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Contradictory effects of erythropoietin on inhibitory synaptic transmission in left and right prelimbic cortex of mice

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ABSTRACT

Erythropoietin (EPO) has been shown to improve cognitive function in mammals as well as in patients of psychiatric diseases by directly acting on the brain. In addition, EPO attenuates the synaptic transmission and enhances short- and long-term synaptic plasticity in hippocampus of mice, although there are still many discrepancies between different studies. It has been suggested that the divergences of different studies take root in different *in-vivo* application schemata or in long-term trophic effects of EPO. In the current study, we investigated the direct effects of EPO in slices of prelimbic cortex (PrL) by acute *ex-vivo* application of EPO, so that the erythropoietic or other trophic effects could be entirely excluded. Our results showed that the EPO effects were contradictory between the left and the right PrL. It enhanced the inhibitory transmission in the left and depressed the inhibitory transmission in the right PrL. Strikingly, this lateralized effect of EPO could be consistently found in individual bi-lateral PrL of all tested mice. Thus, our data suggest that EPO differentially modulates the inhibitory synaptic transmission of neuronal networks in the left and the right PrL. We hypothesize that such lateralized effects of EPO contribute to the development of the lateralization of stress reaction in PFC and underlie the altered bilateral GAGAergic synaptic transmission and oscillation patterns under stress that impact the central emotional and cognitive control in physiology as well as in pathophysiology.

1. Introduction

The prefrontal cortex (PFC) plays an essential role in the integration of cognitive and affective behavior and regulating autonomic and neuroendocrine functions. It has extensive connections with the limbic areas and with other cortical areas (Uylings et al., 2003) that directly controls the regulation of emotional responses (Bandler et al., 2000) as well as socio-affective and visceromotor behaviors (Damasio, 2000; Damasio et al., 2000). Within the PFC, the prelimbic cortex (PrL) and infralimbic cortex (IL) project to different nuclei of amygdala (Gabbott et al., 2005; Vertes, 2004) that play different roles in central fear control (Anglada-Figueroa and Quirk, 2005; Herry et al., 2008; Maren and Quirk, 2004; Pape and Paré, 2010). In addition, the distribution of PV^+- and NPY^+-interneurons is different in the dorsal and ventral parts of PrL, suggesting their different functional impacts in central control of emotion (Saffari et al., 2016).

Patients with damage in the PFC fail to show autonomic responses to emotionally charged stimuli and exhibit greatly impaired emotional and social functioning, decision-making and risk assessment (Damasio et al., 2000). Interestingly, studies in PFC lesioned patients suggest that decision-making abilities are lateralized to the right PFC areas (Clark and Manes, 2004). In addition, the lateralization of the PFC occurs very early during neurodevelopment in rodents and interhemispheric interactions (activation/inhibition) between the left and the right PFC are especially important when emotional processing is involved (Cerqueira et al., 2008).

The hemispheric lateralization is likely to be of relevance to the pathogenesis of psychiatric diseases, as lateralized disturbances of brain structure or function, most notably in the PFC, have been reported in patients suffering from major depressive and anxiety disorders (Davidson et al., 1999; Johnstone et al., 2007). Furthermore, patients with strokes in the left frontal lobe tend to have a disproportionate incidence of depression, while comparable damage to the right frontal lobe often leads to indifference, hypomania or mania (Robinson et al.,

Abbreviations: EPO, erythropoietin; EPOR, receptor for erythropoietin; PFC, prefrontal cortex; PrL, prelimbic cortex; ACC, anterior cingulated cortex; IL, infralimbic cortex; IPSC, inhibitory postsynaptic current; GABA, Gamma-Aminobutyric acid; GAD67, glutamic acid decarboxylase 67

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1984). It has been, thus, suggested that this lateralized difference is likely to reflect the influence of both PFC hemispheres in emotion and behavior (Wittling, 1997). Furthermore, neurochemical and anatomical lateralization have also been found in animal studies and, in some instances, have been associated with the behavioral asymmetries as well as after chronic stress (Czéh et al., 2008), while the left PFC seems to be more responsive to high levels of corticoid and more vulnerable to stress than the right PFC (Cerqueira et al., 2008). It has also been found that extra stimulation in early life can enhance existing asymmetries or can induce asymmetries where none previously existed (Denenberg, 1983).

Erythropoietin (EPO) is a glycoprotein that belongs to the cytokine superfamily. It was originally characterized as the main factor of erythropoiesis (Lasne and de Ceaurriz, 2000). In addition to this known function, EPO has been reported to have anti-apoptotic, anti-inflammatory, anti-oxidative, and trophic properties (Aoshiba et al., 2009). EPO receptors (EPORs) expression was first characterized in kidney and liver. Since then, it has been receiving increasing attention in neuroscience as its expression have also been found in different regions of the central nervous system (CNS; Maiese et al., 2012). In cell culture, it has been shown that astrocytes and neurons express EPOR (Masuda et al., 1993; Morishita et al., 1997) and produce EPO (Masuda et al., 1994). EPO and EPOR are also expressed in neurons and glia in the brain of rodents and primates (Hasselblatt et al., 2006; Jelkmann, 2005). Moreover, a subpopulation of neural crest cells produce EPO very early in the embryogenesis (Suzuki et al., 2013) and that it is critical for normal brain development (Alnaeeli et al., 2012). Thus, these data suggest EPO exerts a hematopoiesis-independent autocrine, paracrine or synaptic effect in the brain of mammals.

Indeed, EPO has direct effects in the brain (Adamcio et al., 2008). It has been shown neuroprotective effects in hypoxia and ischemic damage induced models (Adamcio et al., 2010; Brzecka, 2005; Undén et al., 2013), and after traumatic brain injury (Adamcio et al., 2010; Mahmood et al., 2007; Y. Zhang et al., 2010). In addition, beneficial EPO effects have also been reported in neurodegenerative diseases of animal models (Esmaeili Tazangi et al., 2015; Hamidi et al., 2013; Maurice et al., 2013) as well as in different animal models of neuropsychiatric diseases (Catania et al., 2002; Kumral et al., 2004; Mogensen et al., 2004; Sirén et al., 2006). In a double-blind, placebo-controlled study in chronic schizophrenic patients, EPO improves schizophrenia-relevant cognitive performance independently of its hematopoietic effects (Ehrenreich et al., 2007b). Similarly, an increase in cognitive performance upon EPO in patients with chronic progressive multiple sclerosis has been occurred independently of changes in hemoglobin levels, and persisted for months after termination of EPO treatment (Ehrenreich et al., 2008, 2007a). Furthermore, EPO has also been shown to improve the cognitive ability in patients with mood disorder (Miskowiak, 2017; Miskowiak et al., 2018, 2010).

On the other hand, published data have also shown that EPO attenuates the excitatory synaptic transmission (Adamcio et al., 2008; Almaguer-Melian et al., 2016; Kamal et al., 2011), whereas the inhibitory synaptic transmission was found to be facilitated (Adamcio et al., 2008; Wójtowicz and Mozrzymas, 2008), and short- and long-term synaptic plasticity was enhanced by EPO in the hippocampus (Adamcio et al., 2008; Kamal et al., 2011; Wójtowicz and Mozrzymas, 2008). In addition, EPO prevents the effect of chronic restraint stress in male rats (Aalling et al., 2018). Furthermore, transgenic expression of a constitutively active EPOR isoform (cEPOR) in pyramidal neurons of cortex and hippocampus exhibit augmentation of spatial learning, cognitive flexibility, social memory, and attentional capacities (Sargin et al., 2011).

