Novel treatment approaches based on identification of molecular determinants in neuropsychiatric diseases

PhD Thesis

In partial fulfilment of the requirements for the degree

"Doctor of Philosophy"

In the Neuroscience Graduate Program

At the Georg August University, Göttingen

Faculty of Biology

Submitted by

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Born in

Istanbul, Turkey

2008

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.

Derya Sargin Göttingen, 16 September 2008

ACKNOWLEDGEMENTS

I am sincerely grateful to Prof. Dr. Dr. Hannelore Ehrenreich first of all for giving me the opportunity to work in her selective laboratory in such an amazing institute. I owe many thanks for her supervision and constant support throughout my PhD years. I would also like to thank for her amazing motivation and guidance in my PhD work.

I owe many thanks to Prof. Anna-Leena Sirén who has helped me during the initial phase of my PhD. I am grateful for her comments, discussion and support.

I would like to acknowledge Prof. Armin-Klaus Nave, Prof. Nils Brose and Prof. Ralf Heinrich for their contributions as members of my thesis committee. I am especially grateful to Prof. Klaus-Armin Nave for his advice, support and discussions.

Many thanks to the members of the Neuroscience Program Coordination office Prof. Michael Hörner and Sandra Drube. I would especially like to thank Prof. Michael Hörner for providing extensive support throughout my PhD thesis.

I would like to thank Dr. Imam Hassouna for his great help on setereological countings and analyses. I owe special thanks to Swetlana Sperling for her great technical assistance. I would like to thank Dr. Kostik Radyushkin for his support and guidance in behavior experiments. I also thank Dr. Martin Begemann, Dr. Claudia Bartels, Dr. Henning Krampe, Dr. Sabina Stawicki, Bartosz Adamcio, Ahmed el-Kordi for their help in many different aspects of my PhD life. I thank Kathrin Hannke and Anja Ronnenberg for assisting me in certain techniques. I acknowledge Wiebke Timner for dealing with all the administrative problems and for her continuous friendly smile. Once more, I thank all the members of the Clinial Neuroscience group for their amazing friendship and support through all these years.

Special thanks to my dearest friends Dr. Benjamin Cooper, Burcu Kasapoglu, Dr. Amit Agarwal and Schanila Nawaz for always being there for me. I would like to thank Ben who is a talented postdoc and a loving boyfriend. I am very happy to have my closest friend Burcu here with me and thank for her amazing friendship and for all the fun we had in and outside Göttingen. I thank Amit for enjoying the cloning with me and thank both Amit and Schanila for their amazing friendship and optimism.

My deepest and warmest gratitude goes to my loving mother and my little sister for always being next to me, for their love and great support.

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1. Introduction to the scientific project

The present cumulative thesis includes three original publications investigating new treatment strategies for neuropsychiatric diseases. The first paper focuses on erythropoietin (EPO) and its mechanisms of action on improving cognition under healthy and disease conditions i.e. schizophrenia. The second paper is a case study of a rapid cycling patient and explores molecular aspects of the disease leading to an alternative treatment approach for stabilization of the cycling behavior. The third paper is based on a traumatic brain injury model that leads to atrophy and cognitive impairment at an older age in mice. It explores the morphological consequences of atrophy induced by lesion and offers EPO as a neuroprotective agent to prevent lesion induced atrophy, cognitive impairment and late morphological changes in the brain.

Two major psychiatric disorders, schizophrenia and bipolar disorder, were first characterized as dementia praecox and manic-depressive insanity respectively by Emil Kraepelin about a hundred years ago (Kendell, 1987). Schizophrenia is a disease in which higher brain functions and as a result, social behavior deteriorate and the patient suffers from auditory hallucinations, delusions, disorganized thinking and cognitive decline. It affects 0.4-0.6% of the population with an age of onset at around mid twenties (Bhugra, 2005). Cognitive deficits and behavioral abnormalities are already present from early childhood but the general characteristics of the disease appear in late teens. Twin studies have shown that heritability as well as environmental factors are important for the outcome of the disease (Prescott and Gottesman, 1993). Thus, it is a multifactorial disorder that has a combination of environmental and genetic causes.

Another common psychiatric disease, bipolar disorder, is characterized by an abnormally elevated mood, referred to as mania, followed by periods of depression. It has a prevalence of 1.3-1.6% and the first major mood episode is

usually experienced during adolescence (Muller-Oerlinghausen et al., 2002). Manic and depressive episodes are mostly separated by periods of normal mood. However, in 10-30% of bipolar disease patients, mania and depression rapidly alternate. This condition is known as rapid cycling (Papadimitriou et al., 2005). In bipolar disorder, symptoms of mania include mood elevation, grandiosity, overconfidence, decreased sleep, rapid speech, increased appetite, and libido and manic episodes are characterized by hospitalization, psychosis and functional impairment. Depressive episodes are usually longer and take over the lives of majority of bipolar patients (Thase, 2005). As in schizophrenia, neurocognitive impairments including disturbances in attention, memory and executive function, are core features of bipolar disorder (Thompson et al., 2005). Recent research has shown that bipolar disorder, like schizophrenia, might be a polygenic disease with more than one gene involved in its etiology (Barrett et al., 2003; Baum et al., 2008).

There are treatments available for reducing the symptoms of both schizophrenia and bipolar disorder but the diseases cannot be cured. Moreover, there is no drug available for preventing the cognitive impairment and thus increasing the quality of life for the patients of both diseases. The first-generation antipsychotic drugs, or typical antipsychotics, function as D2 dopamine receptor antagonists. They are used to treat positive symptoms of schizophrenia, such as hallucinations and delusions. However, they are not effective in treating negative symptoms, such as lack of motivation, anhedonia and cognitive dysfunction and patients suffer from adverse extrapyramidal side effects (Murphy et al., 2006). In 1989, with the introduction of clozapine, typical antipsychotic drugs are replaced by atypical antipsychotics having less side effects and offering more effective treatment (Kane et al., 1988). They act by inhibiting D2 dopamine as well as serotonin ($5HT_{2A}$) receptors (Snyder and Murphy, 2008). After clozapine, the next 15-20 years in schizophrenia research was devoted to developing clozapine-like atypical antipsychotics that are safer and better tolerated. Development of additional atypical drugs like risperidone, olanzapine, quetiapine, and ziprasidone

did not fulfill the expectations since these drugs as well as clozapine are unable to treat negative symptoms of the disease (Keefe et al., 1999). Moreover, though they do not lead to extrapyramidal side effects, atypical antipsychotics still have their own side effect burden such as weight gain (Allison et al., 1999) and metabolic syndrome (Newcomer et al., 2002).

For bipolar disorder, the most commonly used mood-stabilizing drug is lithium. It has been effective in reducing the symptoms of the disease and preventing suicide (Fountoulakis et al., 2007). A group of anticonvulsants and anti-epileptic drugs, such as valproic acid and carbamazepine, are among the most commonly used mood-stabilizers in bipolar disease (Sachs and Thase, 2000). Second-generation atypical antipsychotic drugs have increasingly begun to be used for stabilization of manic phases (Surja et al., 2006). Once again, limitations of the medication are inadequate effectiveness, inability to treat target symptoms and adverse side effects (Perlis et al., 2006). Moreover, presence of different subtypes of the disease, such as rapid cycling disorder, provides more challenge for development of an effective treatment. Rapid cycling patients are poor responders to treatment with either lithium or anticonvulsants (Calabrese et al., 2005; Tondo et al., 2003). Interestingly, there are even increasing yet inconclusive evidence that antidepressants may trigger or worsen rapid-cycling disorder (Ghaemi et al., 2003; Wehr et al., 1988).

Understanding mechanisms and molecular determinants underlying pathophysiology of neuropsychiatric disorders is important for development of novel therapies for these diseases with high prevalence and increased risk of mortality. In order to study the pathophysiology, it is inevitable to combine genetic and environmental approaches for these multifactorial disorders for developing animal models. Traumatic brain injury (TBI) is one of the epigenetic risk factors contributing to the outcome of personality changes (Gualtieri and Cox, 1991; Lannoo et al., 1997), depression (Fedoroff et al., 1992; Rosenthal et al., 1998), schizophrenia (Malaspina et al., 2001; Silver et al., 2001), mania (Shukla et al.,

1987; Starkstein et al., 1987) and neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Bower et al., 2003; Nemetz et al., 1999; Stern, 1991). About 15% of those afflicted with TBI in their lives appear to be symptomatic for other neurological disorders (Alexander, 1995). The outcome of TBI would depend on previous vulnerabilities, the extent of the injury and the reaction of the brain to the injury. Studies show that the young and the old are at higher risk for suffering the consequences of TBI (Kraus and Nourjah, 1988).

Mechanisms leading to cognitive impairment and cognitive related disorders after TBI remain to be elucidated. Depending on the extent of concussion, damage might include swelling of the brain, hypoxia or diffuse axonal injury. Primary brain injury mechanisms might involve damage on axons and microvasculature leading to formation of microhemorrhages that disrupt blood supply in certain regions of the brain. Damage on axons might lead to Wallerian degeneration or neuronal degeneration, and subsequent depolarization of neurons in affected areas or even at remote sites (Hall et al., 2008; Hurley et al., 2004; Povlishock, 1993). This might lead to an increase in the activity of N-methyl-D-aspartate (NMDA) receptors and result in an excess release of glutamate, excitotoxicity, free radical formation and oxidative stress (Faden et al., 1989; Giza et al., 2006). Neuronal death at the site of injury might mediate release of cytokines initiating pro-inflammatory reactions with the induction in expression of interleukins and other pro-inflammatory mediators leading to an inflammatory reaction. Inflammation initiates the beginning of the second stage of brain injury or secondary brain injury. Increased permeability of the blood-brain barrier followed by invasion of macrophages are additional events in the secondary injury cascade of TBI (Potts et al., 2006).

Neuroimaging studies in posttraumatic patients have reported cortical and subcortical atrophy, ventricular enlargement and white matter injury (Anderson et al., 1996; Arciniegas and Silver, 2001; Bigler, 2003). These findings supported the notion that TBI is associated with reductions in the volumes of cerebral

structures important for maintenance of cognitive functions. Events occurring in response to acute injury are extensively studied however, at present, efficient pharmacotherapy for TBI is lacking and most importantly, mechanisms leading to the late consequences of TBI and/or cognitive impairment and neurological disorders are unkown.

Despite having different disease characteristics, TBI and cognitive disorders share common pathophysiological features such as neuronal or axonal injury, oxidative stress and inflammation. Because of these common but diverse functional disturbances, cognitive disorders remain untreated by the application of a single drug treatment. Therefore, it is essential to develop therapeutic strategies that aim at treating more than one aspect of the disease and supporting the regenerative potential of the brain. Such a natural protein hormone, which has been used to treat anemia since more than a decade, is the glycoprotein erythropoietin (EPO). EPO is a 30.4 kDa glycoprotein that regulates red blood cell differentiation by inhibiting apoptosis of erythroid progenitors in bone marrow (Koury and Bondurant, 1992). It is a member of the class I cytokine superfamily which also includes interleukins (IL-2, -3, -4, -5, -6), granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factor (GM-CSF), leukemia inhibitory factor and ciliary neurotrophic factor. EPO is predominantly produced in the fetal liver and adult kidney (Dame et al., 1998; Zanjani et al., 1977). The finding that EPO and its receptor (EPOR) are expressed in the brain (Sasaki et al., 2001) led to the notion that EPO exerts direct, hematopoiesis-independent effects on the nervous system.

EPO functions by binding to an erythropoietin receptor (EPOR), which is a glycosylated and phosphorylated transmembrane polypeptide with the molecular weight of 72-78 kDa. Like other cytokine receptors, EPOR contains 2 β sandwich domains, D1 (N-terminal) and D2 (carboxy-terminal) (Syed et al., 1998). Due to the unique structural features of the N-terminal and C-terminal domains, EPOR belongs to a novel class of receptors called the hematopoietin receptor

superfamily, which includes human growth hormone and prolactin receptors (Krantz, 1991). After EPO binding, EPO receptors homodimerize triggering the activation of several signal transduction pathways. The first step of EPO-induced intracellular signaling is the activation of the Jak2 tyrosine kinase, which is constitutively associated with the EPOR. Jak2 activation leads to the phosphorylation of 8 tyrosines located within the intracellular domain of EPOR. These serve as docking sites for other various intracellular proteins, which then can also become phosphorylated and activated. This phosphorylation cascade activates several intracellular pathways, including signal transducer and activator of transcription (STAT), Ras–mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (Brines and Cerami, 2005).

Discovery of EPO to increase neuromuscular strength (Sobh et al., 1992), cognitive functions (Nissenson, 1989) and quality of life (Wolcott et al., 1989) in anemic patients has led to the notion that EPO might be involved in neuronal protection. One of the first studies to show EPO-induced neuroprotection was increased neuronal survival after hypoxia by EPO addition in culture (Konishi et al., 1993). Different neuroprotective properties of EPO has rendered this growth hormone to be used as an ideal pharmacotherapeutic agent to offer neuroprotection in neurological disorders with a variety of pathophysiological features resulting in a common characteristic i.e. cognitive impairment. EPO has been shown to have anti-apoptotic (Siren et al., 2001), anti-oxidative (Chattopadhyay et al., 2000; Genc et al., 2002), anti-inflammatory (Agnello et al., 2002; Villa et al., 2003), neurotrophic (Campana and Myers, 2001) and angiogenetic (Sasaki et al., 2001) properties . Moreover, it is known to induce stem cell differentiation (Chen et al., 2007; Gonzalez et al., 2007; Jelkmann, 2000; Shingo et al., 2001). Development of animal models of neurological diseases in order to directly evaluate neuroprotective functions of EPO *in vivo* and deciphering mechanisms of action of this multifactorial compound will open a new era in treatment and understanding of neuropsychiatric disorders.

2. Focus of the present work

The thesis includes 3 original publications that investigate mechanisms leading to neuropsychiatric disorders and present novel treatments for these diseases.

2.1 Aims of project I

The first original publication focuses on mechanisms of EPO action and how it improves cognition in neuropsychiatric disorders like schizophrenia. For the experiments, young (28 days old) C57BL6 mice, primary hippocampal neuronal cultures from mice at embryonic day 17 (E17) and autaptic hippocampal cultures from postnatal day 0 (P0) mice were used.

The first aim of the study was to develop a model to show that EPO improves cognition under healthy conditions. This would allow us to search for the mechanisms of EPO action in the healthy brain where interference from disease associated conditions could be excluded. Young healthy mice at the age of 28 days old were subjected to EPO injections for 3 weeks every other day. Mice were then run through an extensive behavioral test battery at different time points to assess EPO action on hippocampus dependent memory and to rule out EPO effect on other parameters.

The second aim was to find out how EPO improves cognitive function. Effect of EPO on synaptic transmission and synapse numbers was investigated using hippocampal slices and sections from EPO-treated mice. Synaptic function was investigated at the network level with primary hippocampal cultures and at the single cell level with autaptic hippocampal neurons.

2.2 Aims of project II

The second original publication focuses on a rare form of bipolar disorder, rapid cycling syndrome. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of a rapid cycling patient at different disease stages and episodes. RNA isolated from PBMC was used for expression analysis.

The first aim of the study was to identify genes that were differentially regulated at manic and depressed phases. RNA isolated from PBMC of the patient at different episodes throughout years was subjected to microarray analysis. Genes identified in this way were further confirmed by quantitative real-time polymerase chain reaction (qRT-PCR).

The second aim was to develop a treatment approach based on differential gene expression in manic and depressed phases. The treatment was designed to keep the patient at the most stable condition by improving depressed as well as manic symptoms. The patient was controlled through psychopathological ratings before and during the treatment period.

2.3 Aims of project III

The third original publication investigates the morphological consequences of a unilateral parietal lesion in mice which develop chronic neurodegeneration devoid of gliosis over time. Pathophysiological findings in this mouse model mimics those observed in schizophrenia and serves as a model to understand the mechanisms leading to the outcome of this disease. C57BL6 mice at the age of 28 days were used for lesion experiments. Mice were analyzed at time points 24 hours and 11 months after lesion.

The first aim of the study was to analyze the histological aspects of global atrophy induced by a unilateral cortical lesion at a young age in mice. Chronic neurodegenerative changes appear many months after lesion and mimic those in schizophrenia. A detailed histological analysis based on stereology would serve to understand the mechanisms leading to the observed atrophy.

EPO treatment right after lesion provided beneficial effects in preventing the outcome of cortical atrophy and the cognitive impairment observed in lesioned mice. The second aim of the study was based on understanding how EPO prevented atrophy and which pathophysiological aspects on the way to atrophy and cognitive impairment could be overcome by early EPO treatment. This study has once more shown the beneficial effects of EPO on cognitive disorders like schizophrenia.

3. Effect of EPO on hippocampus dependent memory

Understanding mechanisms of EPO-induced cognitive improvement under healthy and disease conditions

3.1 Overview of project I

EPO is a hematopoietic growth factor that was initially used for treating anemic patients. Observation of improved cognition in EPO-treated patients and that EPO and its receptor are expressed in the brain opened up a new era in EPO research. Since then, many studies showed that EPO acts in an anti-apoptotic, anti-inflammatory, anti-oxidative and stem-cell modulatory fashion under pathological conditions. EPO has also been shown to promote neurite outgrowth and axonal repair (Campana and Myers, 2001; Celik et al., 2002; Konishi et al., 1993; Shingo et al., 2001; Siren et al., 2001). These studies further supported the notion that EPO exerts neuroprotective effects independent of its hematopoietic function.

