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Erythropoietin Increases $GABA_A$ Currents in Human Cortex from TLE Patients

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Abstract—Erythropoietin (EPO) is a hematopoietic growth factor that has an important role in the erythropoiesis. EPO and its receptor (EPO-R) are expressed all over in the mammalian brain. Furthermore, it has been reported that EPO may exert neuroprotective effect in animal models of brain disorders as ischemia and epilepsy. Here, we investigate whether EPO could modulate the GABA-evoked currents (I_{GABA}) in both human epileptic and non-epileptic control brain tissues.Therefore, we transplanted in *Xenopus* oocytes cell membranes obtained from autoptic and surgical brain tissues (cortex) of seven temporal lope epilepsy (TLE) patients and of five control patients. Two microelectrodes voltage-clamp technique has been used to record I_{GABA}. Moreover, qRT-PCR assay was performed in the same human tissues to quantify the relative gene expression levels of EPO/EPO-R. To further confirm experiments in oocytes, we performed additional experiments using patch-clamp recording in slices obtained from rat cerebellum.We show that exposure to EPO significantly increased the amplitude of the I_{GABA} in all the patients analyzed. No differences in the expression of EPO and EPO-R in both TLE and control patients have been found. Notably, the increase of I_{GABA} has been recorded also in rat cerebellar slices. Our findings show a new modulatory action of EPO on $GABA_A$ receptors ($GABA_A$-Rs). This effect could be relevant to balance the GABAergic dysfunction in human TLE.

Key words: $GABA_A$ receptor, epilepsy, human brain tissue, oocytes, slices.

INTRODUCTION

Erythropoietin (EPO) is a 34 kDa glycoprotein hormone that has a central role in erythropoiesis by inhibiting apoptosis and stimulating the proliferation of erythroid progenitors (Jelkmann, 1992). However, several lines of evidence suggest other alternative roles of EPO based on the finding that both EPO and its receptors (EPO-R)s are expressed at different sites, including the central nervous system (CNS) (Morishita et al., 1997; Cerami, 2001; Cerami et al., 2001; Nagai et al., 2001; Csete et al., 2004). Different studies have shown that EPO exerts potent neuroprotective effects in animal models of CNS disorders both *in vitro* (Bartesaghi et al., 2005; Brines et al., 2000; Dame et al., 2001; Genc et al., 2004) and *in vivo*, especially by enhancing neurogenesis (Shingo et al., 2001; Xiong et al., 2008; Kadota et al., 2009). Several mechanisms seem to be involved in neuroprotective actions of EPO, including: activation of kinase pathways involved in neuroprotection (Sirén et al., 2001), activation of transcription factors as the nuclear factor-κB (NFκB) (Digicaylioglu and Lipton, 2001) and of anti-apoptotic genes (Renzi et al., 2002), reduced inflammation (Villa et al., 2003). Furthermore, there is some evidence of a protective action of EPO against glutamate-induced neuronal damage (Kamal et al., 2011; Garzón et al., 2018).

Recombinant human Epo (rHuEpo) is able to cross the blood–brain barrier (BBB) (Brines et al., 2000; Ferriero, 2005) and, when systemically delivered, it is able to reduce tissue damage in animal model of ischemic stroke (Sirén et al., 2001).

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Moreover, the modulatory effect of EPO on neurogenesis has been also observed in epilepsy; both endogenous and exogenous EPO have been implicated in reduction of brain damage related to generalized seizure (Nadam et al., 2007), and in a rodent model of temporal lope epilepsy (TLE), EPO showed a significant effect in antagonizing the development of status epilepticus (SE) (Uzum et al., 2006).

Notably, one study (Ott et al., 2015) demonstrated that EPO-R is upregulated in some specific areas of the hippocampus obtained from surgical resection in a single patient afflicted from pharmaco-resistant complex-focal seizures of temporal-mesial origin, indicating thus a possible, not yet fully clarified, role of EPO system in focal epilepsy.