In summary, the currently available data clearly indicate that EPO can improve cognitive function of mammals by directly acting on the brain, although there are still many discrepancies between different studies whether the cellular effects of EPO are just long-term consequences of erythropoiesis (Adamcio et al., 2008; Kamal et al., 2011; Wójtowicz and Mozrzymas, 2008). It has been suggested that the divergences of different studies take root in different *in-vivo* application schemata or in long-term trophic effects of EPO (Adamcio et al., 2008; Kamal et al., 2011; Wójtowicz and Mozrzymas, 2008). Our previous data showed EPO effects in hippocampus two weeks after *in-vivo* application (Adamcio et al., 2008; Wójtowicz and Mozrzymas, 2008). In the current study, we investigated the direct effects of EPO on inhibitory transmission in the left and the right PrL of mice. In our experiments, EPO was applied *ex-vivo*, so that erythropoietic and other long-term effects can be entirely excluded. Our results demonstrated that the EPO effects on inhibitory synaptic transmission were contradictory on the left and the right PrL. Interestingly, these lateralized effects of EPO could be consistently found in the individual bi-lateral PrL of tested mice, suggesting that EPO differentially modulate the inhibitory synaptic transmission of neuronal networks in the left and the right PrL.

2. Methods

2.1. Ethical approval

The experiments were performed in accordance with the European Communities Council Directive (86/EEC) and were approved by the Federal State Office for Consumer Protection and Food Safety of North Rhine-Westphalia, Germany (LANUV NRW).

2.2. Animals

For most immunohistochemistry and electrophysiology, adult male wild-type C57BL/6 mice (10–16 weeks old) were used (details s. Supplemental Information). To further analyze the distribution of EPOR and the EPO effects on GABAergic interneurons in PFC, transgenic GAD67-GFP mice (10–16 weeks old) were tested.

2.3. Immunohistology

Coronal sections of 25 µm in thickness were cut using cryostat (Leica CM3050 S, Leica Microsystems Nussloch, Nussloch, Germany) from the PFC of the brains (from 1.98 to 1.54 mm anterior to Bregma) for immunohistochemistry and immunofluorescent procedures. For detection of EPOR and GAD67, standard immunohistochemistry and immunofluorescent staining procedures were performed (details provided in Supplemental Information) with two different primary antibodies including, anti-EPOR (Sc-5624; Santa Cruz biotechnology; 1:500) and anti-GFP (sc-101536; Santa Cruz biotechnology; 1:500; s. Saffari et al., 2016). We also did negative control, where the immunohistochemistry was conducted exactly as the normal procedure, except the primary antibody was excluded. In such cases, no stained cells could be detected (data not shown).

2.4. Image acquisition and analysis of immunohistochemistry and immunofluorescent

For quantification, the brain areas and the layer borders were defined according to the mouse brain atlas (Paxinos G, Franklin KBJ) and based on cytoarchitectural features as described before (Saffari et al., 2016) Multiple alignment of images taken with × 4 and × 10 magnifications was performed with Cell^P software (Olympus). Distributions of positively stained cells were analyzed using ImageJ software (NIH, Bethesda, MD, USA) in the anterior cingulated cortex (ACC), PrL and IL. For each region, mean numbers of cells as well as cell density (cell × mm^{-2}) were calculated across all the layers in above regions.

To count and quantify GAD67 positive neurons and EPOR positive cells for immunofluorescent procedure, sections were viewed with confocal imaging 700-AX10 laser scanning microscope (Carl Zeiss). GAD67 positive neurons and EPOR positive cells were counted across

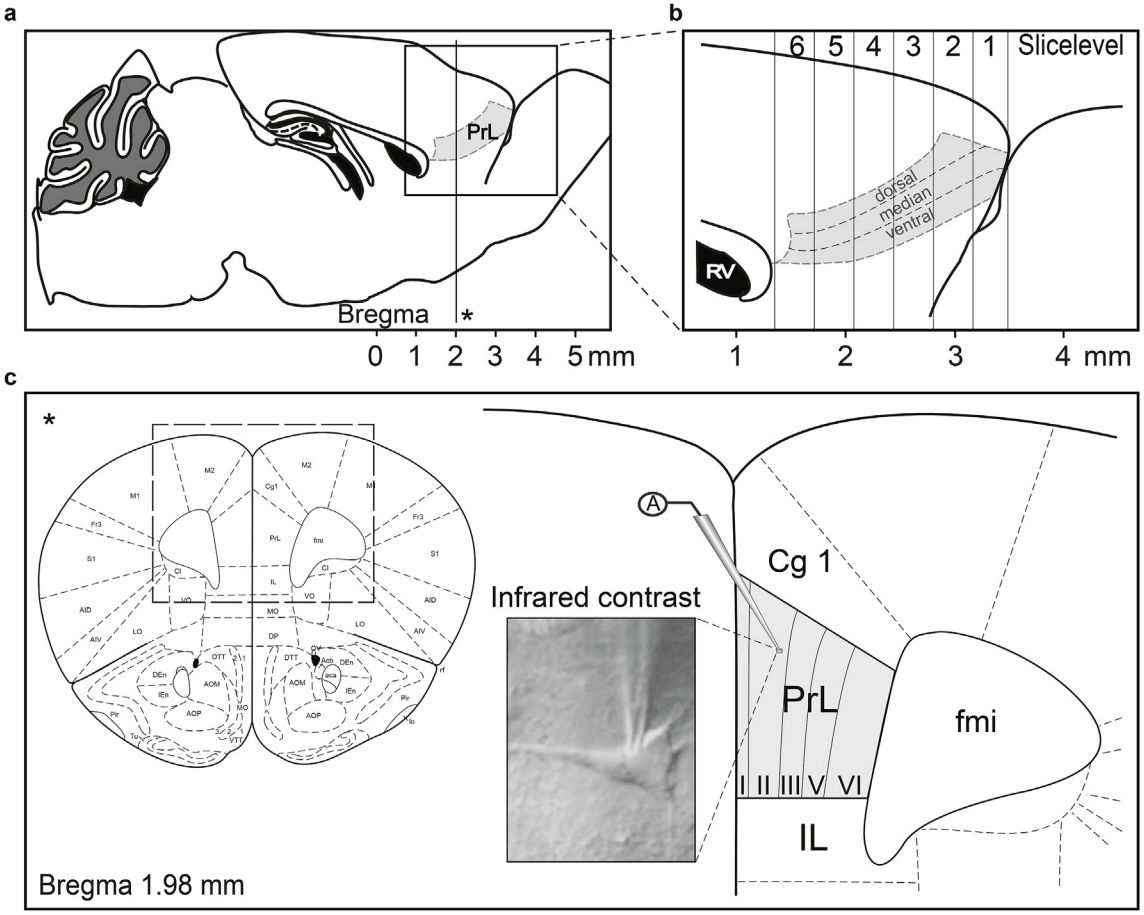

Fig. 1. Schematic drawing of the brain area where the neurons have been recorded. (**a**) longitudinal view of brain of mouse and the location of PrL; (**b**) schematic drawing of dorsal, medial and ventral part of PrL; (**c**) schema of coronal view of brain of mouse, where pyramidal neurons located in layer II of PrL have been recorded. (modified after mouse brain atlas, Franklin and Paxinos, 2007).

all the layers in medial prefrontal cortex in order to calculate the percent co-localization of both markers. (more details about procedures s. Supplemental Information).

