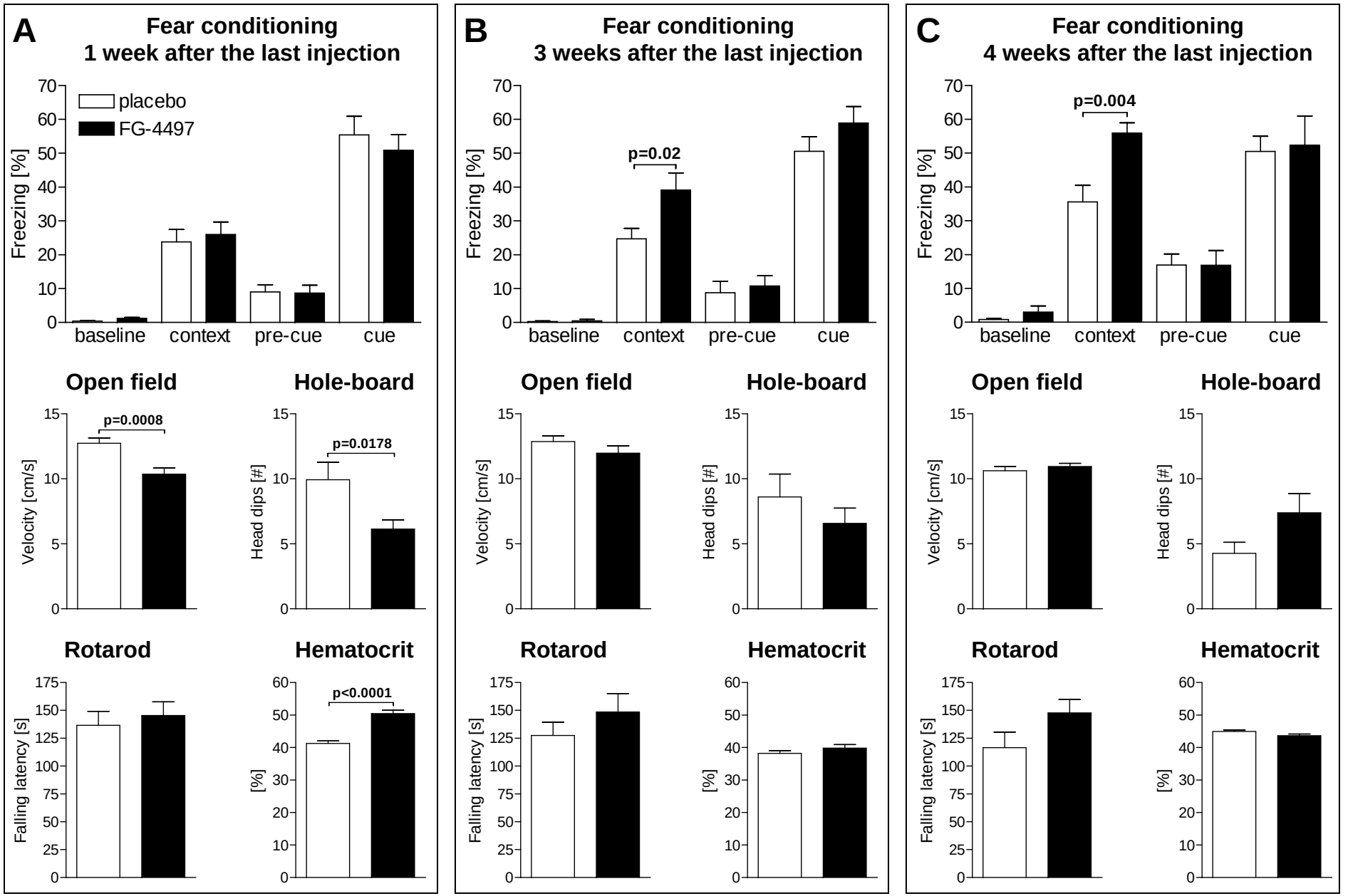

Hippocampal extracts
A
Placebo
FG-4497
Normalized to GAPDH
HIF1 alpha
B
p=0.026
Intensity (% of control)
HIF1 alpha
C
p=0.028
Normalized to GAPDH
EPO
D
p=0.034
Normalized to GAPDH
VEGF-A
Primary hippocampal neurons
E
p=0.011
Normalized to beta-actin
EPO
F
p=0.026
Normalized to beta-actin
VEGF-A