Human TLE has shown to be associated with a dysfunction of the inhibitory signaling mediated by $GABA_A$-Rs (Loup et al., 2000; Palma et al., 2005a,b; Loup et al., 2009). In detail, continuous activation by GABA of epileptic $GABA_A$-Rs induces a use-dependent decrease of I_{GABA} amplitude (*i.e.*, rundown) that it is not present in control non-epileptic tissues (Palma et al., 2004; Goodkin et al., 2007). The GABAergic current rundown in TLE is prevented by pretreatment with phosphatase inhibitors, neurotrophic factor BDNF, and adenosine derivatives (Palma et al., 2004, 2005b; Roseti et al., 2008, 2009), suggesting that the phosphorylation state of $GABA_A$-Rs, and/or its accessory proteins are relevant players of this phenomenon (Laschet et al., 2007; Palma et al., 2007). Here, we wondered whether EPO could play a modulatory role of $GABA_A$-Rs function in human brains. Specifically, we investigated the effects of EPO on the epileptic human $GABA_A$-Rs micro-transplanted in *Xenopus* oocytes from patients affected by drug-resistant TLE (Miledi et al., 2002). For comparison, we decided to use cortical tissues from individuals without any sign of recurrent or occasional seizures. We found that EPO positively modulates $GABA_A$-Rs by increasing I_{GABA} amplitude. The EPO effect was washable, time-dependent, and blocked by the broad spectrum kinases inhibitor, staurosporine, suggesting an involvement of phosphorylation system.

Therefore, EPO could represent an interesting agent able to modulate $GABA_A$-Rs function, especially in all the epileptic conditions characterized by a strong GABAergic impairment.

EXPERIMENTAL PROCEDURES

Patients

The surgical cases of TLE patients were selected by the neurologists and neurosurgeons from the Department of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam). AMC Research Code provided by the Medical Ethics Committee and approved by the Science Committee of the VUMC Biobank.

We used seven surgical specimens (temporal cortex) from patients who underwent extensive temporal lobectomy for drug- resistant epilepsy (patients 1–7; Table 1). All patients were clinically evaluated before their surgery but no invasive tests were performed. The cases that were subjected to chronic invasive monitoring with subdural strip and/or grid electrodes before resection were not included in the study. Our patients mainly suffered from drug-resistant complex partial seizures, not controlled with maximal doses of antiepileptic drugs (Table 1). Epilepsy duration was calculated as the years elapsed from the onset of seizure; all patients did not present seizures in the 24 h before surgery. Two neuropathologists reviewed independently all the cases confirming the diagnosis according to the international consensus classification (Blümcke et al., 2013). For comparison we used human brain samples from autopsies of individuals with no sign of neurological pathologies nor brain inflammation (patients 8–9; Table 1; they died of heart failure and myocardial infarction respectively); and peritumoral samples from meningioma surgeries (WHO III, patients 10–12; Table 1). In these two patients the absence of seizures was determined using 60 min of awake EEG recordings while in the epileptic patients, seizures were classified according to the Engel classification (Engel, 2011). All autopsies were performed within 10 to 24 h of death. Histological examination of control autopsies indicated a pattern of immunoreactivity (IR) similar to that observed in surgical controls, suggesting an antigen preservation in autopsies (Roseti et al., 2015).

Tissue was snap-frozen over liquid nitrogen, stored at −80 °C and subsequently shipped in dry ice by courier to University of Rome. The use of human tissues was in

Table 1. Clinical characteristics of patients.