2.5. Electrophysiology

For PFC slice preparation of wild-type mice the brain was quickly removed after decapitation and immersed for 2–3 min in ice-cold cutting solution (3 mM KCl, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 0.2 mM $CaCl_2$, 10 mM glucose, 124 mM sucrose). Transverse slices (300 µm) containing PrL and IL (Schema see Fig. 1) were cut with a 752M vibroslicer (Campden Instruments, Loughborough, UK). The slices were then transferred to an incubation chamber and allowed to recover for at least 60 min at 33 °C, followed by incubation at RT. The recording chamber was continuously perfused with artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 10 mM glucose, aerated with 95% O_2 and 5% CO_2 (by flow rate of 3–4 ml/min).

All recordings were performed in neurons of PrL, where the sites (left or right PrL) and the locations of recorded neurons (dorsal, medial or ventral PrL) were carefully noticed based on mouse atlas, and the data were analyzed according to their locations (Fig. 1). Pipette solution contained 140 mM KCl, 1 mM $CaCl_2$, 10 mM EGTA, 2 mM $MgCl_2$, 4 mM Na_2ATP, 0.5 mM Na_2GTP, 10 mM HEPES, pH 7.2.

Spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) were recorded at a holding potential of −70 mV in the presence of the 10 µM AMPA-receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 µM NMDA-receptor antagonist 2-amino-5-phosphonovaleric acid (AP5), and 2 µM glycine receptor antagonist strychnine. Signals with amplitudes at least twofold above the background noise were analyzed. There were no significant differences in noise levels between all tested animals. Patches with a serial resistance of > 20 MΩ, a membrane resistance of < 0.8 GΩ, or leak currents of > 150 pA were excluded. The membrane currents were filtered by a four-pole Bessel filter at a cut-off frequency of 2 kHz, and digitized at a sampling rate of 5 kHz using the DigiData 1322A interface (Axon Instruments/Molecular Devices, Sunnyvale, CA). Data acquisition was performed using commercially available software pClamp 10.1 (Axon Instruments/Molecular Devices). MiniAnalysis 6.0.9 (Synaptosoft, Decatur, GA) and Prism 5 (GraphPad Software, San Diego, CA) were used to perform amplitude, frequency and other statistical analysis of sIPSCs.

2.6. Drug application

Epoetin alfa (final concentration: 5000 I.U./l) was obtained from Janssen-Cilag GmbH (ERYPO® Janssen-Cilag GmbH, Germany). For testing the direct effect of EPO, spontaneous inhibitory synaptic activities were recorded 30 min after *ex-vivo* application of EPO (5 I.U./ml) in all experiments. 7-nitroindazole (7-NI, 100 mM), a selective inhibitor of neuronal NO synthase, was obtained from Sigma-Aldrich (St Louis, MO). 7-NI was incubated for 15 min before the recording started. All other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