Figure 2


A
Fear conditioning
1 week after the last injection
placebo
FG-4497
Freezing [%]
baseline
context
pre-cue
cue
Open field
Velocity [cm/s]
p=0.0008
Hole-board
Head dips [#]
p=0.0178
Rotarod
Falling latency [s]
Hematocrit
[%]
p<0.0001
B
Fear conditioning
3 weeks after the last injection
p=0.02
C
Fear conditioning
4 weeks after the last injection
p=0.004

6. Summary

In the first original publication, we investigated late consequences of chronic psychosocial stress on pre-pulse inhibition of startle response in a mouse model and explored a potential modifying influence of social deprivation versus social support.

Using the resident-intruder paradigm, 28 days old C57Bl/6NCrl mice, housed individually or in groups, were subjected daily for 3 weeks to social defeat. Ten weeks after the termination of stress procedure we performed tests measuring the most important basic behavioral readouts, including: open field (spontaneous activity), hole-board test (exploratory activity) and rota-rod (motor function, balance and coordination). Next, in order to examine potential influence of stress on sensorimotor gating deficits we performed pre-pulse inhibition test.

We found, similarly to other studies, that chronic psychosocial stress led to temporal decrease in body weight gain in both housing conditions. At the time of behavioral testing this effect had become smaller. Ten weeks after the termination of stress procedure, neither stress nor type of housing had caused any effects on mice spontaneous activity. In contrast, both stress and individual housing increased the anxiety level, indicated by reduced time spent in the central zone of the open field. Additionally, we found that individual housing, independently on stress, significantly reduced exploratory behavior in the hole-board test. Taking into account that hole-board test is also believed to reflect anxiolytic/anxiogenic states in mice (Takeda et al., 1998) these data suggest that social deprivation by itself can be a stressor and ultimately alter emotionality.

Most importantly, we found that in individually housed mice chronic psychosocial stress led to significant decrease in pre-pulse inhibition of startle response. In contrast, this effect of stress was not measurable in mice that were housed in groups.

In summary, this study indicated (1) that there are additive effects of psychosocial stress and single housing (a stressor in itself) on anxiety, (2) that there are effects of single housing on exploratory activity, with no additional effect caused by psychosocial stress, and (3) that only a combination of psychosocial stress and individual housing causes persistent impairment in basic information processing/neuronal network function in adulthood (PPI). These findings, when translated to man, underline the importance of immediate and continuous social support for preventing downstream damage after psychotrauma.

In the second original publication, we investigated the mechanism of EPO action on cognitive performance in healthy mice. For this we developed a mouse model of a robust cognitive improvement by EPO.

We have shown here that a 3-week treatment of young mice with EPO leads to significant improvement of hippocampus-associated memory in fear conditioning test 1 week after the cessation of treatment. Interestingly, this effect was still measurable 3 weeks after the last EPO injection and was independent of changes in hematocrit.

In order to examine the possible influence of EPO on synaptic plasticity we performed electrophysiological measurements in hippocampal slices obtained from mice at the time point of EPO-induced enhancement of memory (1 week after the termination of treatment). We have found that EPO treatment resulted in increased long-term potentiation (LTP), a cellular correlate of learning processes, in the CA1 region of the hippocampus. Additionally, EPO enhanced short term potentiation (STP), short term depression (STD) and paired-pulse facilitation. Next, performing whole-cell patchclamp recordings on CA1 pyramidal neurons, we were able to show that EPO increases the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) and decreases frequency of spontaneous excitatory postsynaptic currents (sEPSCs).

Moreover, using multi-electrode arrays (MEA), we examined network activity in primary hippocampal cultures. MEA recordings confirmed the direct effects of EPO on neural cells and have shown that EPO treatment leads to lower overall spiking activity but enhanced bursting activity. These results are in agreement with our *in vivo* data, which showed that EPO decreases excitatory synaptic transmission and increases inhibitory synaptic transmission.