Effects of EPO on cognition has been shown in different animal models of disease including ischemia/hypoxia and traumatic brain injury (Catania et al., 2002; Kumral et al., 2004; Siren et al., 2006). A double-blind, placebo-controlled, proof-of-principle (phase II) study has shown for the first time beneficial effects of EPO in improving cognitive functions in chronic schizophrenic patients (Ehrenreich et al., 2007b). Moreover, an exploratory open label study (phase I/IIa) in chronic progressive multiple sclerosis (MS) patients has shown improvement in motor and cognitive function upon EPO treatment. In these studies, there was hardly any detectable hematopoietic response to EPO (Ehrenreich et al., 2007a).

Despite EPO's proven beneficial role in neurological diseases, its mechanisms of action to improve cognitive function and its effect on cognition under healthy conditions are still unknown. In order to investigate EPO's action on cognitive

functions under healthy as well as disease state, we have developed an experimental condition where systemic EPO treatment for 3 weeks in mice has led to a significant improvement in hippocampus dependent memory task, fear conditioning. For all the experiments, young (28 days old) and healthy C57BL6 mice were used. EPO was applied intraperitoneally every other day (11 injections in total). EPO-treated mice showed a significant improvement in hippocampus dependent contextual memory task assessed by fear conditioning test 1 week after the last injection. EPO-treated animals were still significantly better in this task compared to the placebo-treated controls 3 weeks after cessation of the treatment. Interestingly, during this time, hematocrit levels were comparable between the groups. Moreover, EPO's action on cognition was rather selective since EPO-treated mice did not differ from controls in terms of motor performance, anxiety and exploratory behavior.

In order to understand the mechanism of EPO action on cognitive function, we performed electrophysiological, histological as well as biochemical analysis to assess EPO-induced changes in synaptic function. Hippocampal slices from mice treated with EPO for 3 weeks and killed 1 week after cessation of treatment were used for electrophysiological analysis. Field excitatory postsynaptic potentials (fEPSPs) were evoked by placing the stimulation electrode in stratum radiatum of the CA3/CA1 region and the recording electrode in the stratum radiatum of the CA1 region. Electrophysiological analysis revealed a significant enhancement of long term potentiation (LTP), short term potentiation (STP) and short term depression (STD) in slices obtained from EPO-treated mice compared to controls. Whole-cell patch-clamp recordings on pyramidal cells of CA1 hippocampal region showed that, upon EPO treatment, the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) increased whereas the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) decreased.

The results obtained in hippocampal slices were further supported by multi-electrode array (MEA) measurements *in vitro* using primary hippocampal

cultures. Hippocampal neurons obtained from mice at E17 were treated with EPO for 3 weeks starting from day 5 in culture. Upon maturation of the culture, the number of silent channels decreased in the placebo-treated cultures whereas EPO treatment prevented this decrease. Moreover, 2-3 weeks after cessation of EPO treatment, the number of bursting channels were higher in EPO-treated cultures compared to controls. Whole-cell patch-clamp recordings in autaptic hippocampal neurons treated with EPO at day 7 in culture and analyzed at day 9-14 showed a significant reduction in EPSC amplitude and readily-releasable pool size upon EPO treatment.

To assess whether EPO has any effects on synapse numbers, we counted synapses on hippocampal sections and in autaptic cultures at the same time points used for electrophysiological analysis. Analysis of synapsin1 positive presynaptic density in different regions of hippocampus and in autaptic cultures revealed no difference upon EPO and placebo treatments. Thus, EPO exerted its effects on hippocampal synaptic function not by affecting the synapse number but rather by shifting the balance between excitatory and inhibitory transmission. EPO might selectively enhance the efficiency of selected neuronal networks whereas keeping the others silent.

In summary, with this publication, we have shown, for the first time, action of EPO on modulating synaptic plasticity by increasing inhibitory and decreasing excitatory transmission. EPO exerts its effects on synaptic transmission in parallel to increasing LTP and improving cognition. By enhancing the activity of selected synapses while keeping the others silent and in this way, affecting the synaptic function but not the number, EPO works in rather an unexpected way to improve cognition. Further experiments that would help us to understand EPO action on synapse in more detail will also help to decipher the mechanisms leading to cognitive disruption in neuropsychiatric disorders and to develop new treatment approaches.

3.2 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 Biology **(In press)**

* Indicates equal contribution for the publications

Personal contribution:

I was involved in the design of the studies, interpretation of results, preparation of the manuscript, setting up the experimental design for EPO injection paradigms and behavioral tests. I also performed brain dissection, section preparation, immunohistochemistry, confocal analysis, immunostaining of autaptic neurons, counting synapses on both sections and cultures, western blot and qRT-PCR analyses. Electrophysiological analyses were performed by our collaborators.

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.

CONCLUSIONS: 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 analyzed 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 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-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.

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 contrast, 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 e, 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 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 analyzed 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 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 disease 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].

Conclusions

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 locomotor 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 formation 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 10x 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 4ºC 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 analyzed 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 63x 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 34ºC.

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 1Hz. 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 1h. 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 4ºC 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 x 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://www.brainworks.uni-freiburg.de/projects/mea/meatools/overview.htm) 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 x 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 4ºC 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 4ºC 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 40x 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 300nM 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 three 5 min periods 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 4ºC 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℃, followed by denaturation at 95℃ for 10 min. The amplification was carried out by 45 cycles of 95℃ for 15 s and 60℃ 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.

Acknowledgements

This study has been supported by the Max-Planck-Society, by several private donations, as well as by the DFG Center for Molecular Physiology of the Brain (CMPB). AS was supported by an EC fellowship for Neuroscience Early Stage Research Training NEUREST. LM was supported by a Lichtenberg Stipend of the State of Lower-Saxony.

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Figure legends

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.

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)**.

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 x 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).

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).

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.

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.

Additional files

Additional file 1:

File name: Adamcio et al – Additional file 1

File format: pdf

Title of data: Additional file 1

Description of data: Analysis of single-sample MEA recordings

Additional file 2:

File name: Adamcio et al – Additional file 2

File format: pdf

Title of data: Additional file 2

Description of data: Mean conditional firing rates for EPO and control samples

Additional file 3:

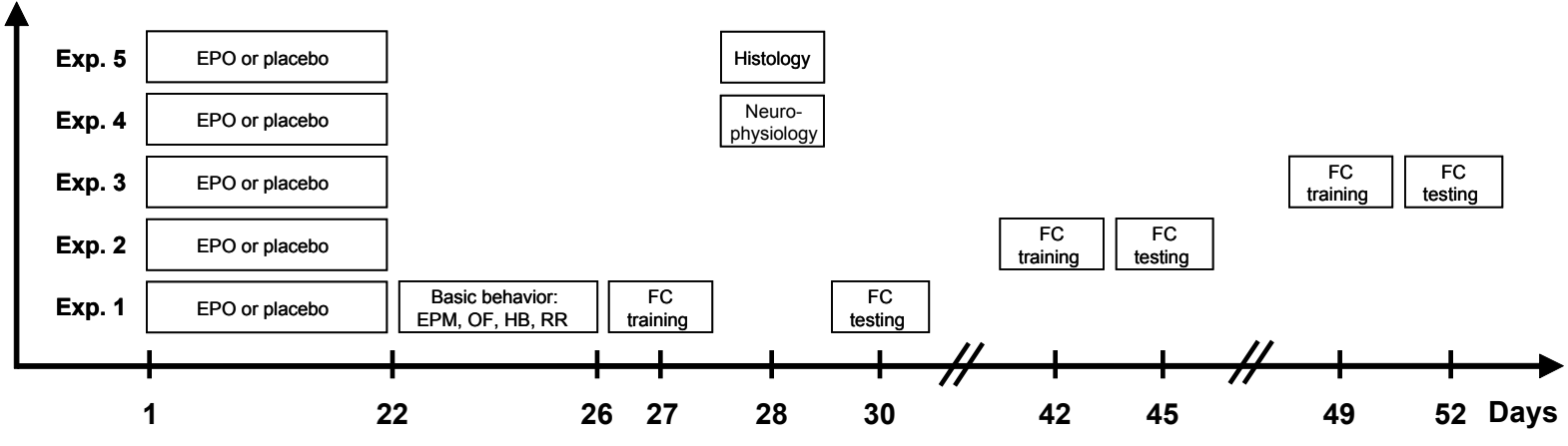
File name: Adamcio et al – Additional file 3

File format: pdf

Title of data: Additional file 3

Description of data: Spike-rate (1/s) for EPO-treated and control dishes

Figure 1


Exp. 5
EPO or placebo
Histology
Exp. 4
EPO or placebo
Neuro-
physiology
Exp. 3
EPO or placebo
FC
training
FC
testing
Exp. 2
EPO or placebo
FC
training
FC
testing
Exp. 1
EPO or placebo
Basic behavior:
EPM, OF, HB, RR
FC
training
FC
testing
1
22
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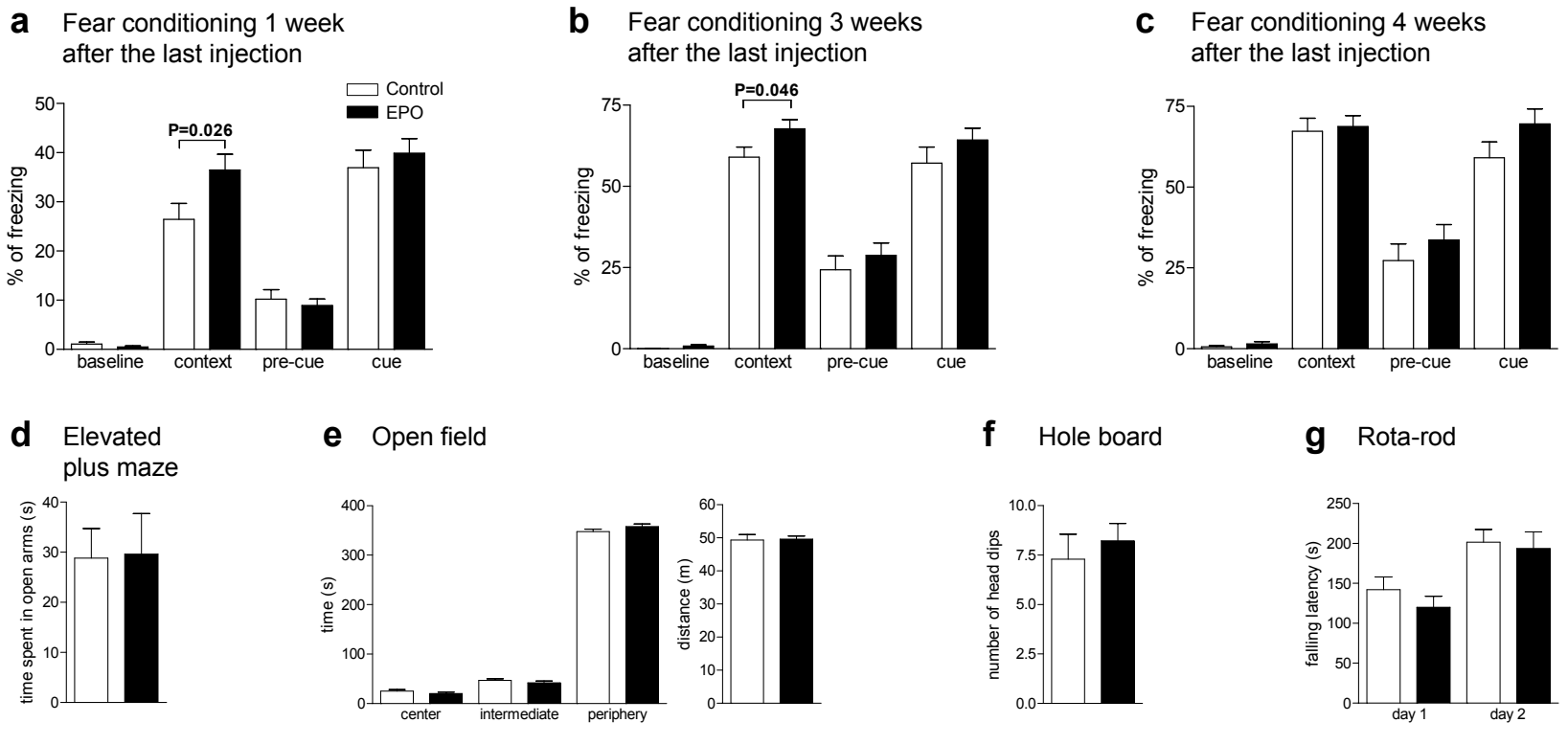
52
Days

Figure 2


a
Fear conditioning 1 week after the last injection
Control
EPO
P=0.026
% of freezing
baseline
context
pre-cue
cue
b
Fear conditioning 3 weeks after the last injection
P=0.046
% of freezing
baseline
context
pre-cue
cue
c
Fear conditioning 4 weeks after the last injection
% of freezing
baseline
context
pre-cue
cue
d
Elevated plus maze
time spent in open arms (s)
e
Open field
time (s)
center
intermediate
periphery
distance (m)
f
Hole board
number of head dips
g
Rota-rod
falling latency (s)
day 1
day 2

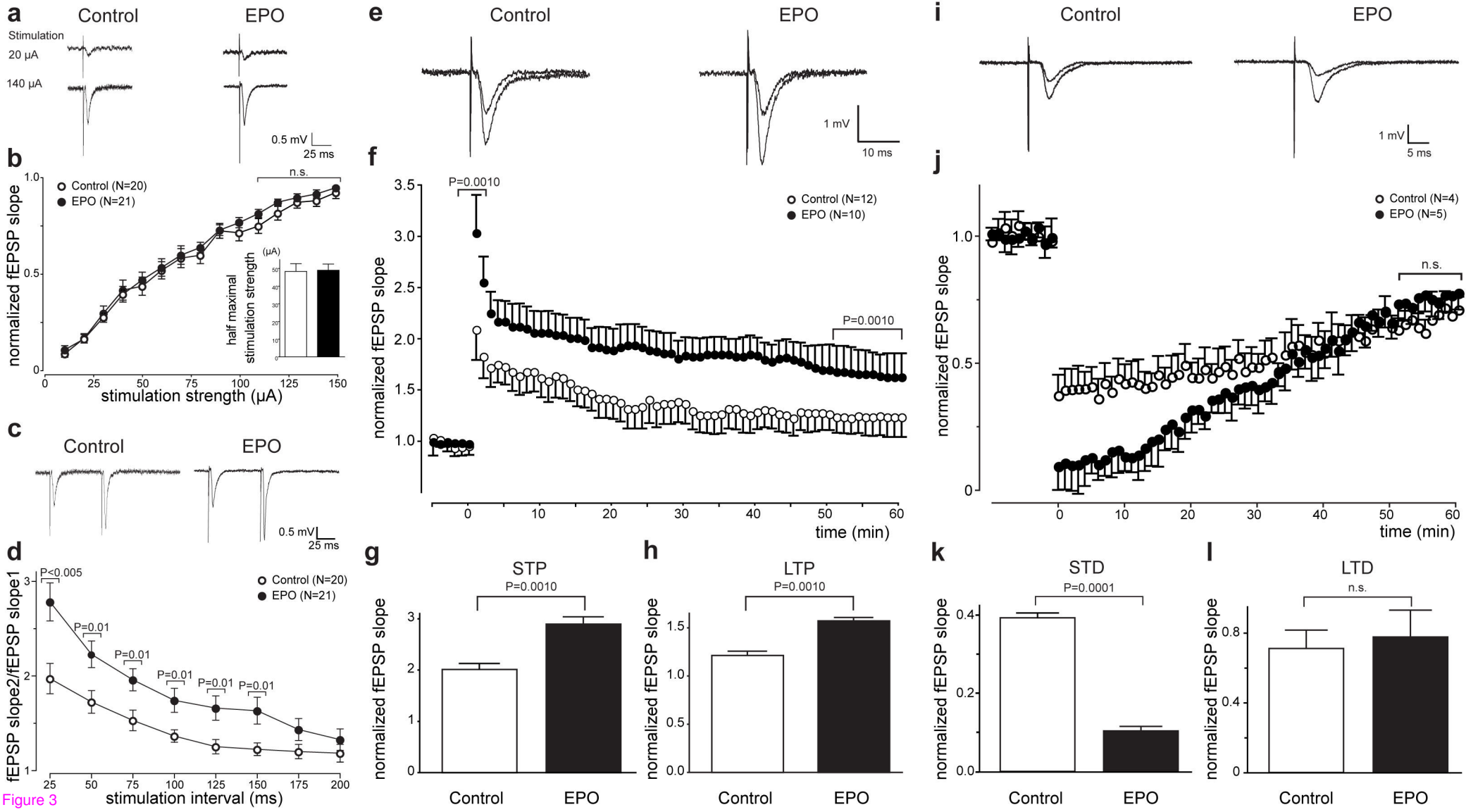

a
Control
EPO
Stimulation
20 μA
140 μA
0.5 mV
25 ms
b
Control (N=20)
EPO (N=21)
n.s.
normalized fEPSP slope
stimulation strength (μA)
half maximal stimulation strength
(μA)
c
Control
EPO
0.5 mV
25 ms
d
P<0.005
P=0.01
Control (N=20)
EPO (N=21)
fEPSP slope2/fEPSP slope1
stimulation interval (ms)
e
Control
EPO
1 mV
10 ms
f
P=0.0010
Control (N=12)
EPO (N=10)
normalized fEPSP slope
time (min)
g
STP
P=0.0010
normalized fEPSP slope
Control
EPO
h
LTP
P=0.0010
normalized fEPSP slope
Control
EPO
i
Control
EPO
1 mV
5 ms
j
Control (N=4)
EPO (N=5)
n.s.
normalized fEPSP slope
time (min)
k
STD
P=0.0001
normalized fEPSP slope
Control
EPO
l
LTD
n.s.
normalized fEPSP slope
Control
EPO