2.7. Statistical analysis

Data were presented as mean ± SEM with number of cells per

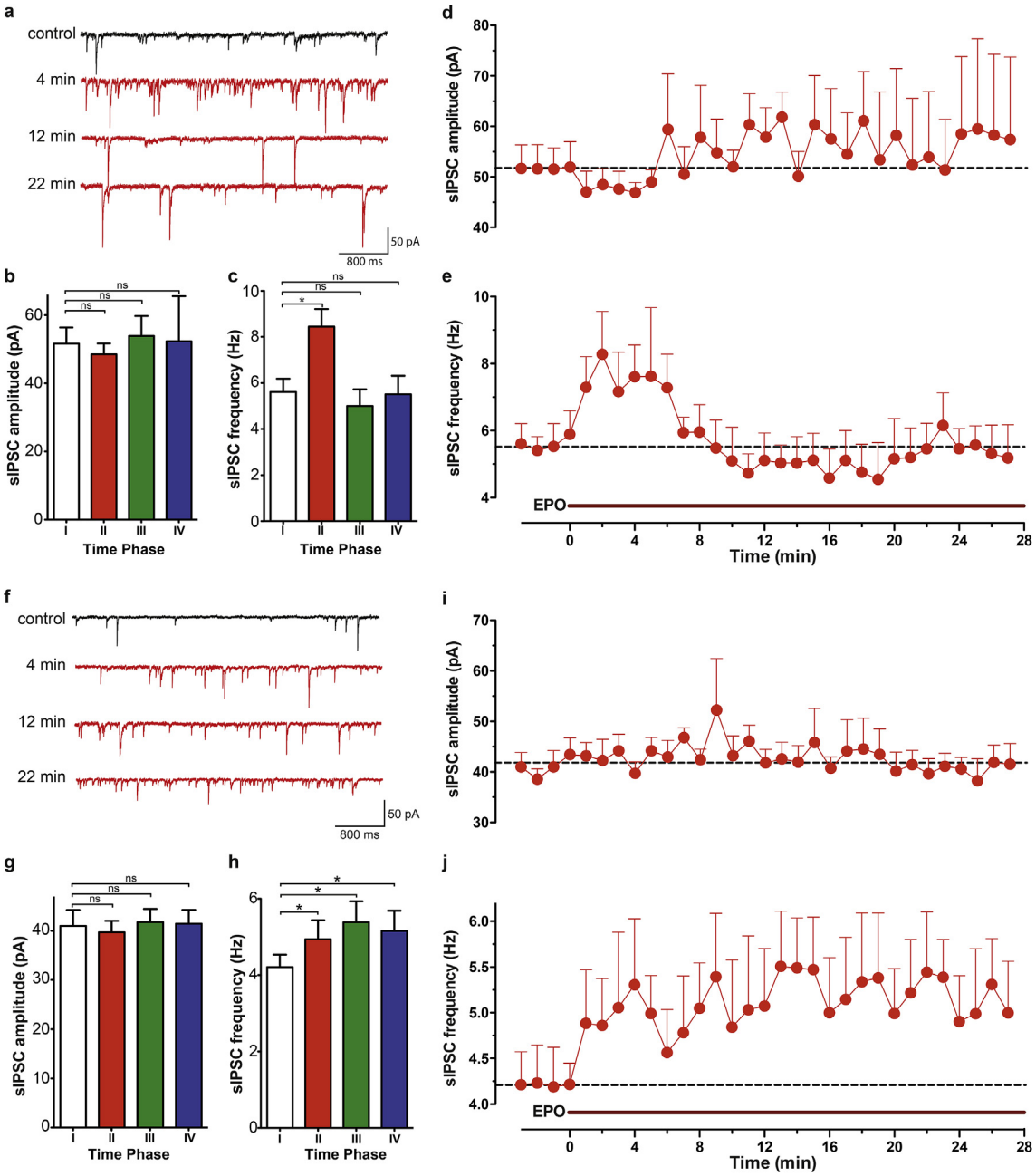

Fig. 2. Type I: EPO transiently enhances the inhibitory synaptic transmission in the left PrL. (**a**) Sample traces of sIPSCs before and 4, 12 and 22 min after *ex-vivo* application of EPO; (**b**) the averaged amplitudes and (**c**) frequency of spontaneous IPSCs in layer II pyramidal neurons of PrL before and after *ex-vivo* application of EPO; Each filled circle within the histogram indicates the averaged frequency of one individual recording; (**d**) time trace of averaged changes of IPSC amplitude before and after *ex-vivo* application of EPO; (**e**) time trace of averaged changes of IPSC frequency before and after *ex-vivo* application of EPO. **Type II**: EPO enhances the inhibitory synaptic transmission in left PrL. (**f**) Sample traces of sIPSCs before and 4, 12 and 22 min after *ex-vivo* application of EPO; (**g**) the averaged amplitudes and (**h**) frequency of spontaneous IPSCs in layer II pyramidal neurons of PrL before (I) and after *ex-vivo* application of EPO (II, III, IV); Each filled circle within the histogram indicates the averaged frequency of one individual recording; (**i**) time trace of averaged changes of IPSC amplitude before and after *ex-vivo* application of EPO; (**j**) time trace of averaged changes of IPSC frequency before and after *ex-vivo* application of EPO. (I-IV: time phase before (I) and 4 (II), 12 (III) and 22 min (IV) after *ex-vivo* application of EPO).

animals indicated in parentheses. One-way ANOVA test were used for time phase analysis in Figs. 1–2. Data on EPO positive cell numbers (Fig. 4) were analyzed 2-way ANOVAs followed Boferroni post-test. Statistical significance is indicated as * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$.

3. Results

3.1. EPO enhances the inhibitory synaptic transmission in the left PrL

We first test the effects of EPO on GABAergic inhibition of pyramidal neurons in layer II of left and right of PrL. By each experiment, the site and the location of patched neurons were carefully noticed based on mouse atlas, and the data were analyzed according to their locations (Fig. 1). The results showed that, in all slices obtained from

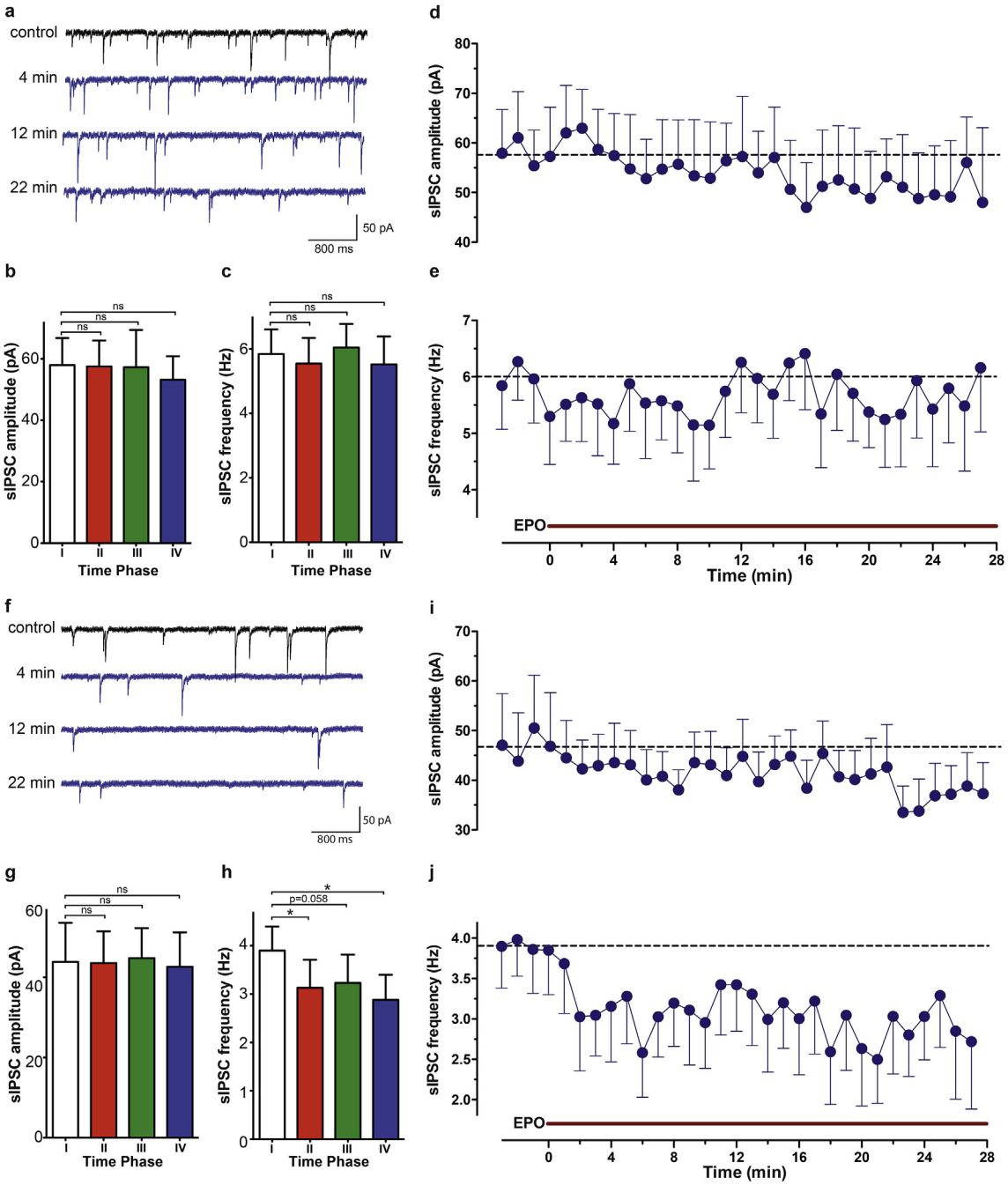

Fig. 3. Type III: EPO does not significantly alter the inhibitory synaptic transmission in right PrL. (**a**) Sample traces of sIPSCs before and 4, 12 and 22 min after *ex-vivo* application of EPO; (**b**) the averaged amplitudes and (**c**) frequency of spontaneous IPSCs in layer II pyramidal neurons of PrL before (I) and after *ex-vivo* application of EPO (II, III, IV); Each filled circle within the histogram indicates the averaged frequency of one individual recording; (**d**) time trace of averaged changes of IPSC amplitude before and after *ex-vivo* application of EPO; (**e**) time trace of averaged changes of IPSC frequency before and after *ex-vivo* application of EPO. **Type IV**: EPO decreased the inhibitory synaptic transmission in right PrL. (**f**) Sample traces of sIPSCs before and 4, 12 and 22 min after *ex-vivo* application of EPO; (**g**) the averaged amplitudes and (**h**) frequency of spontaneous IPSCs in layer II pyramidal neurons of PrL before and after *ex-vivo* application of EPO (II, III, IV); Each filled circle within the histogram indicates the averaged frequency of one individual recording; (**i**) time trace of averaged changes of IPSC amplitude before and after *ex-vivo* application of EPO; (**j**) time trace of averaged changes of IPSC frequency before and after *ex-vivo* application of EPO (I-IV: time phase before (I) and 4 (II), 12 (III) and 22 min (IV) after *ex-vivo* application of EPO).

the left PrL, application of EPO enhanced the frequency, but did not alter the amplitude of sIPSCs (Fig. 2). Interestingly, the EPO effects in the left PrL could be summarized in two different types that were referred to as type I and type II.