Finally, electrophysiological and immunocytochemical analyses of individual autaptic hippocampal neurons have shown that EPO leads to a reduction in the amount of primed vesicles without altering number of synapses or efficiency of vesicle fusion and vesicle dynamics.

In summary, this study showed that treatment with EPO improves hippocampus dependent memory. The results suggest that this cognitive improvement is caused by neuroplastic changes that may be based on a more efficient bursting activity of selected synapses together with persistent silencing of other synapses. In order to develop new treatment strategies for neuropsychiatric disorders these mechanisms of action of EPO have to be further investigated.

In the third original publication, we investigated whether upregulation of endogenous EPO in the brain by HIF stabilization would increase hippocampal memory similar to exogenous EPO. For stabilization of HIF-1α we used FG-4497, a HIF prolylhydroxylase inhibitor.

First, in order to confirm FG-4497 ability to stabilize HIF and upregulate HIF dependent transcription in brain, we injected mice with FG-4497 and dissected their hippocampi 6h later. We found that FG-4497 treatment was indeed proficient to stabilize HIF-1α subunit, which was indicated by an increase in HIF-1α protein concentration (Western blot). In addition, quantitative RT-PCR of hippocampal extracts revealed elevated expression of EPO and VEGF mRNA upon FG-4497. These findings were then replicated in primary hippocampal

neuronal cultures indicating that the effects of FG-4497 on gene expression are also attributable to neurons.

To test the effects of HIF stabilization on cognitive performance, healthy male 28 day-old mice were injected intraperitoneally with FG-4497 or Placebo every other day for 3 weeks. Similarly to the EPO study, three independent groups of mice were examined in the fear conditioning test at either 1, 3 or 4 weeks after termination of FG-4497 treatment We found that 3 and 4 weeks after the last injection, respectively, FG-4497 treated mice compared to Placebo mice had improved hippocampal memory in fear conditioning without a change in hematocrit. In contrast, no improvement in memory was detected at 1 week when the hematocrit was increased, indicating that cognitive improvement and hematocrit are not directly related.

In summary, this study indicated that FG-4497 application for 3 weeks leads to delayed (compared to exogenous EPO treatment) but lasting enhancement of hippocampal memory, making HIF stabilization an attractive target for pharmacological manipulation of cognition.

7. Literature

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8. List of publications

1) **Adamcio B**, Sperling S, Hagemeyer N, Walkinshaw G, and Ehrenreich H. Hypoxia inducible factor stabilization leads to lasting improvement of hippocampal memory in healthy mice. ***(submitted)***

2) **Adamcio B**, Havemann-Reinecke U and Ehrenreich H. Chronic psychosocial stress in the absence of social support induces pathological pre-pulse inhibition in mice. ***Behav Brain Res. 2009 Dec 1;204(1):246-9.***

3) **Adamcio B*,** Sargin D*, Stradomska A*, Medrihan L, Gertler C, Theis F, Zhang M, Müller M, Hassouna I, Hannke K, Sperling S, Radyushkin K, el-Kordi A, Schulze L, Ronnenberg A, Wolf F, Brose N, Rhee JS, Zhang W, Ehrenreich H. Erythropoietin enhances hippocampal long-term potentiation and memory. ***BMC Biol. 2008 Sep 9;6:37.***

* Indicates equal contribution for the publications

4) Bespalov A, Dravolina O, Belozertseva I, **Adamcio B**, Zvartau E. Lowered brain stimulation reward thresholds in rats treated with a combination of caffeine and N-methyl-D-aspartate but not alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate or metabotropic glutamate receptor-5 receptor antagonists. ***Behav Pharmacol. 2006 Jun;17(4):295-302.***

Book chapter:

5) El-Kordi A, Radyushkin K, **Adamcio B** and Ehrenreich H. Rodent models of schizophrenia: Past, present and future. In: Endophenotypes of Psychiatric and Neurodegenerative Disorders in Rodent Models. ***2009 Ed. Granon S. Signpost.***

9. Curriculum vitae

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