Figure 3

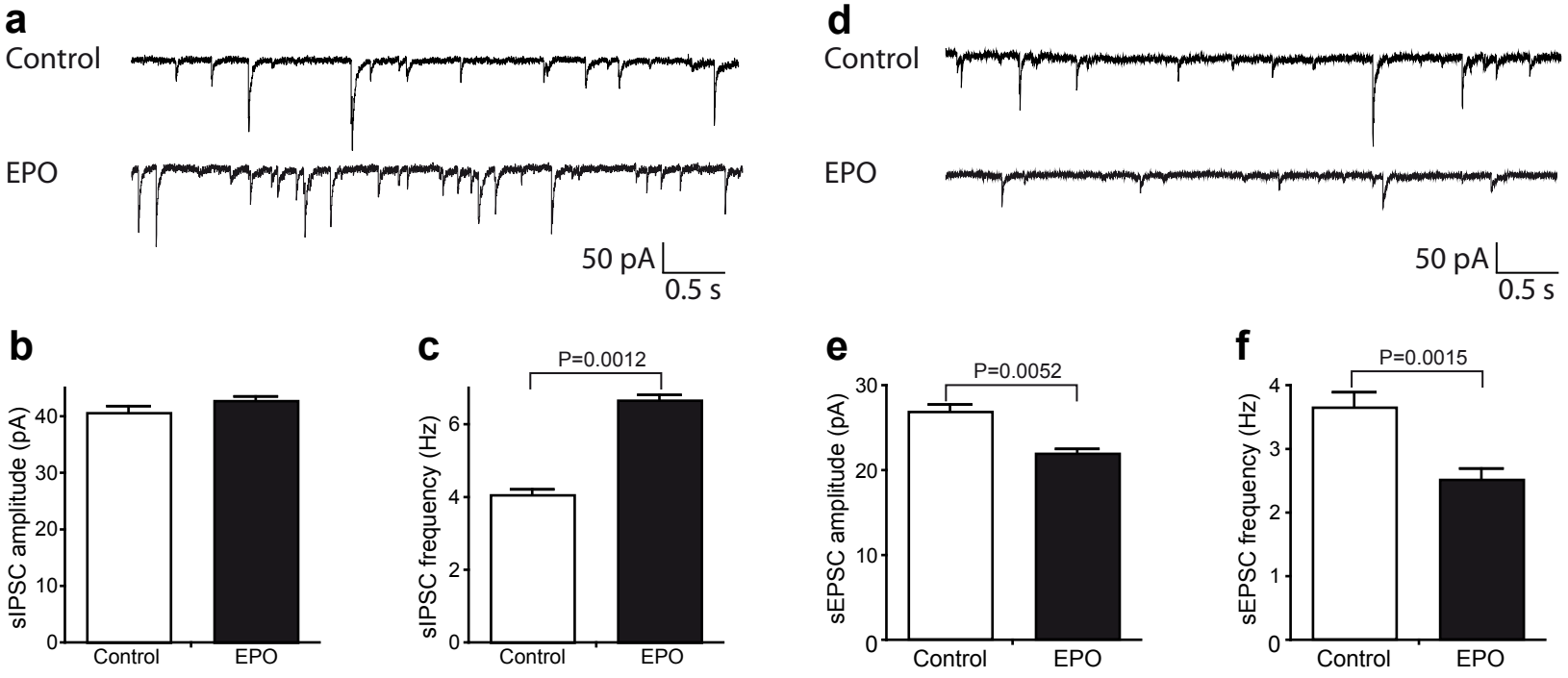

a
Control
EPO
50 pA
0.5 s
d
Control
EPO
50 pA
0.5 s
b
sIPSC amplitude (pA)
0
10
20
30
40
Control
EPO
c
P=0.0012
sIPSC frequency (Hz)
0
2
4
6
Control
EPO
e
P=0.0052
sEPSC amplitude (pA)
0
10
20
30
Control
EPO
f
P=0.0015
sEPSC frequency (Hz)
0
1
2
3
4
Control
EPO

Figure 4

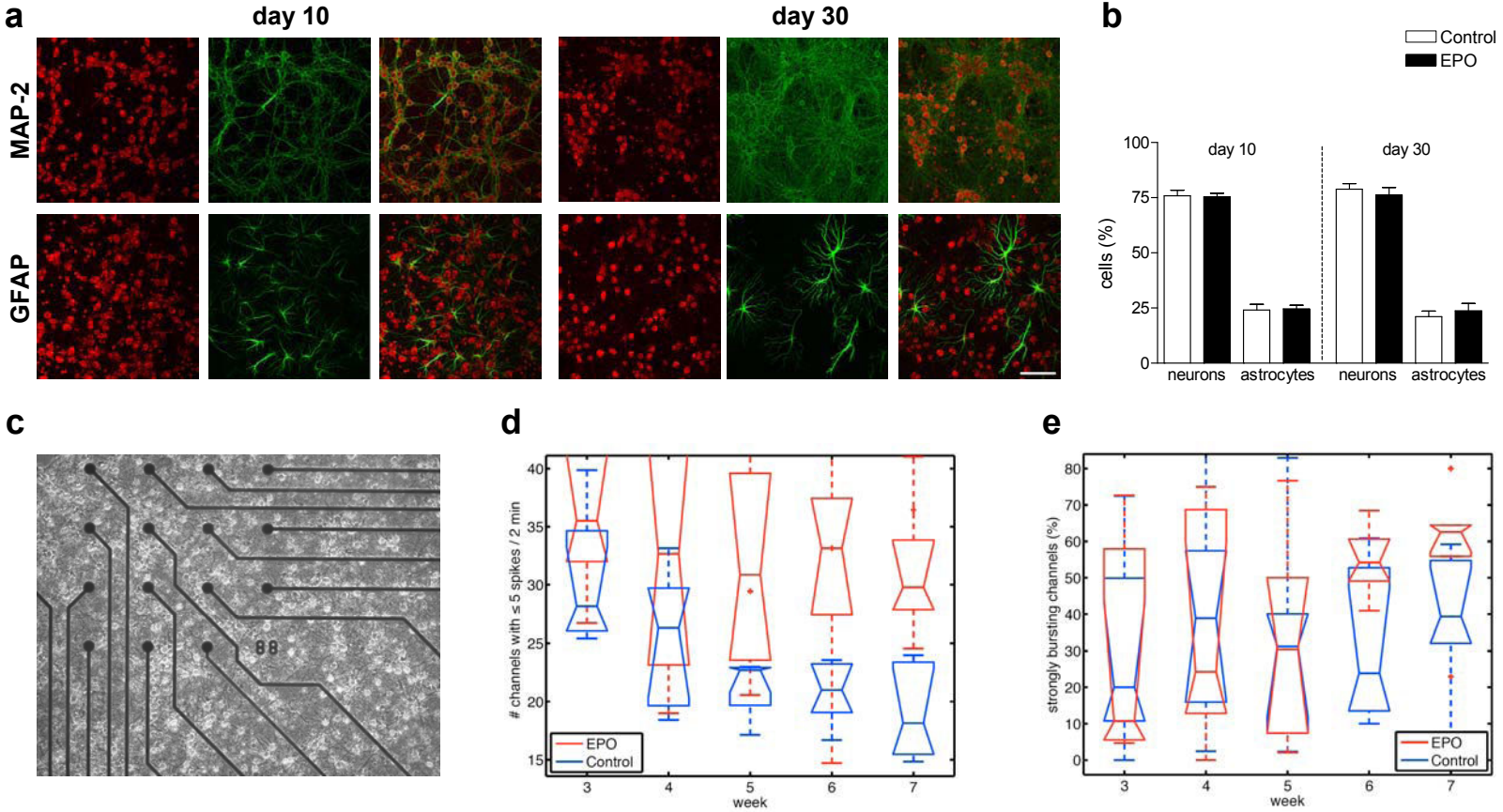
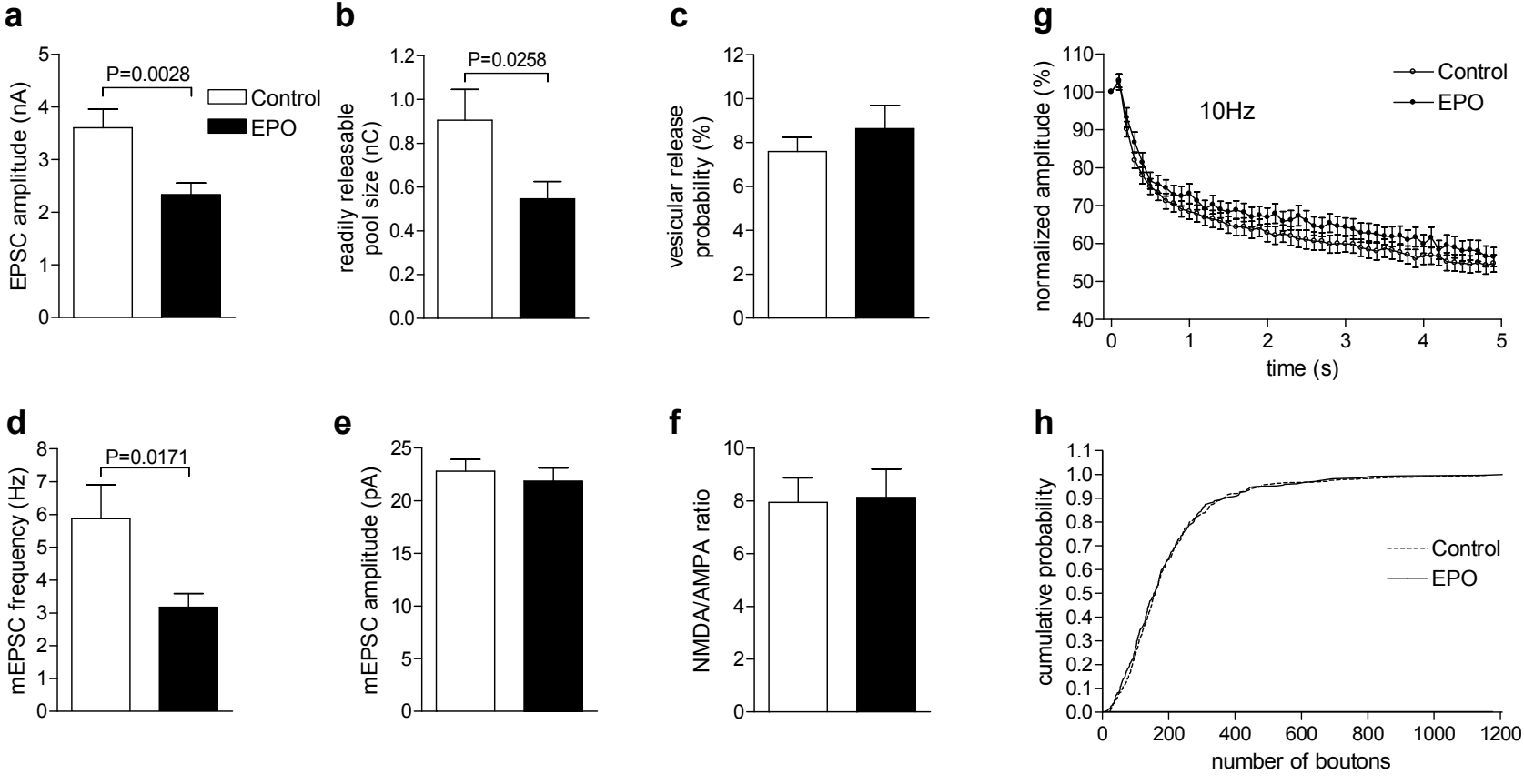

a
day 10
day 30
MAP-2
GFAP
b
Control
EPO
100
75
50
25
0
cells (%)
day 10
day 30
neurons
astrocytes
neurons
astrocytes
c
88
d
channels with ≤ 5 spikes / 2 min
40
35
30
25
20
15
EPO
Control
3
4
5
6
7
week
e
strongly bursting channels (%)
80
70
60
50
40
30
20
10
0
EPO
Control
3
4
5
6
7
week

Figure 5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EPSC amplitude (nA)
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Control
EPO
b
readily releasable pool size (nC)
P=0.0258
c
vesicular release probability (%)
g
normalized amplitude (%)
10Hz
Control
EPO
time (s)
d
mEPSC frequency (Hz)
P=0.0171
e
mEPSC amplitude (pA)
f
NMDA/AMPA ratio
h
cumulative probability
Control
EPO
number of boutons

Figure 6

Additional files provided with this submission:

Additional file 1: adamcio et al - additional file 1.pdf, 479K
http://www.biomedcentral.com/imedia/8277533782153184/supp1.pdf
Additional file 2: adamcio et al - additional file 2.pdf, 392K
http://www.biomedcentral.com/imedia/7336617821531913/supp2.pdf
Additional file 3: adamcio et al - additional file 3.pdf, 138K
http://www.biomedcentral.com/imedia/9020546042153184/supp3.pdf

4. Molecular determinants underlying rapid cycling disorder and new treatment approaches

4.1 Overview of project II

Rapid cycling syndrome is a rare disease affecting 10-30 % of patients with bipolar disorder. According to the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) criteria, a patient can be diagnosed with the rapid cycling syndrome if he/she experiences at least four episodes of major depression, mania, mixed mania, or hypomania in the past year, occurring in any combination or order. The patient should experience a complete manic or a complete depressive episode occurring after one another for at least 1 week. These patients are poor responders to mood stabilizers and currently, no other treatments are available. Moreover, mechanisms underlying bipolar affective disorders and their subgroup rapid cycling syndrome are still unkown.

In this original publication, we studied a rapid cycling patient with a 16 year-old disease history. In order to find out the genes that are differentially expressed in manic and depressed episodes that would help us to get closer to understand the genetic mechanisms leading to the switch between different disease episodes, we exploited RNA isolated at different disease stages from peripheral blood mononuclear cells (PBMC) of the patient. Two recent publications have reported changes in gene expression profiles using microarray analysis on post mortem brain tissue of bipolar patients (Nakatani et al., 2006; Ryan et al., 2006). However, these studies failed to report a single overlapping gene. Keeping in mind the difficulties and challenges arising from postmortem studies, we performed the microarray analysis in a living patient. Since bipolar disorder is a systemic disease, we believe that changes obtained from periphery would as well give us a hint about the cycling changes occurring in the brain. Moreover, global gene expression analysis using PBMC to identify molecular signatures of the

disease pathology has proven to be useful in different brain diseases (Achiron and Gurevich, 2006; Buttarelli et al., 2006; Grond-Ginsbach et al., 2008).

In order to identify genes regulated differentially in manic and depressed phases, we used a three-tiered strategy. First, blood was collected from the patient on two consecutive days at a specific time in the morning (8:00 a.m.). Blood samples were collected when the patient was in the middle of her two consecutive manic or depressed episodes. We performed microarray analysis on RNA isolated from PBMC of the patient which was followed by a two-step bioinformatic processing. In this way, we excluded genes that showed different expression levels between two consecutive days of a certain episode as well as genes that were differentially expressed between two manic or two depressed episodes. Therefore, we could exclude genes that showed daily and monthly variations using this three-tiered experimental strategy. Next, we validated the results of the microchip screening by performing qRT-PCRs and selected the genes that showed the same expression pattern in microarray analysis. We further confirmed the results of qRT-PCRs with additional blood samples collected more than one year later.

After comparing the expression pattern of the remaining genes between the different episodes, we grouped these genes according to the biological categories. After this careful screening approach, we were left with eight different genes that were involved in prostaglandin metabolism, PTGDS (lipocalin-type prostaglandin D synthetase) and AKR1C3 (prostaglandin D2 11-ketoreductase), neurodevelopment, NRG1 (neuregulin 1) and SPON2 (spondin 2), regulation of the immune system, GZMA (granzyme A) and KLRD1 (killer cell lectin-like receptor subfamily D, member1/CD94), as well as hemoglobins A and B (HBA and HBB).