Type I: the *ex-vivo* application of EPO significantly increased the frequency of sIPSCs within 4 min (control phase I: 5.5 ± 0.4 Hz vs. phase II EPO: 7.8 ± 0.6 Hz; n = 5, $p < 0.05$; Fig. 2a,c,e), while the amplitudes were not significantly affected (control phase I: 52.2 ± 4.8 pA vs. phase II EPO: 45.5 ± 3.3 pA; n = 5, n.s.; Fig. 2a,b,d). These effects attenuated quite fast in the further presence of EPO, so that the frequency of sIPSCs return to the basal level within 12 min (Phase III EPO: 5.9 ± 0.5 Hz; n = 5, n.s.; Fig. 2a,c,e) and remained on this level after 22 min (Phase IV EPO: 5.8 ± 0.6 Hz; n = 5, n.s.; Fig. 2a,c,e). During the same time period, the amplitudes of sIPSCs

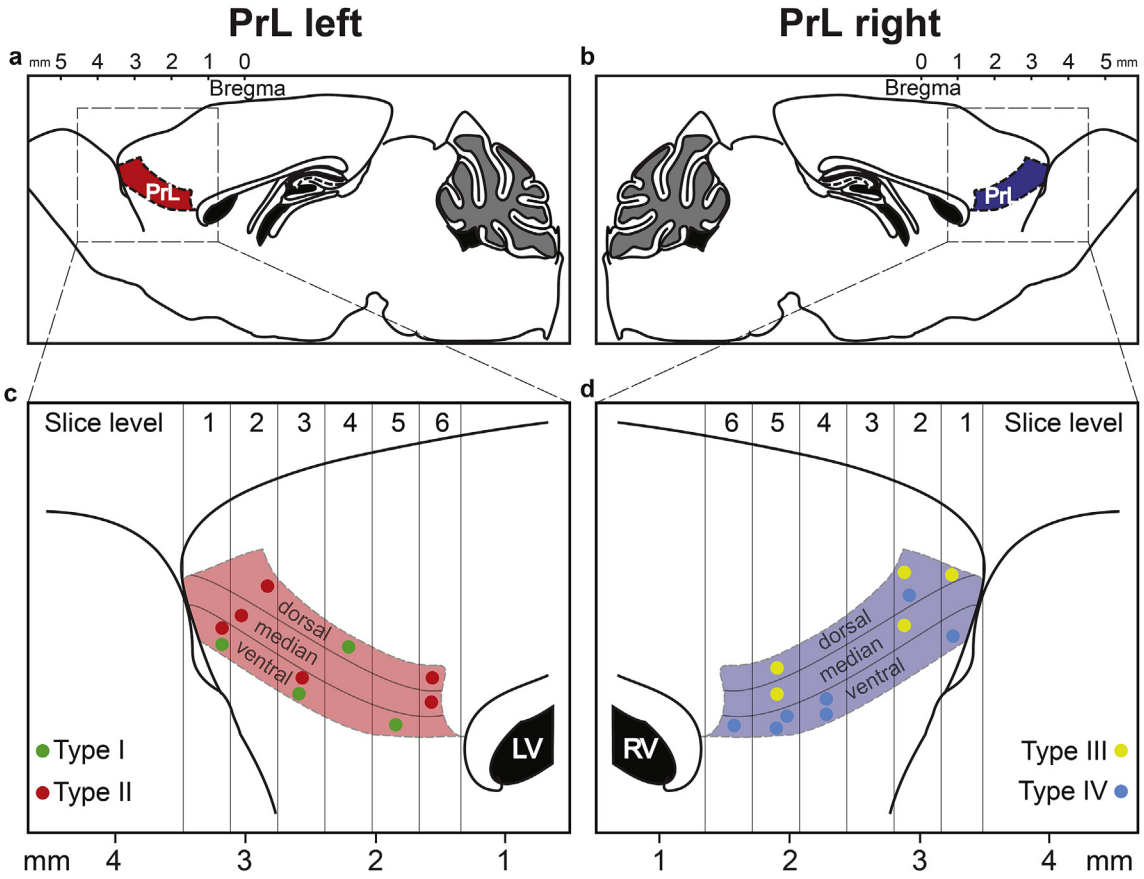

Fig. 4. Schematic drawing of left and right brain area where the neurons have been recorded. (**a**) longitudinal view of left brain of mouse and the location of PrL; (**b**) longitudinal view of right brain of mouse and the location of PrL; (**c**) schematic drawing of left PrL where the type I neurons (green dots) have been recorded in ventral part, whereas type II neurons (red dots) in dorsal and medial part of PrL; (**d**) schematic drawing of right PrL where the type III neurons (yellow dots) have been recorded in dorsal and medial part, whereas type IV neurons (blue dots) in ventral part of PrL; (modified after mouse brain atlas, Franklin and Paxinos, 2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were unaffected (Fig. 2a,b,d).

Type II: here, the *ex-vivo* application of EPO significantly increased the frequency of sIPSCs within 4 min (control phase I: 4.8 ± 0.1 Hz vs. phase II EPO: 6.2 ± 0.2 Hz; n = 10, p < 0.001; Fig. 2f,h,j), while the amplitudes were not significantly affected (control: 40.4 ± 3.1 pA vs. phase II EPO: 39.8 ± 2.6 pA; n = 10, n.s.; Fig. 2f,g,i). But, the elevation of frequency of sIPSC remained after 12 min (phase III EPO: 5.9 ± 0.1 Hz; n = 10, p < 0.001; Fig. 2f,h,j) and even 22 min in the presence of EPO (Phase IV EPO: 6.4 ± 0.1 Hz; n = 10, p < 0.001; Fig. 2f,h,j). At the same time period, the amplitudes of sIPSCs remained unaffected (Fig. 2f,g,i).

It is quite interesting that all recorded type I neurons were located in more ventral part of the left PrL, while all recorded type II neurons were located in more dorsal part of the left PrL (Fig. 4c). In all experiments on the left PrL, the results were quite consistent and the described two types of EPO effects remained until we lost the cell (about 20–40 min, data not shown).

3.2. EPO attenuates the inhibitory synaptic transmission in the right PrL

In contrast, our results obtained from slices of the right PrL showed that *ex-vivo* application of EPO either decreased or had no significant effects on frequency, and in all cases, it did not alter the amplitude of sIPSCs (Fig. 3). Similarly, the EPO effects on the right PrL could be summarized in two different types that were referred as type III and type IV.

Type III: the *ex-vivo* application of EPO showed no significant effect on frequency of sIPSCs within 4 min (control phase I: 5.9 ± 0.5 Hz vs. phase II EPO: 5.8 ± 0.6 Hz; n = 7, n.s.; Fig. 3a,c,e), and the amplitudes were not altered (control phase I: 58.9 ± 8.6 pA vs. phase II EPO: 58.5 ± 8.2 pA; n = 7, n.s.; Fig. 3a,b,d). The frequency of sIPSCs remained unaltered 12 min (phase III EPO: 6.2 ± 0.6 Hz; n = 7, n.s.; Fig. 3a,c,e) and 22 min after the drug application (phase IV; EPO: 5.9 ± 0.7 Hz; n = 7, n.s.; Fig. 3a,c,e). In phase III and IV, the amplitudes of sIPSCs remained unaffected (Fig. 3a,b,d).

Type IV: the *ex-vivo* application of EPO significantly decreased the frequency of sIPSCs within 4 min (control phase I: 4.1 ± 0.1 Hz vs. phase II EPO: 3.4 ± 0.2 Hz; n = 10, p < 0.01; Fig. 3f,h,j), while the amplitudes were not significantly affected (control phase I: 45.4 ± 9.4 pA vs. phase II EPO: 43.6 ± 7.9 pA; n = 10, n.s.; Fig. 3f,g,i). The frequencies of sIPSC remained depressed 12 min (phase III EPO: 3.5 ± 0.1 Hz; n = 10, p < 0.05; Fig. 3f,h,j) and 22 min after the drug application (phase IV EPO: 3.5 ± 0.1 Hz; n = 10, p < 0.05; 3f,h,j). At same time period, the amplitudes of sIPSCs were unaffected (Fig. 3f,g,i).

It is worth mentioning that all recorded type III neurons were located in more dorsal part of the right PrL, while all recorded type IV neurons were located in more ventral part of the left PrL (Fig. 4, right panel). In all experiments on the right PrL, the results were quite consistent and the described two types of EPO effects remained until we lost the cell. (about 20–40 min, data not shown).

The lateralized effects of EPO on inhibitory synaptic transmission exist in the same brain.

The above results were obtained from pooled data of neurons in left and right PrL of different mice. To further clarify whether the difference between left and right also occurred in same brain we performed a

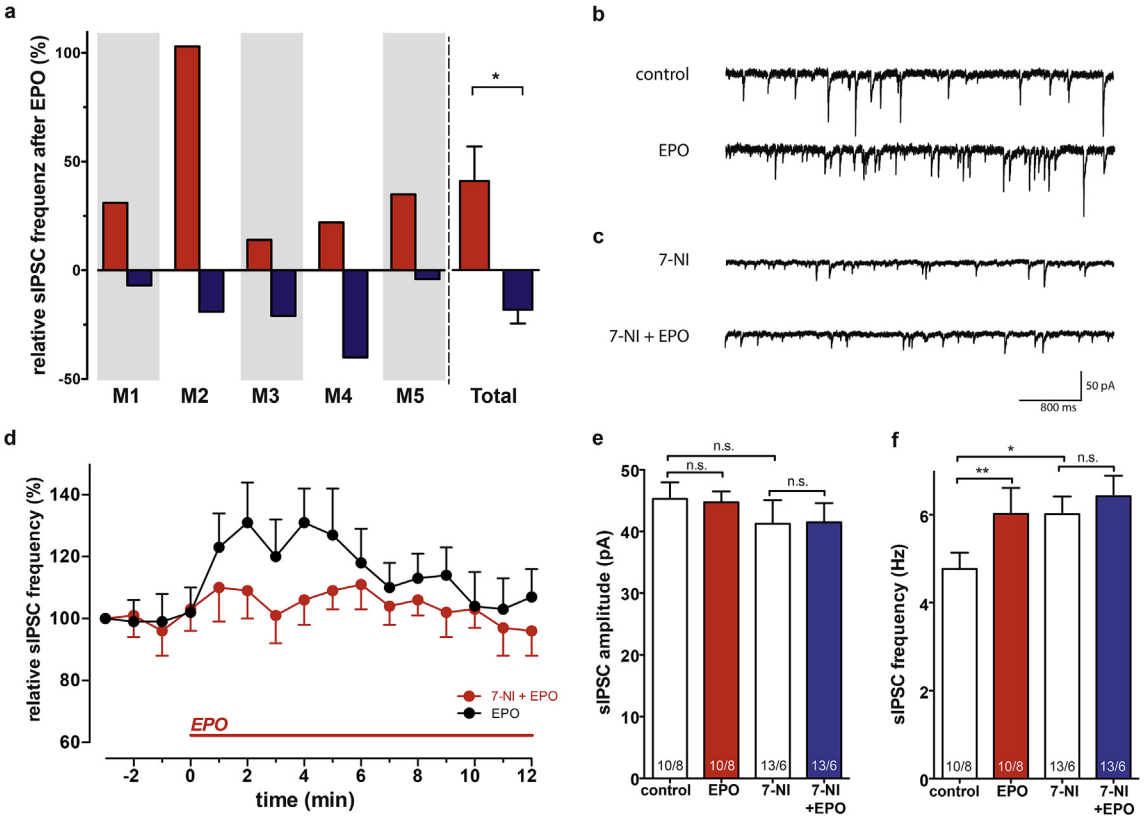

Fig. 5. The EPO-induced facilitation of sIPSCs is abolished in the presence of 7-NI. (**a**) The histogram shows effects of EPO on sIPSCs in left (red; enhanced IPSC) and right PrL (blue; reduced IPSC) of the same brain slice (M_n indicates individual mices); (**b**) Sample traces of sIPSCs before, after EPO; after 7-NI and after 7-NI and EPO; (**c**) time trace of averaged changes of IPSC frequency after 7-NI and after 7-NI and EPO; (**e**) the averaged amplitude and (**f**) frequency of spontaneous IPSCs in layer II pyramidal neurons of PrL before, after EPO; after 7-NI and after 7-NI and EPO (n). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

further series of experiments, where the EPO effects on sIPSCs were tested consecutively on the left and the right of PrL obtained from the same slice of the same individual mouse brain. Our results clearly demonstrated that the lateralized effects of EPO on sIPSCs did exist between the left and the right PrL of the same individual brain (Fig. 5a; see Figs. 1 and 4).

In endothelial cells, EPO has been shown to activate the nitric oxide (NO) bioavailability by interaction with β common receptor, although the definitive mechanisms are still not fully understood (Su et al., 2011). We next examined whether NO-system might at least partly be involved in the EPO effects on sIPSCs. In our experiments on the left PrL, incubation of 7-NI (100 mM) significantly increased the frequency of sIPSCs (control: 4.7 ± 0.4 Hz vs. 7-NI: 6.0 ± 0.4 Hz; n = 13, $p < 0.05$; Fig. 5b,c,f), while the amplitude of IPSCs did not alter (control: 45.3 ± 2.7 pA vs. 7-NI: 41.2 ± 3.8 pA; n = 13, n.s.; Fig. 5b,c,e). In the presence of 7-NI, additional *ex-vivo* application of EPO (5 min) did neither significantly alter the frequency (7-NI: 6.0 ± 0.4 Hz vs. 7-NI + EPO: 6.4 ± 0.5 Hz; n = 13, n.s.; Fig. 5c,d,f), nor the amplitude of IPSCs (7-NI: 41.2 ± 3.8 pA vs. 7-NI + EPO: 41.5 ± 3.1 pA; n = 13, n.s.; Fig. 5c,e). Thus, these results indicated that the NO signaling might likely be involved by the fast EPO-induced facilitating effect on sIPSCs in the left PrL.

EPO receptors are abundant in the left and the right hemispheres of PFC.

To test the expression of EPO receptors (EPORs), a so far commercially available antibody was used. Immunoreactivity of EPOR was abundant across different tested brain areas while there were no obvious differences between stained cell in the left and the right hemisphere (Fig. 6a,d), although it is not quite clear, how specific was the immunoreactivity (Elliott et al., 2014; Jelkmann, 2005). Higher-magnification images showed that the EPORs were expressed on the somata of cells in different layers (Fig. 6b). On average, the densities of $EPOR^+$-cells were 810 ± 80 mm^{-2} in the left, but 907 ± 55 mm^{-2} in the right ACC (n = 5, p = 0.086). Similarly, the densities of $EPOR^+$-cells appeared slightly higher in the right as in the left PrL (right PrL:1040 ± 40 mm^{-2}; left PrL: 983 ± 52 mm^{-2}; n = 5, p = 0,102) and IL (right IL:1055 ± 39 mm^{-2}; left IL: 982 ± 49 mm^{-2}; n = 5, p = 0.0525), although the differences were statistically not significant (Fig. 6c).