The most striking finding was the association of two prostaglandin genes, PTGDS and AKR1C3, with rapid cycling disorder. Both genes showed higher

expression in depressed episodes compared to manic episodes. PTGDS catalyzes conversion of PGD2 from PGH2 (the product of oxygenation of arachidonic acid by cyclooxygenases) and is preferentially expressed in CNS where it is involved in various functions including sleep, pain and odor responses, may serve an anti-apoptotic role for oligodendrocytes (Taniike et al., 2002) and was shown to be induced upon pathological conditions (Kagitani-Shimono et al., 2006; Kawashima et al., 2001; Mohri et al., 2006; Taniguchi et al., 2007). AKR1C3 is also involved in prostaglandin synthesis by catalyzing conversion of PGF2α from PGD2. Prostaglandin synthesis has been shown to be involved in homeostasis, regulation of sleep, allergic responses and inflammation (O'Hara et al., 1999; Qu et al., 2006). Interestingly, altered prostaglandin metabolism has been reported in major affective disorders (Calabrese and Gulledge, 1984; Lowinger, 1989) and altered levels of prostaglandins have been implicated to cause mood disorders (Bishop et al., 1987; Lloyd, 1992). Moreover, prostaglandins, especially PTGDS, have important roles in hibernation cycle of mammals. Hibernation cycle is accompanied with dramatic physiological changes including periodic eating, sleeping and high psychomotor activity. Based on association of prostaglandins with hibernation and keeping in mind that the hibernation involves periodic and alternating behavior, we have developed the hibernation hypothesis of rapid cycling which might reflect an evolutionary ancient behavioral program in humans and become reactivated under pathological conditions.

In order to investigate the relevance of our findings in differential prostaglandin gene expression, we started a clinical experiment in which we offered the patient a treatment approach using cyclooxygenase-2 (COX2) inhibitor, celecoxib. Interestingly, celecoxib has previously been used in clinical trials on patients with major monopolar depression (Muller et al., 2006) and bipolar depression (Nery et al., 2008) both of which showed promising results. Moreover, increased prostaglandin E2 (PGE2) and COX2 production in schizophrenic and depressive patients has been discussed in a recent review which suggests inhibitors of this

enzyme to be used in clinical studies involving patients of the respective diseases (Muller and Schwarz, 2008). COX2 treatment on our patient, with a daily dose of 400mg, was continued for about 5 months and was well tolerated. Before and during treatment period, we performed psychopathology ratings on the patient to follow the disease course. Celecoxib treatment led to a significant reduction in overall manic and depressed rating scores over five months. The positive effect of celecoxib on our patient, who previously had been treated with many other drugs and had never benefited from any, made us emphasize the role of the prostaglandin metabolism in rapid cycling disorder and offer this new treatment approach to be used in more patients suffering from the same disease.

4.2 Original publication

Begemann M*****, **Sargin D***, Rossner MJ, Bartels C, Theis F, Wichert SP, Stender N, Fischer B, Sperling S, Stawicki S, Wiedl A, Falkai P, Nave KA, Ehrenreich H. (2008). Episode-specific differential gene expression of peripheral blood mononuclear cells in rapid cycling supports novel treatment approaches. Molecular Medicine 14 (9-10): 546-552

* Indicates equal contribution for the publications

Personal contribution:

I was involved in the design and concept of the experimental work, interpretation of the results, validating the work in the context of the existing literature (creating a literature data base), as well as preparation of the manuscript. I also performed microarray analysis and interpretation; choosing the genes interesting for biomedical analysis; primer design and testing for qRT-PCR. I confirmed the microarray data by qRT-PCR and performed the statistical analysis.

Episode-Specific Differential Gene Expression of Peripheral Blood Mononuclear Cells in Rapid Cycling Supports Novel Treatment Approaches

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[1]Division of Clinical Neuroscience and [2]Department of Neurogenetics, Max Planck Institute of Experimental Medicine, [3]Max Planck Institute of Dynamics and Self-Organization, and [4]Department of Psychiatry and Psychotherapy, University of Göttingen, Germany

Molecular mechanisms underlying bipolar affective disorders are unknown. Difficulties arise from genetic and phenotypic heterogeneity of patients and the lack of animal models. Thus, we focused on only one patient (n = 1) with an extreme form of rapid cycling. Ribonucleic acid (RNA) from peripheral blood mononuclear cells (PBMC) was analyzed in a three-tiered approach under widely standardized conditions. Firstly, RNA was extracted from PBMC of eight blood samples, obtained on two consecutive days within one particular episode, including two different consecutive depressive and two different consecutive manic episodes, and submitted to (1) screening by microarray hybridizations, followed by (2) detailed bioinformatic analysis, and (3) confirmation of episode-specific regulation of genes by quantitative real-time polymerase chain reaction (qRT-PCR). Secondly, results were validated in additional blood samples obtained one to two years later. Among gene transcripts elevated in depressed episodes were prostaglandin D synthetase (PTGDS) and prostaglandin D2 11-ketoreductase (AKR1C3), both involved in hibernation. We hypothesized them to account for some of the rapid cycling symptoms. A subsequent treatment approach over 5 months applying the cyclooxygenase inhibitor celecoxib (2 × 200 mg daily) resulted in reduced severity rating of both depressed and manic episodes. This case suggests that rapid cycling is a systemic disease, resembling hibernation, with prostaglandins playing a mediator role.

Online address: http://www.molmed.org
doi: 10.2119/2008-00053.Begemann

INTRODUCTION

Rapid cycling syndrome is a bipolar affective disorder, amounting to 10% to 30% of the bipolar population. It is characterized by at least four episodes per year and rapid shifts between cycles. Patients with bipolar affective disorder, as well as patients with rapid cycling syndrome, typically experience their first major mood episode during adolescence (1–5).

Recently, gene expression data from post mortem brains of bipolar patients were compared with those of healthy controls in two independent studies (6,7). While post mortem approaches certainly cannot reveal cyclic changes of gene expression, these studies also failed to yield a single overlapping candidate gene for bipolar disease. Moreover, the lack of an adequate animal model for bipolar disorder demands novel experimental approaches.

We hypothesized that cycling alterations of brain functions in bipolar disease are reflected by systemic physiological changes that have a molecular genetic basis. If true, it should be possible to obtain molecular signatures of manic and depressed states even outside the brain, such as in peripheral blood mononuclear cells (PBMC). While not disease causing, such gene expression changes in PBMC may shed light on similar cyclic alterations in brain.

To study quantitative peripheral gene expression, we specifically refrained from comparing larger groups of bipolar patients (who are genetically heterogeneous and differ in baseline gene expression profiles), and aimed instead at monitoring the gene expression in one individual, serving always as her own control, at recurrent stages of the disease.

CASE REPORT

The female patient, born in 1945, had no prior medical illness and no evidence of neuropsychiatric illnesses in her family. In 1991, she became ill with rapid cycling syndrome and kept a diary over her illness, used to reconstruct 108 cycles over a 16-year period. The time series suggests

**MB and DS made equal contribution to the manuscript.*

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Submitted May 1, 2008; Accepted for publication June 4, 2008; Epub (www.molmed.org) ahead of print June 5, 2008. Contributed by: Hannelore Ehrenreich.

complex rhythms in periodicity with mean total cycle lengths of 53 ± 21 d, switching within hours between manic (mean 28 ± 14 d) and depressed (mean 26 ± 14 d) episodes without normal intervals (Supplementary Figure 1). Results of affective rating scales obtained repeatedly during depressed and manic episodes, together with psychopathology, neuropsychological test results, appearance, autonomic, and physical signs are summarized in Table 1.

In addition to typical affective symptoms, the patient has physical and cognitive signs recurring in an episode-specific manner. In the first 2 to 3 d of a manic episode, she is sleepless and restless; in the following d, she sleeps 3 to 4 h per night. The 2 to 3 d before the end of manic episodes, she notes a 'normalization of sleep' with non-interrupted sleep of regular 8 h duration. The patient eats and drinks excessively during manic episodes, leading to alternating weight changes (up to 5 kg) between episodes and hyperhydration, resulting in significant shifts of hematocrit and hemoglobin concentrations. Three d after the onset of manic episodes, the patient regularly develops

Table 1. Psychopathology, physical signs and neuropsychological test results (before celecoxib).

Category	Depressed episode	Manic episode
Psychopathology		
Mood	dysphoric, despair, anxiety	expansive, exuberant, irritable
Drive	impaired: - most of the time in bed - social withdrawal - neglected personal hygiene - reduced self-care	increased: - seeking contacts - loss of inhibition - unnecessary purchases - booking of travels
Thought process	brooding, difficulty making decisions, slowed thinking	logorrhea, distractibility, poor concentration, racing thoughts
Suicidality	passive death wishes	*joie de vivre* ("Lebensfreude")
Gestures and mimic	mask-like face and slow limb movements oculomotoric decreased sad appearing withdrawn	rich in gestures and facial expression oculomotoric increased searching for eye contact and attention
Voice	low pitched, monotonous	high pitched, melodious
Handwriting	jittery and slow	orthographic mistakes and corrections
Dotting	moderately impaired	normal
Tapping	moderately impaired	moderately impaired
HAM-D[a]	range between 31 and 38	range between 3 and 4
BDI[b]	range between 44 and 52	range between 3 and 5
YMRS[c]	range between 1 and 3	range between 21 and 24
PANSS[d]	range between 84 and 92	range between 40 and 41
Autonomic and physical signs		
Sleep	increased requirement for sleep and daytime in bed	reduced requirement for sleep and disrupted sleep
Appetite	decreased	increased
Weight	decreased	increased
Libido	absent	increased
Allergy	no allergies	susceptibility to allergies
Edema	no edema	edema on lower extremities
Neuropsychology		
Short-term memory	mildly impaired	mildly impaired
Long-term memory	normal	normal
Working memory	moderately impaired	severely impaired
Semantic fluency	moderately impaired	normal
Executive functions	moderately impaired	severely impaired
Psychomotor speed	moderately impaired	moderately impaired

[a]Hamilton Depression Rating Scale.
[b]Beck Depression Inventory.
[c]Young Mania Rating Scale.
[d]Positive and Negative Syndrome Scale.

edema in her lower extremities that recover immediately after onset of depression. Only during manic episodes does she become susceptible to seasonal allergies (hay fever). This allergic response is rarely observed during depressed episodes. Witnesses describe a change of her voice in the last 2 to 3 d of manic episodes to raspy and less melodious. At the end of depressed episodes, her voice becomes more cheerful and richer in tonal inflections. The patient is not aware of these changes. Because there was an episode-specific susceptibility to allergens, lymphocyte subpopulations were studied by fluorescence-activated cell sorting in different episodes. Subtle shifts between CD4-helper and CD8-suppressor cells were noted (Supplementary Figure 2).

The cyclic pattern of the patient's affective disorder has had a poor response to pharmacologic treatment over the past 16 years, such as lithium, venlafaxine, chlorprothixene, citalopram, paroxetine, carbamazepine, valproic acid, trimipramine, lamotrigine, olanzapine, or flupentixol, and no response to psychotherapy and hypnosis. Antipsychotic medication, such as flupentixol, somewhat reduced the severity of symptoms but not the cyclic behavior of the disorder. During the time of all analyses presented here, the patient was on continuous lamotrigine medication (400 mg), resulting in comparable serum levels of 4.1–8.9 μg/mL upon repeated controls (therapeutic range: 2-10 μg/mL). No other medication was allowed 2 wks before or during the time of each testing, or before and during the treatment approach, reported here, using the cyclooxygenase inhibitor celecoxib (Celebrex, Pfizer, Karlsruhe, Germany, 2 × 200 mg daily per os).

MATERIALS AND METHODS

Strategy of Episode-Dependent Gene Shift Detection

A three-tiered approach was used to identify candidate genes that are expressed in an episode-specific fashion. In the first step, eight blood samples for PBMC isolation (see below) were collected (always at 8:00 a.m. after overnight fasting conditions) in the approximate middle of two different consecutive depressed and manic episodes on two consecutive days each. All sample collection was done well outside the hay fever season and in complete absence of allergic symptoms. The screening identified genes that showed at least two-fold differences in expression in manic compared with depressed episodes, and *vice versa*. After screening by microarrays, the data set was submitted to two bioinformatic processing steps (see below). The data were normalized and analyzed to identify and exclude genes that differed between the two consecutive days within a particular episode (arbitrary daily variation). Further genes were excluded that were differentially expressed within the two depressed or within the two manic episodes (arbitrary monthly variation). The expression pattern of the remaining depressed and manic episode genes was subsequently compared. Using this approach, several genes were identified that showed high expression in depressed and low in manic episodes, and *vice versa*. Because the patient never had periods where she was free of symptoms, samples could not be obtained within an euthymic episode.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were collected applying the standard Ficoll-Paque Plus isolation procedure (Amersham Biosciences, Freiburg, Germany). RNA was prepared using Trizol and Qiagen RNAeasy columns (Qiagen, Hilden, Germany). The RNA samples were used to synthesize cDNAs (SuperScriptIII, Invitrogen, Karlsruhe, Germany).

DNA Microarray Analysis

Transcriptome analysis was performed using the GeneChip Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) (8) according to the published protocols (9). All cDNAs used for microarrays were one-round amplified. GeneChip data were analyzed using the software GCOS version 1.2 (Affymetrix). Detailed data analysis was performed with R open-source software, and the open-source Bioconductor platform.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Genes found to be differentially regulated by DNA microarray analysis were subsequently validated independently using qRT-PCR. Cyclic changes in the expression of these genes were confirmed further on PBMC of additional blood samples obtained up to two years after the initial screen. qRT-PCR was performed with the aid of SYBR Green detection on Applied Biosystems 7500 Fast System. CT (cycle threshold) values were standardized to CT values of GAPDH. Primers are listed in Supplementary Table 1.

Statistical Analyses

All numerical results are presented as mean ± SD in the text and mean ± SEM in the Figures. Statistical analyses (10,11) and Fast Fourier Transformations (12) were performed as published using MATLAB R2007a software. Nonparametric independent Mann-Whitney *U* test (two-tailed) and Student *t* test (two-tailed) were calculated using SPSS 16.0 for Windows.

All supplementary materials are available online at molmed.org.

RESULTS

A three-tiered approach was employed to identify genes that were regulated in an episode-specific fashion (Figure 1A). We grouped the genes in biological categories and present their mean expression pattern over six to ten separate time points during either depression or mania (Figure 1B–1E).

Notably, the genes involved in prostaglandin metabolism, PTGDS (lipocalin-type prostaglandin D synthetase), and AKR1C3 (prostaglandin D2

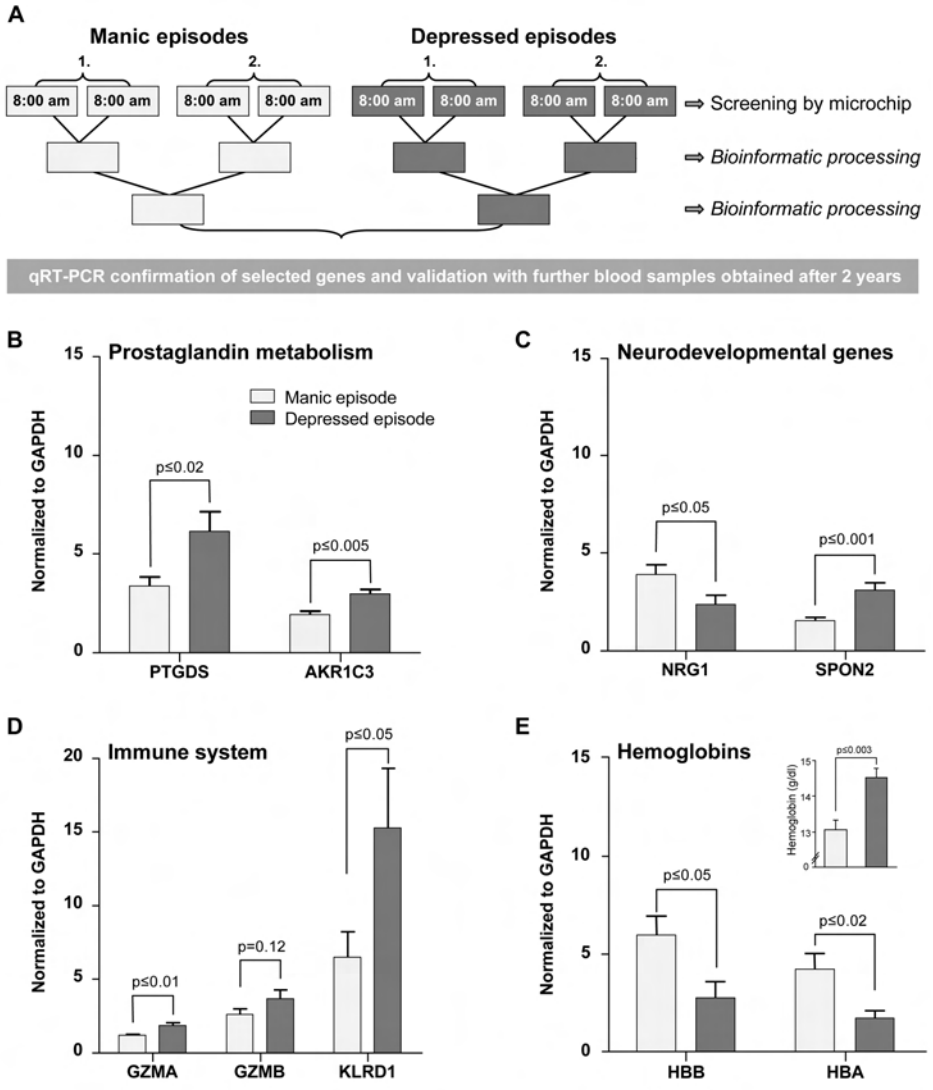

Figure 1. (A) Strategy of episode-dependent gene shift detection using microchip analysis. A three-step strategy was used to identify candidate genes that are expressed in an episode-specific fashion: Eight blood samples were collected (always at 8:00 a.m.) in two consecutive depressed and manic episodes on two consecutive days each. After screening by microarrays, the data set was submitted to two bioinformatic processing steps. Genes were subtracted that differed between the two consecutive days within a particular episode (arbitrary daily variation). Further genes were excluded that were differentially expressed within the two depressed or within the two manic episodes (arbitrary monthly variation). The expression pattern of the remaining depressed and manic episode genes was subsequently compared. (B–E) Episode-specific PBMC gene expression involves different groups of genes. Genes found to be differentially expressed by microchip screening were confirmed by qRT-PCR in all samples. Moreover, blood sampling was extended beyond the initial screening period, and regulated genes were again validated more than one year later. Genes were grouped (B–E) according to biological categories. Each bar represents mean ± SEM of 6 to 12 determinations from cDNAs obtained from PBMC on different days in independent manic (n = 6; open bars) and depressed (n = 5; filled bars) episodes. The chart for hemoglobins contains an insert with hemoglobin (protein) concentrations in whole blood (n = 7 samples of independent manic and depressed episodes, respectively). Mean values were compared using independent Student t test (two-tailed).