Co-localization of EPOR and GAD-GFP immunoreactivity of was tested in GAD67-GFP mice (Fig. 6e). The overall percentage of EPOR and GAD67 co-localization was significantly higher in the left as compared to the right PFC (Fig. 6f, total PFC; left: 53.7 ± 0.4% vs. right: 47.4 ± 0.1%; n = 6; $p < 0.05$). In ACC, there was no difference between left and right (Fig. 6f; left: 50.68 ± 0.8% vs. right: 50.71 ± 0.9%; n = 6; n.s.), while in PrL (Fig. 6f; left: 58.84 ± 1.4% vs. right: 39.0 ± 0.5%; n = 6; $p < 0.01$) and IL (Fig. 6f; left: 52.37 ± 0.4% vs. right: 43.46 ± 1.2%; n = 6; $p < 0.05$), the percentages of co-localization were significantly more abundant in the left as in the right hemisphere (Fig. 6f). Thus, although the commercially available antibody used in the present study was not very specific, the percentages of EPOR and GAD67 co-localization were significantly different between the left and the right PFC (Fig. 6f), that might provide a good hint for the bilateral different EPO effects.

4. Discussion

To the best of our knowledge, the current study provided the first evidence that EPO rapidly regulates GABAergic transmission in the PFC in a lateralized manner, where the erythropoietic effects could be

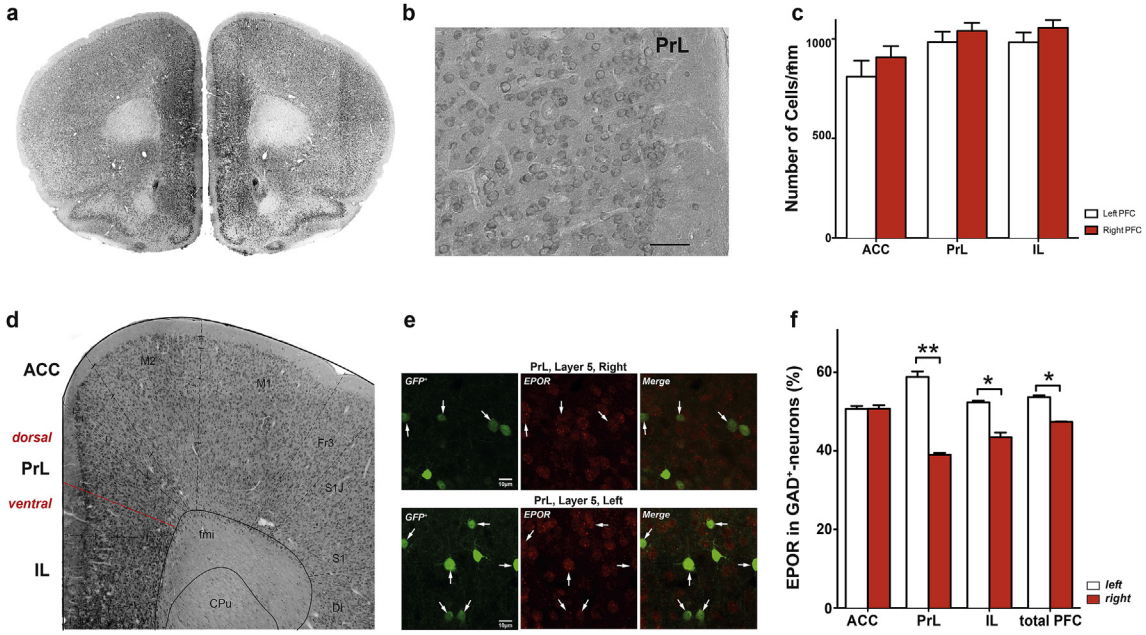

Fig. 6. The distribution of EPOR$^+$-cells and co-localization of EPOR & GAD67 immunoreactivities in ACC, PrL and IL. (**a**) Overview of EPOR -stained coronal sections of PFC in 1.70 mm anterior to Bregma; (**b**) Expression of EPOR on the soma of cells; (**c**) Quantification of the overall density of EPOR$^+$-cells on the left and the right halves of ACC, PrL and IL; (**d**) EPOR$^+$ – stained coronal sections of right PFC in higher magnification. (**e**) samples of GAD67 (green) and EPOR (red) stained cells in coronal sections of layer V of PrL in 1.70 mm anterior to Bregma. Only part of them are co-localized; (**f**) Percentage of GAD/EPOR co-localization on the left and the right of ACC, PrL, IL and in PFC. ACC: anterior cingulate cortex; IL; infralimbic cortex; PrL; prelimbic cortex. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

entirely excluded. Our study provides three major findings: (1) EPO showed fast effects on inhibitory transmission in the PFC, and the NO-mediated signaling might likely be involved; (2) EPO enhanced the inhibitory transmission in the left and depressed the inhibitory transmission in the right PrL; (3) the expression of EPOR in GAD$^+$-neurons is different between the left and right PFC. Given the importance of the neuronal network of the PFC in central control of emotion and cognition under stress, the lateralized effects of EPO would contrarily activate the reciprocal left and right PrL-related networks and differently modulate the bilateral down-stream limbic areas. Such lateralized effects of the EPO would, thus, have enormous consequences on shaping the central emotional and cognitive control in the physiology and the pathophysiology.

4.1. EPO effects on inhibitory synaptic transmission by activating membrane-binding EPORs

Several lines of evidences indicate that the function of hippocampus can be influenced by EPO, as it is protective against neuronal death in the CA1 region after global cerebral ischemia (F. Zhang et al., 2006), as well as after epilepsy *in vivo* (Yang et al., 2007), and it transiently increases adult hippocampal neurogenesis (Ransome and Turnley, 2007). In addition, *in-vivo* EPO treatment improves hippocampus-associated memory, and enhances hippocampal short- and long-term plasticity in a hematopoiesis-independent manner (Adamcio et al., 2008; Kamal et al., 2011; Wójtowicz and Mozrzymas, 2008).

The effect of EPO on synaptic transmission has also been confirmed by analyses of spontaneous synaptic activity in acute hippocampal slices obtained from EPO-treated mice. Published data showed that EPO attenuates the excitatory synaptic transmission (Adamcio et al., 2008; Almaguer-Melian et al., 2016; Kamal et al., 2011), and facilitates the inhibitory synaptic transmission in hippocampal neuronal network (Adamcio et al., 2008; Wójtowicz and Mozrzymas, 2008). In addition, the analysis of individual autaptic hippocampal neurons reveal that EPO possibly leads to a reduction in the amount of primed vesicles without altering number of synapses or efficiency of vesicle dynamics, and it reduces overall spiking activity of neurons and enhances bursting efficiency of selected neuronal networks (Adamcio et al., 2008).