11-ketoreductase), showed higher expression in depressed episodes (Figure 1B). We also identified two neurodevelopmental genes that revealed opposing gene expression: NRG1 (neuregulin-1) was expressed higher in manic, SPON2 (spondin-2) in depressed episodes (Figure 1C). Several other genes, involved in regulation of the immune system, were episode-specifically expressed. These include GZMA (granzyme A) and KLRD1 (killer cell lectin-like receptor subfamily D, member 1/CD94) with higher expression in depressed as compared with manic episodes (Figure 1D). Hemoglobin genes A and B (HBB and HBA) were higher in manic, compared with depressed episodes. This contrasts the hemoglobin (protein) concentration in whole blood (insert) showing opposite regulation (Figure 1E).

As discussed below, the most intriguing finding was the association of prostaglandin-synthesizing genes PTGDS (lipocalin-type prostaglandin D synthetase) and AKR1C3 (prostaglandin D2 11-ketoreductase) with rapid cycling. We undertook a clinical experiment to explore the relevance of this observation and to distinguish between a disease marker and mediator. We offered to the patient a treatment approach applying (off-label) the cyclooxygenase inhibitor celecoxib (Celebrex, Pfizer, 2 × 200 mg daily oral).

Treatment with celecoxib was started with 100 mg and increased by 100 mg daily to reach the final dose of 400 mg (2 × 200 mg daily) at d 4. This dose has been continued for more than 5 months and is well tolerated. Figure 2 illustrates the clinical course before and during celecoxib, including psychopathology ratings that revealed a significant improvement of depressed as well as of manic symptoms.

DISCUSSION

In our first molecular-genetic approach to alternating gene expression in bipolar disorders, we used PBMC of a woman with 16-year history of an extreme form of rapid cycling, and obtained an episode-specific gene expression pro-

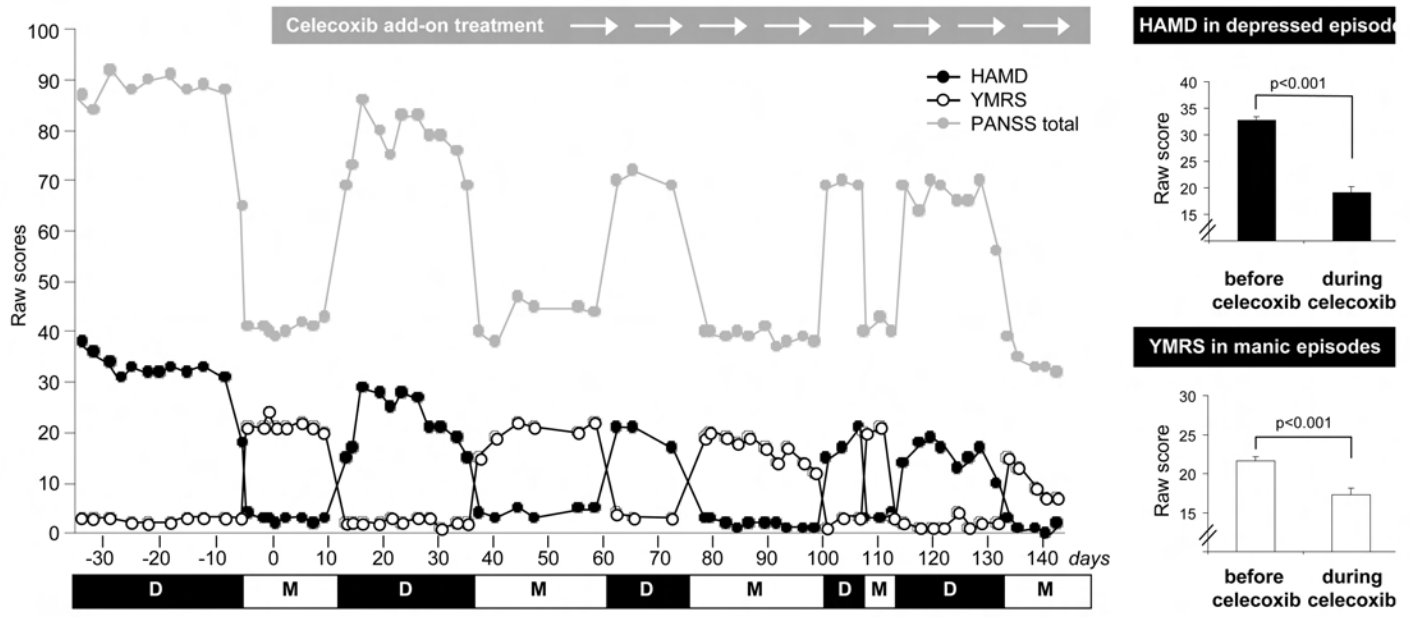

Figure 2. Clinical course of psychopathology ratings before and during treatment with the cyclooxygenase inhibitor celecoxib. The course of the Hamilton Depression Rating Scale (HAM-D) scores, the Young Mania Rating Scale (YMRS) scores, and the Positive and Negative Syndrome Scale (PANSS) scores, presented in line graphs, show a pronounced cycling pattern. Day 0 denotes start of celecoxib intake. The magnitude of cyclic changes appears to gradually decrease during treatment. Bar graphs give scores (mean ± SEM) of $n = 6$ depressed and $n = 6$ manic episodes (two before and four after onset of treatment each). HAM-D score in depressed and YMRS score in manic episodes decrease significantly upon treatment. Mean values are compared using independent Student t test (two-tailed). M = manic episode, D = depressed episode.

file on an identical genetic background. Our strategy was set up to minimize the risk of identifying false positive genes, due to daily or monthly variations in gene expression that are disorder-unrelated. The gene expression differences among episodes are small but significant, and would not have been recognized in a pool of patients or in comparison with healthy controls, due to different genetic background and modifiers of gene expression. In principle, such an approach could be employed as a screening strategy for genes in any condition with temporal periodic behavior, such as sleep or seasonal phenomena/disorders.

Follow-up studies on larger numbers of bipolar patients will have to follow, to confirm the general disease-relevance of the identified gene expression shifts. Specifically, patients with a more typical age of onset and course of bipolar disease will have to be screened. An age of onset of 46 years and cycling over decades without euthymic episodes, as in our patient, clearly is an exception (3–5).

The patient's impairments comprise psychopathological symptoms in combination with physical signs and symptoms, including the immune system, and a variety of cognitive domains evident upon neuropsychological testing. Accordingly, episode-specific gene expression involved different biological systems, such as blood, metabolism, immune functions, as well as neuronal genes, confirming rapid cycling as a systemic disorder. Like all association studies, we can make no claim whether or not a particular gene expression shift is a cause or consequence of rapid cycling, and whether similar gene expression changes occur in the brain. Moreover, it is currently unknown whether some of the observed alterations in gene expression partly reflect the shifts in immune cell subsets observed here.

Lamotrigine has been shown to alter expression of certain genes, such as GABA-A receptor β3 subunit in rat hippocampal cells (13). During the entire observation period (analysis and experimental treatment) reported in this paper, however, the patient was always on the same dose of lamotrigine (episode-independent), making an influence of this pharmacological treatment on the alternating gene expression shown here very unlikely. No other medication was used.

Some regulated genes identified in PBMC are known to be expressed in the nervous system. For example, NRG1 is a neuronal growth factor regulating differentiation, synaptogenesis, and myelination of the nervous system (14). NRG1 also is expressed in activated monocytes (15). Similarly, SPON2 (spondin, mindin) originally was characterized in zebrafish as a protein involved in outgrowth of hippocampal

neurons (16). It is expressed abundantly in lymphoid tissue and involved in inflammation (17).

Granzymes A and B (GZMA and GZMB) and several natural killer cell receptors were higher in depressed episodes. We speculate that the observed episode-specific gene expression contributes to the pathogenesis seen in our patient, including her allergic diathesis. In contrast, the elevated globin gene expression in manic episodes found here may reflect a physiological counterregulation to the relative increase in extracellular fluid (and decrease in hemoglobin level) due to massive drinking.

Interestingly, two genes were identified that are essential for prostaglandin synthesis, PTGDS and AKR1C3. PTGDS is preferentially expressed in the central nervous system and mediates synthesis of PGD_2 from PGH_2 (the cyclooxygenase-mediated product of arachidonic acid), and AKR1C3 mediates synthesis of $PGF_{2\alpha}$ from PGD_2. $PGF_{2\alpha}$ is a $PPAR_\gamma$ antagonist, in contrast to two other PGD_2 metabolites that are spontaneously converted from PGJ_2: Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 that are $PPAR_\gamma$ agonists. Prostaglandin synthesis plays a pivotal role in metabolic homeostasis, sleep regulation, adipogenesis, allergic response, and inflammation (18–22). Altered levels of prostaglandins have been detected in different body fluids in patients with major affective disorders (23–28). Also pharmacological evidence (29–31) suggests that altered prostaglandin metabolism may lead to mood disorders. All this is consistent with episode-specific differential gene expression of PTGDS and AKR1C3 as revealed in the present case.

Alterations in inflammatory markers in serum of bipolar patients, such as cytokines, c-reactive protein and components of the complement system, have been reported previously (32–38). There might well be an interaction between the prostaglandin system found to be episodically regulated here, in the described case, and these inflammatory molecules, also in the CNS (35,39,40).

Intriguingly, altered expression of PTGDS marks the hibernation cycle, and accumulation of prostaglandins during hibernation season has been described in hibernating animals (18,22). Based on our molecular data, we speculate that the rapid cycling syndrome in humans may reflect an evolutionary ancient behavioral program resembling the hibernation cycle (with periodic eating, high psychomotor activity and nesting behavior, alternating with episodes of rest and sleep) (41,42) that becomes pathologically re-activated by unknown triggers, thereby creating rapid cycling.

The gene products involved in prostaglandin metabolism might give clues to potential treatment targets, but to confirm their general relevance in bipolar disease, more patients will have to be examined for cycling-associated alterations in prostaglandin gene expression. Nevertheless, the positive result of the clinical experiment with celecoxib in our patient, showing considerable attenuation of both depressed and manic rating scores, supports a mediator role of prostaglandins in rapid cycling. We are fully aware of the limitation of a case report in comparison to a clinical trial. However, we note that the 16-year clinical history of our patient has never shown benefits from any of the many pharmacological treatments, suggesting that a potential placebo effect in our experiment would be minor.

Supporting our findings, a recent proof-of-concept trial in Germany including patients with major monopolar depression found beneficial effects on mood upon 6-wk add-on treatment with the cyclooxygenase-2 inhibitor celecoxib. In contrast to our gene expression-based "hibernation hypothesis of bipolar disease," this trial was based on the hypothesis that inflammatory processes might be involved in the pathogenesis of depression (43). Following the same inflammation hypothesis of depression, another small 6-wk study was performed in the United States of America, exploring the effect of celecoxib as adjunctive agent in bipolar depression, also with promising results (44). Bringing hibernation and inflammation together, it is intriguing to speculate that rapid cycling bipolar disorders are characterized by episodic self-limiting inflammatory processes that, unlike other inflammatory conditions in the brain, do not lead to overt neurodegeneration.

ACKNOWLEDGMENTS

The study was supported by the Max Planck Society, by several private donations, as well as by the DFG Center for Molecular Physiology of the Brain (CMPB). The authors would like to thank Jeannine Dietrich and Kathrin Hannke for excellent assistance and Fred Wolf for helpful discussions.

DISCLOSURE/CONFLICTS OF INTEREST

It is herewith declared that none of the authors have any conflicts of interest in publishing our data.

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5. Morphological and cellular consequences of chronic neurodegeneration induced by an early unilateral parietal cryo-lesion and treatment by EPO

5.1 Overview of project III

In this project, we analyzed cellular consequences of chronic neurodegeneration using a mouse model of unilateral parietal cryo-lesion. We have previously shown that upon an early unilateral parietal lesion, mice developed a global progressive brain atrophy and cognitive impairment that could be prevented by early EPO treatment (Siren et al., 2006). Mice lesioned at 28 days showed gray matter loss and cognitive deficits in a hippocampus-dependent learning and memory task, Morris water maze, at a late time point i.e. 9 months after lesion. These neurodegenerative changes were completely prevented when the mice were treated with EPO (5000 IU/kg, i.p.) for 2 weeks every other day starting right after lesion.

The cryo-lesion model in mice represents a mild traumatic insult at an early period in the life of a patient which might seem to be devoid of importance but might lead to the outcome of cognitive disorders like schizophrenia, Alzheimer's Disease and Parkinson's Disease (AbdelMalik et al., 2003; Bower et al., 2003; Koponen et al., 2002; Nemetz et al., 1999) when left untreated. Progressive cortical decline is a common phenomenon observed in these diseases. Therefore, it is important to understand what leads to the observed progressive pathology and how to mediate neuroprotection.

In order to find out the mechanisms that might contribute to the outcome of global brain atrophy induced by this early lesion, we performed a detailed stereological analysis on anterior cingulate cortex and hippocampal subregions of 1 year old mice. This was the time point lesioned mice had already developed atrophy and cognitive deficits. The regions analyzed in this study were chosen according to

their importance for cognitive functions in rodents. The findings of the histological analysis were further supported by immunoblotting experiments.

Unexpectedly, lesioned mice did not have differences with respect to their neuron and astrocyte numbers from sham-operated and EPO-treated mice i.e. they did not show any signs of neuronal loss or gliosis despite the fact that they had global brain matter loss and cognitive decline. This finding was consistent with the pathology seen in cognitive disorders like schizophrenia which is characterized by neurodegenerative changes accompanied by gray matter loss, ventricular enlargement, cognitive impairment in the absence of neuronal loss or gliosis. The relevance of our model for schizophrenia led us to explore the cellular consequences of global atrophy in more detail by focusing on subtypes of neurons and glia, which were consistently reported to be disturbed in schizophrenic post mortem brain tissue.

The early lesion induced a bilateral increase in the number of IBA1 (ionized calcium-binding adapter molecule 1) positive microglia in anterior cingulate cortex and hippocampal subregions 24 hours after lesion. One dose of EPO treatment had already reduced the microglial number in respective areas in lesioned mice. Interestingly, when the mice were analyzed 11 months after lesion, they still had increased microglial numbers in hippocampus. This showed a lesion-induced persistent inflammatory response ongoing in the brains of lesioned mice. Similarly, EPO treated mice showed comparable microglia numbers to the sham operated mice.

In 1 year old lesioned mice, we detected a subtle decrease in the number of Olig1 positive oligodendrocytes and decreased expression of myelin-related proteins, CNPase (cyclic nucleotide phosphodiesterase) and MBP (myelin basic protein) in hippocampus. Early EPO treatment led to an increase in the number of oligodendrocytes and prevented the decrease in myelin protein expression.

A detailed analysis of a subtype of neurons, the Parvalbumin positive GABAergic interneurons, revealed a bilateral increase in the relative number of these neurons in the dentate gyrus of lesioned mice at the age of 1 year. This increase in the relative number of GABAergic interneurons was further supported by the increase in the expression of 67 kDa isoform of GABA synthesizing enzyme, GAD67, in the contralateral hippocampus of lesioned mice detected by Western blot analysis. Once again, EPO-treated mice had comparable numbers of Parvalbumin positive GABAergic interneurons and similar levels of GAD67 protein expression to the sham-operated mice.

The role of synaptic loss in the outcome of brain atrophy and cognitive decline led us to count the number of synapsin1 positive presynaptic boutons in cingulate cortex and hippocampus of different groups of mice. Confocal analysis of presynaptic bouton number revealed no change between sham, lesioned and lesion+EPO groups. However, Western blot analysis of synapsin1 protein in contralateral hippocampus showed a decrease in the expression of this protein in lesioned mice compared to sham-operated and EPO-treated mice. The failure to document this finding histologically might be due to subtle changes between the groups which might be difficult to reveal with the methods used in this study.