In the present study, the effect of EPO was tested shortly after the *ex-vivo* application on PFC slices where hematopoietic and other tropic effects of EPO could be entirely excluded. We showed here that the acute application of EPO elicited a surprisingly rapid change of GABAergic transmission in the PrL of mice. The fast onset of the EPO effect shown in this study is substantially different than the time course of EPO effect described in other papers, where the EPO effects have been tested hours or even weeks after the application (Adamcio et al., 2008; Almaguer-Melian et al., 2016; Kamal et al., 2011). Thus, our results suggest that such rapid effect is very likely exerted by activation of none-genomic and membrane-binding EPOR and its corresponding down-stream signaling pathways, such as the NO-mediated retrograde signaling (Jelkmann, 2005), quite similar as the none-genomic effects of glucocorticoid receptors (Hu et al., 2011; Teng et al., 2013).

4.2. Differences between the left and the right brain

Previous studies demonstrated that EPO univocally enhanced the inhibitory synaptic transmission in hippocampal neurons (Adamcio et al., 2008; Almaguer-Melian et al., 2016; Kamal et al., 2011), whereas the current study showed that EPO elicits an enhancement of GABAergic transmission only in the left PrL, whereas it rather depressed the GABAergic transmission in the right PrL. In our opinion, previous studies have been performed in slices of hippocampus of rats (Adamcio et al., 2008), of mice (Kamal et al., 2011) or in culture of hippocampal neurons (Wójtowicz and Mozrzymas, 2008), where the data could not be further discriminated to their bilateral origins in the brain. In our study (Figs. 1–6), the bilateral localization of recorded neurons of the PFC slices have been carefully labeled, and the data have been subsequently analyzed according to their bilateral localizations. Therefore, careful analysis of EPO effects according to their bilateral origins in the future studies would very likely reveal such lateralized EPO effect in other brain regions.

The activation of EPO-mediated signaling starts by interaction of

EPO with EPOR. The EPOR is a 484 amino acids glycoprotein belonging to the cytokine class I receptor superfamily (Youssoufian et al., 1993). There are at least two functional EPOR isoforms, an homodimeric form (EPOR)2, and a heterodimeric form EPOR/bCR resulting from the interaction of EPOR with the a common receptor (bCR) (Uversky and Redwan, 2016). Depending on its isoforms, the EPOR has several intracellular messenger pathways, including Jak-2/Stat-5, PI3- K/Akt, MAPK and PKC (Jelkmann, 2005). In contrast to the (EPOR)2, which relies on the binding of picomolar concentrations of EPO, the activation of the heterodimeric EPOR/bCR receptor requires higher local concentrations of EPO. Thus, the local EPO concentration would determine, which EPOR isoform is activated (Nairz et al., 2012). Furthermore, the interaction of EPOR with the a common receptor (bCR) (Uversky and Redwan, 2016) has been shown to activate the nitric oxide (NO) bioavailability (Su et al., 2011).

In the present study, the abundance and density of EPOR-positive cells in the left and the right PFC are quite similar (Fig. 4), although our staining results don't differentiate the presence of (EPOR)2 or EPOR/bCR. In addition, the commercially available antibody used in the present study was not very specific (Elliott et al., 2014), and unfortunately, no other trustworthy antibodies are available at present time. Thus, it could not be excluded that lateralized EPO effects might represent the differential distribution of (EPOR)2 and EPOR/bCR in the left and the right PFC. Our results showed that NO-signaling is likely be involved in the fast EPO effects on inhibitory transmission (Fig. 3). This is at least the first indication that EPOR/bCR might be activated in the left PrL (Su et al., 2011).

In the brain of mouse, the major EPO binding sites are found in the hippocampus, capsula interna, cortex, midbrain as well as in retina (Digicaylioglu et al., 1995; J. Zhang et al., 2008), where the distribution of EPOR in the hippocampus of rodents and primates has been detailed investigated (Hasselblatt et al., 2006; Jelkmann, 2005). In the present study, the expression of EPOR in GAD67$^+$-neurons is different between the left and the right PFC (Fig. 6) suggesting that application of EPO would have different impact on activation of GABAergic inhibitory interneurons in the left and the right PFC. Therefore, the lateralized EPO effect in the present study might also represent the differential distribution of (EPOR)2 and EPOR/bCR in GABAergic inhibitory interneurons of the left and the right PFC.

4.3. Possible EPO effects under stress

The mPFC receives diverse afferent inputs from limbic regions, such as the amygdala and ventral hippocampus. It provides direct outputs to hypothalamic and numerous brainstem areas involved in the regulation of emotion and in the physiological response to stress (Bandler et al., 2000). On the other hand, the response of HPA axis to restraint stress is attenuated in rats with mPFC lesions (Diorio et al., 1993), and published data suggest that the left and right mPFC differentially regulates the HPA axis responses (Sullivan, 2004). Importantly, the mPFC-mediated regulation of the HPA axis is impaired when animals are subjected to chronic stress (Mizoguchi et al., 2003, 2001; Sullivan and Gratton, 1999).

In the present study, application of EPO differentially modulated the GABAergic synaptic inhibitions in the left and right PrL. It is thus quite possible that, in this way, application of EPO may at least partly restore the chronic stress-induced impairment of GABAergic networks in PFC (Czéh et al., 2018). In addition, as left and right PFCs are known to differentially modulate the down-stream limbic areas (Cerqueira et al., 2008), EPO-induced enhancement of synaptic inhibition in the left PrL would down-regulated the down-stream HPA axis, and EPO-induced reduction of synaptic inhibition in the right PrL would then enhance the down-stream hippocampal activity and plasticity (illustrated in Graphic abstract). Thus, the lateralized EPO effect in the present study might contribute to the beneficial effects of EPO under stress as well as by mental disorders (Czéh et al., 2018; Ehrenreich et al., 2008, 2007b; 2007a; Miskowiak, 2017; Miskowiak et al., 2018, 2010).

5. Conclusion

In summary, the outlined fast action of EPO provided a potential mechanism that could underlie the beneficial effects of EPO in the physiology and the pathophysiology (Adamcio et al., 2008; Kamal et al., 2011; Wójtowicz and Mozrzymas, 2008), although the cellular mechanisms of the lateralized EPO effects presented in the current study still need to be further elucidated. We hypothesize that such lateralized effects of EPORs contribute to the development of the lateralization of stress reaction in PFC (Cerqueira et al., 2008) as well as the altered bilateral oscillation patterns under stress. Furthermore, as the GABAergic transmission undergoes profound changes during development (Hollrigel and Soltesz, 1997) and that its dysfunction and the subsequent shift of the excitatory inhibitory balance are associated with brain disorders, the differential modulation of bilateral GABAergic networks might accentually alter the excitatory inhibitory balance in the left and the right brain hemispheres. Considering the vital role of GABAergic interneurons in the regulation of network oscillations (Sohal et al., 2009; Somogyi and Klausberger, 2005) and the hemispheric differences in stress reaction of brain (Cerqueira et al., 2008; Czéh et al., 2008), such lateralized EPO effects on GABAergic transmission might underlie the altered bilateral oscillation patterns that impact the central emotional and cognitive control.

Conflicts of interest

The authors do not have any competing interests.

Author contributions

W.Z. initiated and directed the study; A.D., R.S. and M.Z. performed the research; A.D., R.S., M.Z. and W.Z. analyzed the data; W.Z. wrote the manuscript. All authors contributed to and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ynstr.2018.08.008.

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