In summary, besides global cortical atrophy and cognitive impairment, lesion at an early age led to an increased inflammatory process assessed by an early and late increase in microglial numbers, decrease in the expression of myelin-associated proteins, increased expression in determinants of GABAergic neurotransmission and decreased expression of a presynaptic protein, synapsin 1. Interestingly, the late morphological and cellular consequences of this early lesion are similar to those observed in schizophrenic patients. In schizophrenia, increased microglial activation (van Berckel et al., 2008), decreased oligodendrocyte number and myelin-related gene expression (Davis and Haroutunian, 2003; Dracheva et al., 2006; Hakak et al., 2001; Hof et al., 2003), disturbances in Parvalbumin positive GABAergic interneurons as well as GAD67

protein expression (Akbarian et al., 1995; Bernstein et al., 2007; Volk et al., 2000) and decreased expression of synapsin 1 protein (Vawter et al., 2002) have been reported in frontal and temporal regions of the patients. Moreover, parietal cortex, involved in sensorimotor and cognitive functions, has been reported to be the starting point of gray matter loss in early-onset schizophrenic patients (Thompson et al., 2001).

The most striking finding in our study was that the morphological and pathological consequences of lesion could fully be prevented by early EPO treatment. With these findings we conclude that EPO treatment, besides its effects on improving cognition, could be effective in preventing the pathophysiology underlying cognitive disorders like schizophrenia and could be used as an add-on treatment approach in neuropsychiatric diseases.

5.2 Original publication

Sargin D*, Hassouna I*, Sperling S, Sirén AL, Ehrenreich H. Uncoupling of neurodegeneration and gliosis in a murine model of juvenile cortical lesion. Glia **(In press)**

* Indicates equal contribution for the publications

Personal contribution:

I wrote the manuscript, performed alone most of the work and was supported regarding stereological analyses by Imam Hassouna (working often in 2 shifts per day on the device).

Uncoupling of neurodegeneration and gliosis in a murine model of juvenile cortical lesion

Journal:	*GLIA*
Manuscript ID:	draft
Wiley - Manuscript type:	Original Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Sargin, Derya; Max Planck Institute of Experimental Medicine, Division of Clinical Neuroscience Hassouna, Imam; Max Planck Institute of Experimental Medicine, Division of Clinical Neuroscience Sperling, Swetlana; Max Planck Institute of Experimental Medicine, Division of Clinical Neuroscience Sirén, Anna-Leena; University of Würzburg, Department of Neurosurgery Ehrenreich, Hannelore; Max Planck Institute of Experimental Medicine, Division of Clinical Neuroscience
Key Words:	brain atrophy, erythropoietin, oligodendrocytes, microglia, schizophrenia

Uncoupling of neurodegeneration and gliosis in a murine model of juvenile cortical lesion

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Running Title: EPO prevents neurodegeneration

Number of words: 6386

Figures: 3 Table: 1

Key Words:
Neurotrauma, brain atrophy, erythropoietin, oligodendrocytes, microglia, schizophrenia

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Abstract

A small experimental cryolesion to the right parietal cortex of juvenile mice causes late-onset global brain atrophy with memory impairments, reminiscent of cognitive decline and progressive brain matter loss in schizophrenia. However, the cellular events underlying this global neurodegeneration are not understood. Here we show, based on comprehensive stereological analysis, that early *unilateral* lesion causes immediate and lasting *bilateral* increase in the number of microglia in cingulate cortex and hippocampus, consistent with a chronic low-grade inflammatory process. Whereas the total number of neurons and astrocytes in these brain regions remain unaltered, pointing to a non-gliotic neurodegeneration (as seen in schizophrenia), the subgroup of parvalbumin-positive inhibitory GABAergic interneurons is increased bilaterally in the hippocampus, as is the expression of the GABA-synthesizing enzyme GAD67. Moreover, unilateral parietal lesion causes a decrease in the expression of synapsin1, suggesting impairment of presynaptic functions / neuroplasticity. Reduced expression of the myelin protein CNPase (cyclic nucleotide phosphodiesterase), reflecting a reduction of oligodendrocytes, may further contribute to the observed brain atrophy. Remarkably, early intervention with recombinant human erythropoietin (EPO), a hematopoietic growth factor with multifaceted neuroprotective properties (intraperitoneal injection of 5000 IU / kg body weight every other day for three weeks), prevented all these neurodegenerative changes. To conclude, unilateral parietal lesion of juvenile mice induces a non-gliotic neurodegenerative process, susceptible to early EPO treatment. Although the detailed mechanisms remain to be defined, these profound EPO effects open new ways for prophylaxis and therapy of neuropsychiatric diseases, e.g. schizophrenia.

Introduction

Neurotrauma has been implicated as a risk factor for different neurodegenerative diseases like Alzheimer's disease (AD) (Nemetz et al., 1999), Parkinson's disease (Bower et al., 2003; Stern, 1991) and schizophrenia (AbdelMalik et al., 2003; Koponen et al., 2002; Malaspina et al., 2001; McAllister, 1998; Parker and Rosenblum, 1996). Progressive brain atrophy is a common phenomenon in these disorders. However, as neuronal death accounts for the massive atrophy seen in AD, absence of gliosis and documentable neuronal loss prohibited schizophrenia to be classified as a neurodegenerative disorder for many decades. With the development of modern MRI technology and application of whole-brain voxel based morphometry, we now know that schizophrenia is accompanied by gray matter loss (Suzuki et al., 2002; Wilke et al., 2001) and that this loss is indeed progressive (Ho et al., 2003; Hulshoff Pol and Kahn, 2008; Thompson et al., 2001).

We have recently described a model of chronic, slowly progressing neurodegeneration, induced by a *unilateral* parietal cryolesion, applied stereotactically through the intact skull of juvenile (4-week old) mice (Siren et al., 2006). This right parietal lesion leads to *bilateral* global cortical atrophy and cognitive impairment, strikingly mimicking the pathology observed in early-onset schizophrenia. Neurodegeneration, evident by the loss of gray matter and ventricular enlargement, progresses over time and becomes highly significant at 9 months after the lesion. Importantly, early treatment with recombinant human erythropoietin (EPO), a hematopoietic growth factor with a wide range of neuroprotective properties, prevents both brain atrophy and cognitive deficits observed in lesioned mice (Siren et al., 2006).

Nothing is known so far on late cellular / morphological consequences of the early set right parietal cortical lesion, i.e. the morphological basis of the observed global brain atrophy. The present paper has therefore been designed to elucidate

changes in cell numbers and in cellular composition underlying the progressing degenerative process. Due to the obviously similar pathophysiological consequences of juvenile parietal lobe lesion and schizophrenia, we focused on brain regions known to be affected in this disease, such as anterior cingulate cortex and hippocampus. We show here that unilateral parietal lesion at an early age leads to distinct bilateral changes in cellular composition in the absence of gliosis, and that the late morphological changes observed upon lesion can all be prevented by early EPO treatment.

Materials and Methods

Animals

For all experiments, male C57BL6 mice were used. They 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. All experiments were approved by and conducted in accordance with the regulations of the local Animal Care and Use Committee.

Surgery-Neurotrauma

Surgery was performed when mice were 4 weeks old. Before surgery, they were anesthesized with intraperitoneal (i.p.) injection of 0.25% tribromoethanol (Avertin; Sigma-Aldrich, Taufkirchen, Germany) (0.125mg/g of body weight). Surgery was performed on the right parietal cortex after exposing the skull through a scalp incision. A copper cylinder with a tip diameter of 1.0mm was filled with liquid nitrogen (-183°C) and placed stereotactically on the right parietal cortex (coordinates from bregma: 1.5mm posterior, 1.5mm lateral) for 60s. Sham-operated animals went through the same procedure without cooling the copper cylinder. Immediately after surgery, one group of lesioned animals was started on intraperitoneal injections of EPO (Epoetin-alpha, Janssen-Cilag, Neuss, Germany, 5000 IU/kg), whereas another group of the lesioned and all sham-operated mice received placebo (diluent for EPO). The drug treatment

went on every other day for 14 days. Animals were sacrificed 24h and 11 months after surgery for analysis. A total of 9-10 animals per group were used for histological analysis at the 24h time point and 13-15 mice per group were used for histological and Western blot analyses at 11 months after lesion. In all experiments described in the following sections, the experimenter was blinded concerning group assignment.

Histology and immunohistochemistry

Animals were anesthesized with 0.25% tribromoethanol (Avertin) (0.125mg/g, i.p.) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were removed, fixed overnight at 4°C with 4% PFA and placed in 30% sucrose / phosphate buffered saline (PBS) for cryoprotection. They were frozen on liquid nitrogen. Whole mouse brains were cut into 30µm thick coronal sections on a cryostat (Leica, Wetzlar, Germany) and kept in a storage solution (25% ethyleneglycol and 25% glycerol in PBS).

For immunohistochemistry, free floating sections were washed with PBS 3 times and incubated with 0.5% hydrogen peroxide for 30min to quench endogenous peroxidases. They were permeabilized and blocked with 5% normal serum of host species from which respective secondary antibodies were derived for 1h at room temperature. Sections were incubated with rabbit anti-GFAP (1:200, Sigma, St Louis, MI, USA), rabbit anti-IBA1 (1:5000, Wako, Neuss, Germany), mouse anti-parvalbumin (1:2000, Sigma, St Louis, MI, USA), and rabbit anti-Olig1 (1:100, Chemicon, Hampshire, UK) antibodies diluted in 3% normal serum, 0.5% Triton-X in PBS for 48h at 4ºC. After 3 washes with PBS, sections were incubated with biotinylated secondary antibodies for 1.5h. The staining was visualized by a peroxidase-labeled avidin-biotin kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (DAB; Sigma-Aldrich, Taufkirchen, Germany). Sections were floated in 1XPBS and mounted on Super Frost microscopic slides. They were allowed to dry overnight and coverslipped using DePeX (Serva, Heidelberg, Germany).

For Nissl staining, sections were mounted on Super Frost microscopic slides, washed in PBS, and immersed for 25min in a dilute cresyl violet stain (0.01%) in acetate buffer (pH 4.5). After being dehydrated in serial dilutions of ethyl alcohol, sections were coverslipped using DePeX.

Stereology

Serial coronal sections, spaced at regular intervals for a specific marker under investigation, were taken through anterior cingulate cortex (coordinates from bregma: 1.42 anterior, 0.22 posterior) and dorsal hippocampus (coordinates from bregma: 1.34-2.54 posterior) for quantitative analysis. Stereological determination of the total cell number was carried out using the optical fractionator technique (StereoInvestigator, MicroBrightfield, Magdeburg, Germany).

Cells were counted using a light microscope (Olympus BX-50) modified for stereology with 40X and 100X oil immersion objectives, 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). Cell numbers were estimated using the modified optical fractionator technique (Keuker et al., 2001; West et al., 1991).

All stereological analyses for cell counting were performed on both sides of anterior cingulate cortex and hippocampus. Hippocampus CA2/CA3 region is referred to as CA3 in text and figures. Neurons were counted in the pyramidal layers of CA1 and CA3 regions and in the granular layer of dentate gyrus. Non-neuronal cells including astrocytes, microglia and oligodendrocytes were counted in all the subfields of CA1, CA3 regions and dentate gyrus. To avoid artifacts, the first 3Mm of section depth was taken as a guard area where counting was not performed. In this way, surface cutting irregularities that may bias the counting

method were avoided. For all markers counted, the same inclusion and exclusion criteria were applied. The stereological sampling scheme was considered adequate when coefficient of error was less than 0.10 (West and Gundersen, 1990).

Immunofluorescence and confocal analysis

To determine the density of synapsin1 immunoreactive presynaptic boutons, sections were washed in PBS, permeabilized and blocked in 5% normal horse serum for 1h at 4ºC, and incubated at 4ºC 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). After washing with PBS, 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 analyzed within stratum radiatum of area CA1, stratum lucidum of area CA3 of hippocampus and molecular layer of dentate gyrus. Images were obtained at a zoom factor 4 using an inverted confocal laser scanning microscope (LSM 510; Carl Zeiss MicroImaging, Inc., Germany) with a 63X oil Plan-Apochromat objective (NA 1.4). 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 was used to calculate the number and density of immunoreactive punctae.

Protein extraction and immunoblot analysis

For protein analysis, mice were decapitated. Hippocampi were taken out, immediately frozen on dry ice and stored at -80ºC. For protein extraction, left (contralateral) hippocampi of mice were homogenized in 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 phenylmethysulfonyl-fluoride, 10µg/ml aprotinin and 10µg/ml leupeptin, using an Ultra-turrax homogenizer (Kinematica, Luzern, Switzerland). The lysates were centrifuged (1200rpm) at 4°C for 45min. The supernatant was kept at -20°C. For blotting, 20µg of protein 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], and boiled for 10min at 95°C. The protein samples were run on NuPAGE 4-12% Bis-Tris Gel (Invitrogen, Karlsruhe, Germany) for 1h at 180V and transferred to a nitrocellulose membrane. After blocking with 5% milk in Tween 20-Tris-buffered saline (TTBS) at room temperature for 1h, membranes were incubated in primary antibodies for mouse anti-CNPase (1:1000, Sigma, St Louis, MO, USA), rabbit anti-MBP (1:5000, Dako, Carpinteria, CA, USA), mouse anti-GAD67 (1:5000, Chemicon, Hampshire, UK), or mouse anti-Synapsin1 (1:10000, SYSY, Goettingen, Germany) with mouse anti-GAPDH (1:5000, Assay Designs, Ann Arbor, MI, USA) as an internal control. Immunoreactive bands were visualized by using secondary antibodies coupled to horseradish peroxidase by enhanced chemoluminescence (Amersham, Freiburg, Germany). Densitometric analysis of protein bands was performed using ImageJ software.

Statistical analysis

All group data are expressed as mean±S.E.M. Statistical analysis was performed using two-tailed unpaired Student's *t* test. Prism 4 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Differences were considered significant at $p<0.05$.

Results

Unilateral parietal cryolesion of juvenile mice leads to cortical atrophy in the absence of neuronal loss and gliosis

In a first step to understand the morphological basis of the global brain atrophy observed late after unilateral parietal cryolesion, neurons and glial cells were stereologically quantified bilaterally in cingulate cortex and subregions of the hippocampus (CA1, CA3, dentate gyrus) of 12 months old mice. At this age, i.e. 11 months after the lesion, mice have already developed a prominent cortical atrophy, as diagnosable by volumetrical magnetic resonance imaging (Siren et al., 2006). Surprisingly, lesioned animals showed no differences in the total number of neurons (Table 1). Also, the number of non-neuronal cells and the number of GFAP positive astrocytes were comparable between groups in all areas investigated (Table 1). Thus, despite obvious cortical atrophy, lesioned mice do not show neuronal loss or gliosis in selected brain areas.

Unilateral parietal cryolesion of juvenile mice induces distinct changes in the cellular composition that are prevented by early EPO treatment

In a next step, we systematically studied subgroups of cells / associated proteins, suspected to either mediate inflammation/degeneration, to explain brain matter loss, or to contribute to neuronal dysfunction.

EPO prevents lesion-induced increase in microglia numbers. In order to quantify microglial activity in response to lesion, we counted microglial cells expressing IBA1 (ionized calcium-binding adapter molecule 1), which is known to be upregulated in activated microglia (Ito et al., 1998; Ito et al., 2001) . *Unilateral* parietal injury of 4-week-old mice led to a significant *bilateral* increase in the number of IBA1 positive microglia in anterior cingulate cortex, CA1 and CA3 regions of the hippocampus as early as 24 hours after injury (Figure 1A). One single dose of EPO already reduced the microglial number in respective areas to an amount comparable to that observed in sham-operated animals. Since IBA1

antibodies also recognize macrophages, we cannot rule out at this point that a global macrophage invasion all over the brain upon temporary lesion-induced disruption of the blood-brain-barrier partly accounts for the increased numbers of IBA1 positive cells. These cells would then settle and behave morphologically like microglia. Importantly, when animals were analyzed 11 months after lesion, the lesioned group still showed more IBA1 positive cells when compared to sham and lesion+EPO groups, consistent with a chronic, persistent low-grade inflammation (Figure 1B).

In an approach to better understand pathophysiological consequences of the increased number of activated microglia, we employed quantitative PCR to determine expression of markers expected to be induced upon microglial activation. However, inflammation-related genes (e.g. Mac1, C1q , Cathepsin S, MHCII) were not found differently expressed in extracts of whole hippocampus of both ipsi- and contralateral hemispheres of the three experimental groups at 24h or 120h after lesion (data not shown).

Microglia showed a highly ramified morphology at all time points investigated in all experimental groups without further morphological signs of major activation upon lesion (Figure 1A and 1B). Thus, at both early and late time points, the microglia population displays rather subtle, mainly numeric activation, evident by an increase in IBA1 positive cells in response to mild injury. This low-grade persistent microgliosis is completely abolished by early EPO application.

EPO increases oligodendrocyte numbers and myelin proteins in lesioned mice. Since at the age of 12 months, total numbers of neurons and non-neuronal cells did not explain the atrophy observed upon early lesion, quantitative analysis of glial and neuronal subpopulations had to be performed in order to better understand the morphological basis of brain matter loss. Whereas, in both hemispheres, lesion tended to reduce Olig1 positive oligodendrocyte numbers, EPO-treated mice had significantly more of these cells in hippocampal areas and

did not differ from the sham group with respect to oligodendrocyte counts (Figure 2A). These findings are strongly supported by Western blot analysis of myelin proteins, also performed at the 12 months time point. In fact, the non-compact myelin protein CNPase (cyclic nucleotide phosphodiesterase) and the compact myelin protein MBP (myelin basic protein) were both found decreased (significantly or with strong tendency) in lesioned compared to sham and lesion+EPO mice. Underlining the late global effect of the unilateral small lesion on the brain, this was even detected in the contralateral hippocampus, a site far away from the lesioned area (Figure 2B).

EPO prevents changes in determinants of GABAergic neurotransmission. Stereological quantification of inhibitory parvalbumin positive GABAergic interneurons relative to the total neuron number revealed an overall tendency of an increase in the lesioned group within all investigated brain areas. This tendency reached significance in the dentate gyrus (Figure 2C). Western blot analysis of the 67kDa isoform of the GABA synthesizing enzyme, GAD67, in the contralateral hippocampus (again pointing to the global consequences of the unilateral small parietal lesion) showed a significant increase in the lesioned group compared to sham. EPO addition led to levels comparable to sham (Figure 2D).

EPO prevents lesion-induced reduction of synapsin1 protein. Confocal analysis of synapsin1 positive presynaptic bouton density in anterior cingulate cortex and hippocampal regions failed to document significant differences among the groups (Figure 3A). However, Western blot analysis of synapsin1 protein in the contralateral hippocampus showed a significant reduction upon lesion compared to sham and lesion+EPO groups (Figure 3B), pointing to disturbed presynaptic function / neuronal plasticity.

Discussion

A small circumscribed cryolesion, stereotactically applied through the intact skull, onto the right parietal cortex of juvenile mice leads to cognitive impairment and bilateral brain atrophy, highly reminiscent of the pathophysiology observed in schizophrenia, and completely abolishable by early EPO intervention (Siren et al., 2006). Focusing on cingulate cortex and hippocampal subregions of both hemispheres, we report in the present paper that this slowly progressing, subtle, non-gliotic neurodegeneration lacks obvious neuronal loss but is accompanied by (1) early onset and persistent microglial activation, (2) decrease in oligodendrocytes / myelin associated proteins, (3) relative increase in determinants of inhibitory GABAergic neurotransmission and (4) reduction of a presynaptic protein, synapsin1. All changes in cellular composition/protein expression are prevented by early EPO intervention, supporting the development of EPO add-on strategies for prevention/treatment of neurodegeneration in schizophrenia (Ehrenreich et al., 2004; Ehrenreich et al., 2007).

Intriguing although yet unexplained is the finding of a *bilateral* increase in microglia in cingulate cortex and hippocampus 24 hours after *unilateral* lesion which is still present at 11 months after the lesion. This finding is consistent with a low-grade chronic inflammatory process that spreads early over the midline of the brain, and most likely turns on and nourishes the slowly progressing degenerative process. Causal involvement of microglia mediated inflammation has been implicated for different degenerative diseases (Block and Hong, 2005; Hanisch and Kettenmann, 2007; Miller and Streit, 2007; Perry et al., 2007), even though there is increasing evidence of even protective roles of microglia under certain circumstances (Schwartz et al., 2006; Trapp et al., 2007). For example, microglia play a major role in synaptic pruning during normal brain development and the microglia-initiated synaptic stripping in response to axonal injury is viewed as a regenerative response (Cullheim and Thams, 2007; Trapp et al., 2007). In our setting, however, it is likely that the increased numbers of microglia

are detrimental rather than protective, since in the presence of EPO, microglial numbers are decreased as much as downstream neurodegenerative consequences. Even a subtle but persistent microglia activation could favor degeneration by a perpetual removal of synapses as evidenced here by the reduced expression of the presynaptic protein, synapsin1. The increase in microglial number in the absence of an aggressive microglial phenotype in our model might be due to the mild nature of the inducing lesion and matches the lack of both, neuronal loss and gliosis. Also in this respect, the mild pathology is strikingly reminiscent of schizophrenia (Bogerts, 1999; Harrison, 1999). In fact, slightly increased microglia activation has recently been demonstrated in schizophrenic patients using quantitative positron emission tomography (van Berckel et al., 2008).

A single dose of EPO is already sufficient in our model to reduce microglial numbers at the 24h time point, and many months after lesion, EPO treated mice have microglial numbers comparable to the sham group. Interestingly, effects of EPO on microglia activation, inflammation, regulation of major histocompatibility complex (MHC) class II expression and pro-inflammatory cytokine production have been described in rodent models of experimental autoimmune encephalomyelitis (EAE) (Agnello et al., 2002; Li et al., 2004), of ischemia (Sun et al., 2005; Villa et al., 2003), traumatic brain injury (Chen et al., 2007; Yatsiv et al., 2005), and of Parkinson's disease (Xue et al., 2007). In particular, EPO treatment reduced CD11b reactivity / mononuclear cell infiltration in EAE (Agnello et al., 2002; Li et al., 2004), traumatic brain injury (Yatsiv et al., 2005) or ischemia (Villa et al., 2003). In a rat model of Parkinson's disease, induced by 6-hydroxydopamine (6-OH) injection into the striatum, smaller numbers of activated microglia were detected in the ipsilateral substantia nigra of EPO-treated animals (Xue et al., 2007). All of these studies, however, have concentrated exclusively on early time points after EPO administration. To our knowledge, only reports (Gentleman et al., 2004; Nagamoto-Combs et al., 2007) but no systematic studies have been performed on persistent low-grade microglia activation in

conditions of neurotrauma/lesion associated neurodegeneration in general. Nothing up to now has been known regarding late effects of early EPO intervention on chronically activated microglia.

Oligodendrocytes, the myelin-forming glial cells of the central nervous system, have also been implicated to be affected in schizophrenia (Hof et al., 2003) (for review see (Karoutzou et al., 2008)) where, as already stated above, progressive gray matter loss and cognitive impairment occur in the absence of neuronal loss and gliosis. In fact, a reduction in the number of oligodendrocytes in prefrontal areas (Hof et al., 2003) as well as decreased expression of myelin-related genes in prefrontal (Davis and Haroutunian, 2003; Hakak et al., 2001) and anterior cingulate cortices and hippocampus (Dracheva et al., 2006) have been reported in studies on post-mortem brains of schizophrenic patients. Similarly, in the parietal cortical lesion model used here, which leads to mild progressive neurodegeneration, we find reduction in myelin proteins, CNPase and MBP, together with an overall tendency of decreased oligodendrocyte numbers in hippocampus of 12 months old mice. Nevertheless, it remains unclear whether the reduction in myelin protein expression/oligodendrocyte numbers is a cause or a consequence of lesion-induced (schizophrenia-induced) atrophy. We note that Cnp1-deficient mice develop progressive neurodegeneration with enlarged ventricles and reduced gray as well as white matter thickness evident at the age of 12 months (Lappe-Siefke et al., 2003). In the lesion model used here, EPO shows a potent neuroprotective effect by preventing the decrease in CNPase expression and increasing oligodendrocyte cell number. Along the same lines, EPO has been described to facilitate oligodendrocyte maturation in vitro (Sugawa et al., 2002) and increase oligodendrocyte progenitor cell proliferation and subsequent myelination in EAE models in vivo (Zhang et al., 2005).

Disturbances in GABAergic interneurons, especially those bearing the calcium-binding protein parvalbumin, and in the levels of GABA synthesizing enzymes (GAD) have been reported in schizophrenia (Akbarian et al., 1995; Bernstein et

al., 2007; Volk et al., 2000) (for review see (Benes and Berretta, 2001; Blum and Mann, 2002; Lewis et al., 2005)) and various other pathological conditions, e.g. stress (Czeh et al., 2005). GABA, the main inhibitory neurotransmitter of the brain, is critical for working memory functions (Constantinidis et al., 2002; Rao et al., 2000; Wang et al., 2002). Working memory impairment after cortical impact injury of rats is associated with an increase in GAD67 levels in prefrontal cortex and hippocampus, and parvalbumin positive interneurons are the GABAergic neuronal subtype responsible for this increase (Kobori and Dash, 2006). In the parietal lesion model applied here, we found a similar slight increase in the proportion of parvalbumin positive cells, also supported by an increase in GAD67 expression in the hippocampus 11 months after lesion. It is tempting to speculate that the elevated interneuron marker expression indicates alterations in inhibitory neurotransmission and/or changes in overall network activity in the lesioned brain. These alterations in turn might explain some of the observed behavioral/cognitive sequelae of juvenile parietal cortical lesion (Siren et al., 2006).

Decreased expression of synapsins have consistently been reported in cognitive disorders (Ho et al., 2001; Vawter et al., 2002). In the parietal lesion model used here, the reduced expression of synapsin1 protein, pointing to some presynaptic insufficiency and altered neuroplasticity, might be another factor contributing to the observed pathophysiology. The failure to histologically document a significant reduction in synapsin1 positive presynaptic bouton density is best explained by the subtle change in a most abundant morphological structure which may not be detected with counting but rather with protein analysis as shown here.

To conclude, EPO treatment within a time window of two weeks directly after *unilateral* parietal cortical lesion of juvenile mice can completely prevent *bilateral* morphological consequences of progressive neurodegeneration. This suggests that the mechanisms which lead to neurodegeneration and prove susceptible to intervention with EPO, are already turned on early after the lesion. These

mechanisms may involve the transient blood-brain barrier disruption that slowly spreads all over the brain (Siren et al., 2006), as well as the global, early-onset and persistent microglial activation reported here. The mild cryolesion, set at a vulnerable age to a vulnerable brain region, initiates a low-grade inflammatory process, leading to slowly progressing neurodegeneration which, in the absence of neuronal loss and gliosis, is characterized by alterations in myelin and presynaptic protein expression as well as in the proportion of inhibitory neurons. All consequences of this non-gliotic neurodegeneration are prevented by early EPO treatment, supporting the application of this growth factor for prophylaxis and therapy of neuropsychiatric diseases.

Acknowledgements

This study has been supported by the Max-Planck-Society as well as by the DFG Center for Molecular Physiology of the Brain (CMPB). DS has a Max-Planck fellowship.

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Table 1

Quantification of neurons and non-neuronal cells in cingulate cortex and hippocampal subregions of sham, lesion and lesion+EPO groups at 12 months of age did not reveal any significant differences.

	cingulate cortex	CA1	CA3	dentate gyrus
Cresyl violet staining				
total number of neurons (x10^3)				
sham	317±8	122±7	117±6	547±29
lesion	314±27	119±7	127±9	500±40
lesion+EPO	339±22	119±12	149±10	474±41
Cresyl violet staining				
total number of non-neuronal cells (x10^3)				
sham	198±21	230±18	175±7	116±10
lesion	253±11	240±20	190±16	117±13
lesion+EPO	223±13	233±10	232±27	119±10
GFAP staining				
number of GFAP positive cells (x10^3)				
sham	n.d.	78±24	68±20	51±11
lesion	n.d.	63±7	66±5	40±2
lesion+EPO	n.d.	74±14	71±3	47±6

n.d.=not done; numbers refer to n=4-6; mean±S.E.M. presented.

Figure legends

Figure 1

Increase in the number of IBA1 positive microglia 24 hours and 11 months after right parietal cortical lesion and prevention by early EPO treatment. **(A)** A significant bilateral increase in IBA1 positive microglia numbers in hippocampal regions at an early time point, 24 hours after lesion, can be prevented with a single dose of EPO. **(B)** Increase in microglia number upon lesion and prevention by EPO is still visible 11 months after lesion. **(Right column of A and B)** Representative pictures from the CA1 region of the contralateral hippocampus of a lesioned mouse are included. At both time points, microglia show a highly ramified morphology without signs of major activation. This morphology is comparable in all experimental groups. n=9-10 for 24h time point; n=5-6 for 11 months time point, Scale bar=10 Mm.

Figure 2

Decrease in myelin and increase in GABAergic protein expression together with respective cellular changes upon right parietal cortical lesion are prevented by EPO treatment. **(A)** A subtle decrease in the number of Olig1 positive oligodendrocytes 11 months after lesion, fully prevented by EPO, can be observed in the hippocampus of lesioned mice. **(B)** Western blot analysis of myelin proteins in contralateral hippocampus points to a reduction of CNPase and MBP proteins in the lesioned group which is corrected for by early EPO. Sample blot for CNPase is shown below the quantitative bar graph. **(C)** A strong tendency of an overall increase in the relative proportion of parvalbumin positive GABAergic interneurons upon lesion is prevented by EPO. **(D)** A significant increase in GAD67 protein expression in contralateral hippocampus of lesioned mice contrasts the comparable expression levels in sham and lesion+EPO groups. Sample blot is shown below the quantitative bar graph. **(Right column of A and C)** Representative pictures of Olig1 and parvalbumin staining from the CA1 region of the contralateral hippocampus of a lesioned mouse are included. The morphology is comparable in all experimental groups. n=5-6 for histological analysis, n=7-8 for Western blot analysis. PV, parvalbumin. Scale bar= 10 Mm.

Figure 3

Analysis of synapsin1 protein expression and synapsin1 positive presynaptic bouton density in the hippocampus following early right parietal cortical lesion. (A) Synapsin1 positive presynaptic bouton density in cingulate cortex and hippocampus does not significantly differ between sham, lesion and lesion+EPO groups. **(B)** Synapsin1 protein expression is decreased in the contralateral hippocampus of lesioned mice. This decrease is prevented by EPO treatment. Sample blot is shown below the quantitative bar graph. n=5-6 for histological analysis, n=7-8 for Western blot analysis.

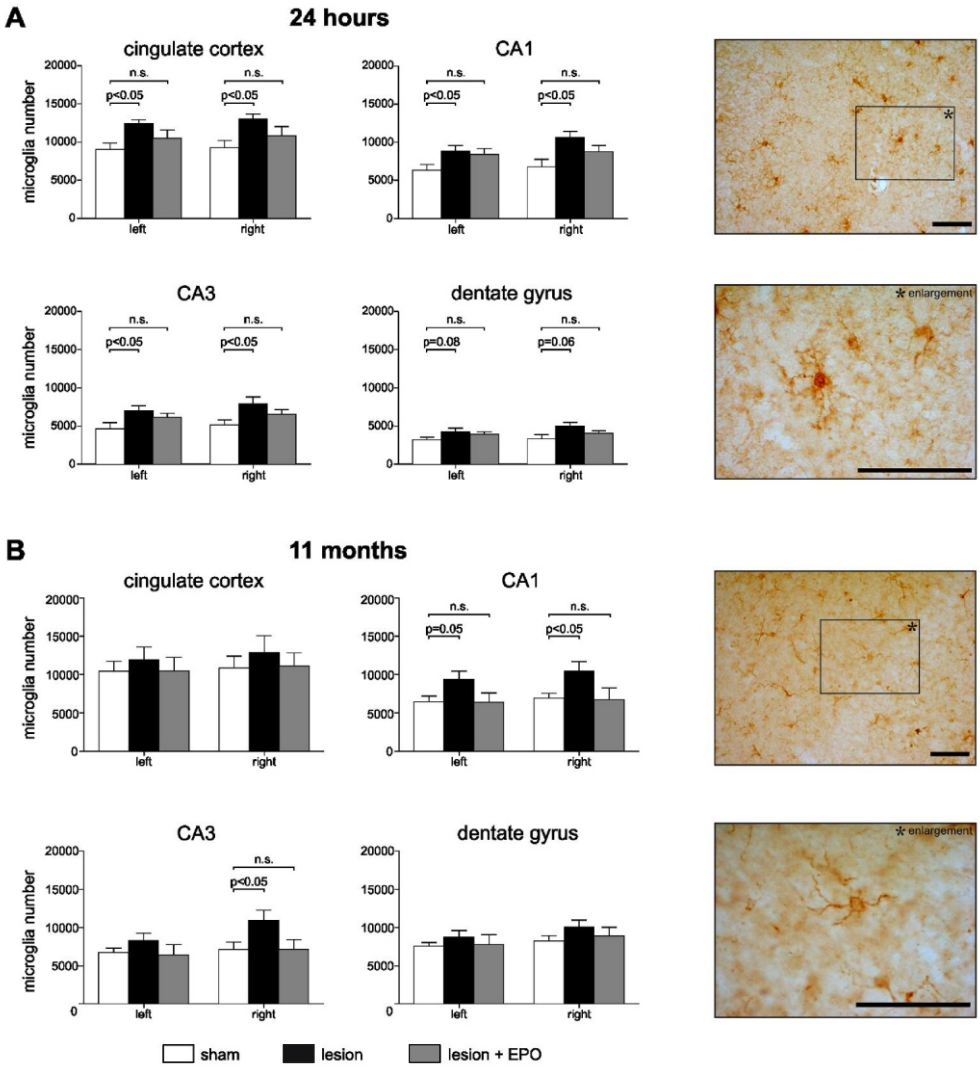
Table 1

Quantification of neurons and non-neuronal cells in cingulate cortex and hippocampal subregions of sham, lesion and lesion+EPO groups at 12 months of age did not reveal any significant differences.

	cingulate cortex	CA1	CA3	dentate gyrus
Cresyl violet staining				
total number of neurons (x10^3)				
sham	317±8	122±7	117±6	547±29
lesion	314±27	119±7	127±9	500±40
lesion+EPO	339±22	119±12	149±10	474±41
Cresyl violet staining				
total number of non-neuronal cells (x10^3)				
sham	198±21	230±18	175±7	116±10
lesion	253±11	240±20	190±16	117±13
lesion+EPO	223±13	233±10	232±27	119±10
GFAP staining				
number of GFAP positive cells (x10^3)				
sham	n.d.	78±24	68±20	51±11
lesion	n.d.	63±7	66±5	40±2
lesion+EPO	n.d.	74±14	71±3	47±6

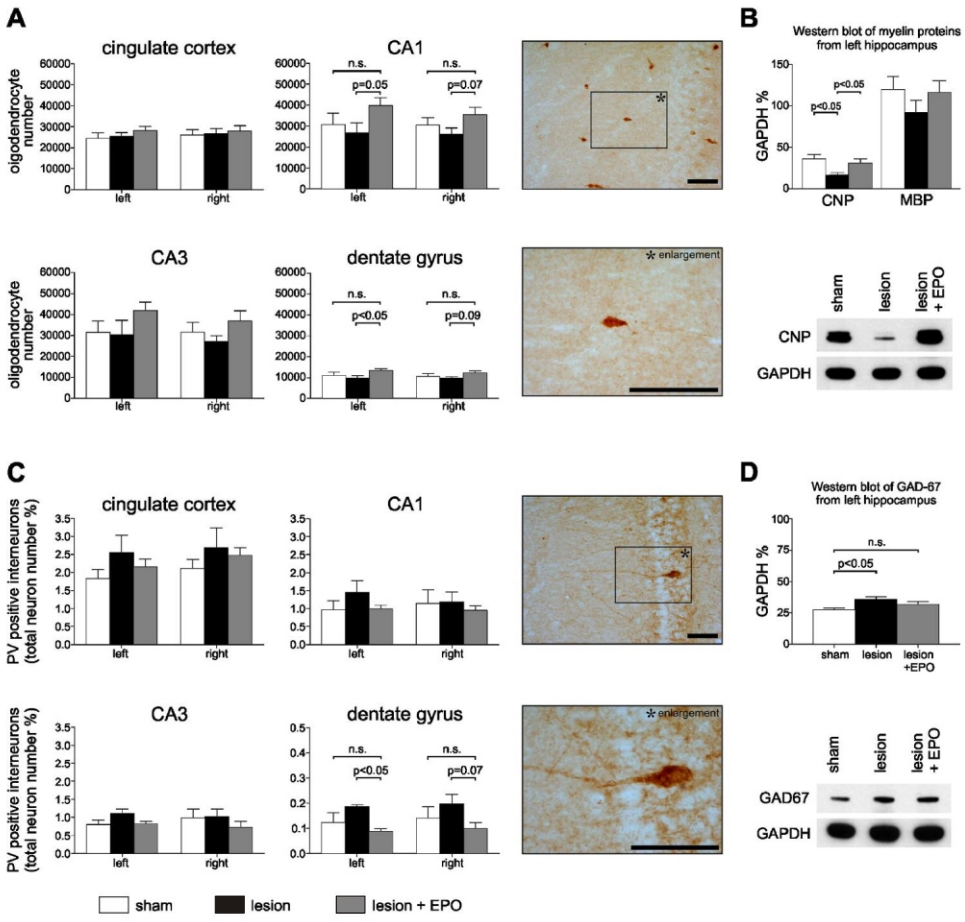
n.d.=not done; numbers refer to n=4-6; mean±S.E.M. presented.

Figure 1



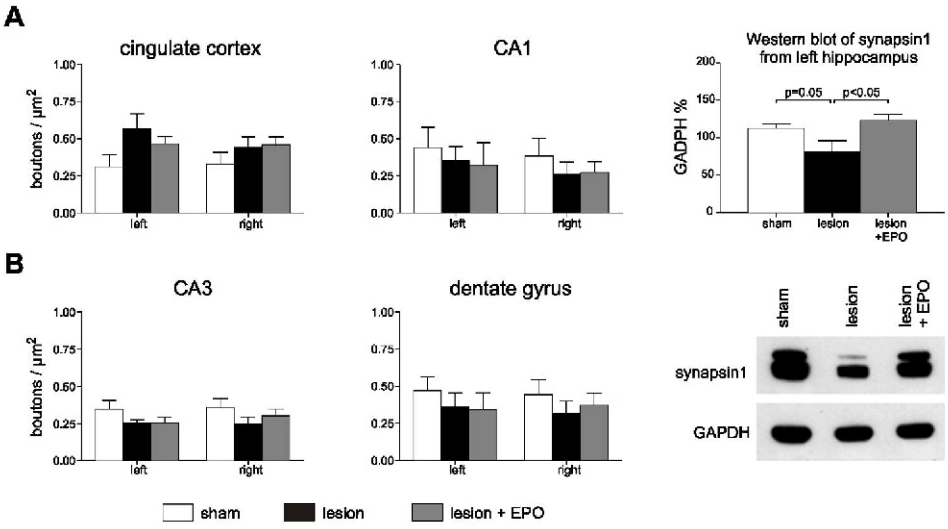
203x230mm (300 x 300 DPI)

Figure 2



254x249mm (300 x 300 DPI)

Figure 3


A
cingulate cortex
CA1
Western blot of synapsin1 from left hippocampus
boutons / µm²
1.00
0.75
0.50
0.25
0.00
left
right
GADPH %
200
150
100
50
0
p=0.05
p<0.05
sham
lesion
lesion +EPO
B
CA3
dentate gyrus
sham
lesion
lesion + EPO
synapsin1
GAPDH
sham
lesion
lesion + EPO

6. Summary

In the first original publication, we investigated effects of EPO on cognition and mechanisms of how EPO improves cognitive functions and affects synaptic plasticity.

We have shown that upon systemic EPO treatment for 3 weeks every other day, young healthy mice performed better in hippocampus-dependent memory tasks compared to the placebo treated group. This effect was observable 1 week after cessation of the treatment and interestingly, it was still maintained for another 3 weeks of EPO treatment-free period. 3 weeks after cessation of EPO treatment, the hematocrit levels were comparable between the 2 mouse groups. This has once more shown that EPO's effect on cognition is independent of its hematopoietic effect.

We performed a detailed analysis to reveal the mechanisms of EPO-induced cognitive improvement by focusing on synaptic function. Slices obtained from mice treated with EPO for 3 weeks and killed 1 week after cessation of the treatment, were subjected to electrophysiological analysis. The most intriguing finding was EPO's effect on increasing LTP. In parallel, magnitudes of STP and STD were significantly greater in EPO-treated mice. Moreover, whole-cell patch-clamp recordings on CA1 pyramidal neurons showed that EPO increased the frequency of sIPSCs and decreased the frequency of sEPSCs. Thus, EPO modulated inhibitory and excitatory transmission inversely. Interestingly, number of synapses in hippocampal subregions did not differ between EPO- and placebo-treated animals.

MEA recordings performed on primary hippocampal neurons in culture confirmed the direct EPO effect on neural cells. Primary hippocampal neurons isolated from mice at E17 were grown on MEA dishes and were treated with EPO every other day starting from day 5 in culture until day 25. Chronic application of EPO similar

to our *in vivo* approach resulted in prevention of a decrease in the number of silent channels upon maturation of the culture. Moreover, EPO treatment led to an increase in the number of bursting-channels. These results were in line with the *in vivo* data which also showed EPO's favourable action on inhibitory transmission.

To reveal EPO's action on single cell level, we used autaptic hippocampal neurons treated with EPO at day 7 and subjected to electrophysiological and immunocytochemical analyses on days 9-14. Single EPO addition led to a decrease in EPSC amplitude and readily-releasable pool size without affecting the total number of synapses.

Based on these findings, we conclude that, in addition to its effect on improving cognition under pathological conditions, EPO leads to an increase in hippocampus-dependent memory in the healthy brain. EPO's mechanism of action on synaptic plasticity seems to be in favor of inhibitory transmission without affecting the total number of synapses. We hypothesize that EPO modulates synaptic plasticity by increasing the activity of selected networks while keeping the others silent.

In the second original publication, we focused on a rare form of bipolar disorder, rapid cycling syndrome, and analyzed gene expression changes in different disease episodes in a rapid cycling patient. Depending on the gene expression results, we performed a clinical experiment offering a new treatment approach for our patient.

RNA isolated from PBMC collected at different manic and depressed episodes from the patient was subjected to a detailed microarray analysis. Genes selected based on microarray analysis, after excluding the ones that showed daily and monthly variations, were confirmed by qRT-PCR. With this approach, we identified a group of genes that showed alterations in different episodes of the

disease. These included genes that were involved in prostaglandin metabolism, neurodevelopment, immune and hematopoietic systems.

Based on our hypothesis that cyclic alterations in rapid cycling syndrome might reflect an ancient evolutionary program similar to hibernation cycle of mammals which is characterized by periodic eating, drinking, sleep and altered metabolism and that this program might be reactivated under certain pathological conditions, we focused on genes involved in prostaglandin synthesis which has been shown to have important roles in hibernation cycle of mammals (O'Hara et al., 1999). We performed a clinical experiment by offering the patient a treatment approach using a cyclooxygenase inhibitor celecoxib. Treatment with celecoxib over 5 months led to stabilization of manic and depressed episodes by reducing manic and depressed rating scores. Keeping in mind that celecoxib has been the only effective drug for our patient who has a 16 year disease history, we believe that more patients should be tested for the efficacy of this treatment in rapid cycling syndrome and to reveal the role of prostaglandin metabolism in this disorder.

In the third study, we investigated the mechanisms of atrophy and EPO-induced recovery upon a discrete cryo-lesion performed on the right parietal cortex of juvenile mice.

Mice lesioned at an early age (28 days old) developed a progressive neurodegenerative process characterized by gray matter loss, ventricular enlargement and cognitive decline which was highly significant 9 months after injury. EPO treatment right after the lesion for 2 weeks every other day prevented lesion-induced brain atrophy and cognitive decline (Siren et al., 2006). In order to investigate the mechanisms of lesion-induced atrophy, we performed a detailed histological analysis based on stereology on 1 year old mice. At this age, brain atrophy and cognitive decline induced by lesion were already evident.

Histological analysis was performed on anterior cingulate cortex and subregions of hippocampus. Interestingly, despite progressive global brain atrophy, lesioned mice had comparable number of neurons and astrocytes to the sham-operated and EPO-treated mice. This indicated a degenerative process going on without neuronal loss and gliosis similar to the characterization of brain atrophy in schizophrenic patients. We next focused on differences in neuronal and glial subpopulations between different groups of mice. 24 hours after a discrete unilateral lesion, a bilateral increase in the number of microglia was observed in all brain areas investigated. Interestingly, 11 months after lesion, increase in the microglial number was still observable in hippocampus. This showed a chronic persistent inflammatory response going on in the brains of lesioned mice. Early EPO treatment prevented the increase in microglial number at both time points. Lesioned mice at the age of 1 year had a slight reduction in the number of oligodendrocytes accompanied by reduced expression in myelin proteins. EPO treatment increased the number of oligodendrocytes and prevented the reduction in myelin protein expression. Moreover, EPO prevented lesion-induced increase in the ratio of Parvalbumin positive interneurons and GAD67 protein expression. Decrease in the expression of synapsin 1 protein upon lesion was also prevented by early EPO treatment.

Morphological and pathological consequences of an early brain injury are similar to those observed in schizophrenic brain. Thus, our parietal injury model serves a good basis to study the mechanisms of progressive atrophy and EPO treatment in cognitive disorders such as schizophrenia. Based on our findings, we conclude that EPO treatment in cognitive disorders might be beneficial in preventing the pathological changes leading to the outcome of these diseases.

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8. List of publications

1) Begemann M*, **Sargin D***, Rossner MJ, Bartels C, Theis F, Wichert SP, Stender N, Fischer B, Sperling S, Stawicki S, Wiedl A, Falkai P, Nave KA, Ehrenreich H. Episode-specific differential gene expression of peripheral blood mononuclear cells in rapid cycling supports novel treatment approaches. Molecular Medicine 14 (9-10): 546-552.

2) 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, Ehrenrich H. Erythropoietin enhances hippocampal long-term potentiation and memory. BMC Biology (In press).

3) **Sargin D***, Hassouna I*, Sperling S, Sirén AL, Ehrenreich H. Uncoupling of neurodegeneration and gliosis in a murine model of juvenile cortical lesion. Glia (In press).

***Indicates equal contribution for the publications**

4) Sirén AL, Radyushkin K, Boretius S, Kämmer D, Riechers CC, Natt O, **Sargin D**, Watanabe T, Sperling S, Michaelis T, Price J, Meyer B, Frahm J, Ehrenreich H. Global brain atrophy after unilateral parietal lesion and its prevention by erythropoietin. Brain 2006 Feb; 129(Pt2): 480-9.

5) Ehrenreich H, Bartels C, **Sargin D**, Stawicki S, Krampe H. Recombinant human erythropoietin in the treatment of human brain disease: focus on cognition. J Ren Nutr. 2008 Jan; 18(1): 146-53 (Review article).

6) Ehrenreich H, **Sargin D**, Sirén AL. The role of erythropoietin in neuroprotection and neuroregeneration. Chapter for the review book in the area of ‘Cell Death and Disease’ by Prof. Turgay Dalkara (In preparation).

9. Curriculum Vitae

PERSONAL DATA

Address	Papendiek 29 37073 Göttingen/Germany
Phone number	+49-551-3899605 +49-176-23527756
E-mail	sargin@em.mpg.de
Date of birth	October 16th 1980
Citizenship	Turkish

EDUCATION

Oct 2003 - present	MSc/PhD program in Neurosciences Max Planck International Research School, Göttingen
Sept 1998- June 2003	Bachelors degree in Molecular Biology & Genetics Bogazici (Bosphorus) University, Istanbul, Turkey GPA: 3,49 (out of 4,00) Major SPA: 3,9
Sept 1991 – June 1998	Beyoglu Anatolian English High School Graduated as Highest Honour Student

TEACHING EXPERIENCE

August 2008 – Dec 2008	Supervision of a Bachelor diploma student in the project ‘Analysis of constitutive erythropoietin receptor (cEPOR) overexpressing transgenic mice’ in the Division of Clinical Neuroscience, Max-Planck Institute of Experimental Medicine
6-8 June 2008	Junior lecturer in the interdisciplinary workshop: Translational Neuroscience, Block I, ‘Schizophrenia’ offered by the Division of Clinical Neuroscience, Max-Planck Institute of Experimental Medicine

PROFESSIONAL SKILLS AND TECHNIQUES

Molecular biology and genetics	Creating transgenic mouse lines, extraction and restriction enzyme digestion of DNA, ligation, cloning, agarose gel electrophoresis, PCR, RNA extraction, quantitative real-time PCR, sequencing, maintenance and growth of bacterial cultures, transformation
Cell culture	Experience with primary neuron cultures, PC12 cell line and microglia, transfection, immunocytochemistry
Biochemistry	Protein isolation, immunoblotting, immunoprecipitation, western blot analysis
Histology	Isolation of different brain regions from mice, perfusion, cryo- and paraffin sectioning, immunohistochemistry on frozen and paraffin sections, experience with laser-scanning microscopy and optical fractionator (stereology)
Mouse behavior	Experience with mouse handling, surgery, eight arm radial maze, fear conditioning, hole board, rota-rod, elevated plus maze, prepulse inhibition, open field, object recognition and Morris water maze
Computer skills	Knowledge and experience of Microsoft Office (Word, Excel, PowerPoint), image processing (Adobe Photoshop, Adobe Illustrator, ImageJ), statistics (GraphPad Prism), reference library (EndNote), genetic analysis (Lasergene)

AWARDS AND STIPENDS

2004 - present	Max Planck stipend
2003 - 2004	International Max Planck Research School stipend
1998 - 2003	Graduation with a High Honour degree from Department of Molecular Biology and Genetics, Bogazici University
1991 - 1998	Highest Honour degree in Beyoglu Anatolian English Highschool

ATTENDED CONFERENCES/SYMPOSIA

12-16 July 2008 Geneva, Switzerland	6th FENS Forum of European Neuroscience **Poster presentation**
17-20 May 2008 Magdeburg, Germany	International Symposium on Neuroprotection and Neurorepair **Poster presentation**
31 May-2 June 2007 Göttingen, Germany	International PhD Student Symposium Neurizons **Poster presentation**
5-8 April 2007 Istanbul, Turkey	New Frontiers in Understanding and Treating ALS
29 March-1 April 2007 Göttingen, Germany	31st Göttingen Neurobiology Conference **Poster presentation**
14-16 March 2007 Garmisch-Partenkirchen, Germany	The Annual Global Conference on Neuroprotection and Neuroregeneration **Poster presentation**
6-9 Sept 2006 Luebeck, Germany	7th International Luebeck Conference on the Pathophysiology and Pharmacology of Erythropoietin and other Hemopoietic Growth Factors **Poster presentation**
17-20 February 2005 Göttingen, Germany	30th Göttingen Neurobiology Conference
15-16 September 2005 **14-15 September 2007** **18-19 September 2008** Göttingen, Germany	International Symposia on Schizophrenia of the Göttingen Research Association for Schizophrenia (GRAS) **(in the Organizing Committee)**

LANGUAGES SKILLS

Turkish (native)

English (advanced)

German (intermediate)

EXTRACURRICULAR ACTIVITIES

Sports (gymnastics, swimming-licensed in highschool, basketball-licensed in highschool, step-licensed in university, windsurfing)

Reading