P	Age (y)/Sex	Epilepsy Onset	Surgical Zone	Seizure type	Number of seizures/month	Pathology	AEDs
1	41/M	20	R-T	CPS/GTCS	12	HS	CBZ, TPM
2	29/F	13	R-T	SPS/GTCS	32	HS	LEV, CBZ
3	43/M	28	L-T	CPS/GTCS	15	HS	CLB, TPM, PHT
4	42/M	15	R-T	CPS	18	HS	LEV, TPM, LMT
5	52/M	40	L-T	CPS	5	HS	CBZ,PB,VGB
6	38/F	12	R-T	CPS/GTCS	8	HS	CBZ,LMT,PB
7	36/F	6	R-T	CPS/GTCS	9	HS	LEV, CBZ
8	47/F	Absent	–	–	–	None	
9	64/M	Absent	–	–	–	None	
10	47/F	Absent	R-T	–	–	Meningioma	
11	52/F	Absent	R-T	–	–	Meningioma	
12	37/M	Absent	R-T	–	–	Meningioma	

Patients 1–7: patients with TLE; *Patients 8–9:* non-epileptic tissues from autopsies; *Patients 10–12*: surgical specimens from non-epileptic patients with meningioma (III WHO); R, right; L, left; T, temporal; HS, Hippocampal Sclerosis. AEDs, anti-epileptic drugs.
Seizures Type: SPS, simple partial seizures; CPS, complex partial seizures; GTCS, generalized tonic/clonic seizures.
Drugs: CBZ, Carbamazepine; TPM, Topiramate; LEV, Levetiracetam; CLB, Clobazam; PHT, Phenytoin; LMT, Lamotrigine; PB, Phenobarbital;VGB, Vigabatrin.

accordance with the Declaration of Helsinki. The Ethics Committees of University of Rome "Sapienza" approved all the selection processes and procedures. Informed consent was obtained from all the patients to use part of the biopsy material for experiments. For more details about patients and screening analysis see Table 1.

Membrane preparation, injection procedures and electrophysiological recordings from oocytes

Membranes were prepared as previously described using tissues from human temporal cortex. Tissue specimens were homogenized using a Teflon glass homogenizer and 2 ml of glycine buffer of the following composition (in mM): 200 glycine, 150 NaCl, 50 EGTA, 50 EDTA, 300 sucrose; plus 20 µl protease inhibitors (cat. no. P2714; Sigma, Milan, Italy); pH 7.4 adjusted with NaOH. The homogenate was centrifuged for 15 min at 9500 × *g* in a Beckmann centrifuge (C1015rotor; Palo Alto, CA, U.S.A.). The supernatant was collected and centrifuged for 2 h at 105 × *g* in a TL-120 rotor at 4 °C. The pellet was washed, re-suspended in assay buffer (glycine 5 mM) and used directly, or aliquoted and stored at −80 °C for later use. Preparation of *Xenopus laevis* oocytes and injection procedures were as detailed elsewhere (Miledi et al., 2006). Evoked currents were recorded from 12 to 48 h after injection, using the intracellular voltage-clamp technique (Miledi et al., 2006). Oocytes were placed in a recording chamber (volume, 0.1 ml) perfused continuously with oocyte Ringer (OR) at controlled temperature (21–23 °C). To apply GABA or OR, we used a gravity driven multi-valve perfusion system (9–10 ml/min) controlled by computer (Biologique RSC- 200; Claix, France). From 0.5 to 1 s are needed to reach the complete replacement of applied solution.

Unless otherwise indicated, GABA 500 µM (freshly dissolved in OR) was applied to oocytes to elicit inward currents (I_{GABA}). Oocytes were first exposed to GABA alone (two repetitive applications at 5 min intervals) and only those where I_{GABA} was stable (less than 5% reduction of peak amplitude after the second application), were used either for co-application of GABA and EPO (10 UI/ml), or for GABA applications after 4, 10 or 60–180 min pre-incubation with EPO. In all the experiments EPO pre-incubation immediately followed the recordings of control GABA current amplitude. We calculated the percentage change in I_{GABA} amplitude with respect to the current evoked before EPO application in the same cells.

In some experiments the oocytes were tested also after a 3 h wash out in OR in order to verify the reversibility of the observed effect.

The GABA current *rundown* ($I_{\%}$) was defined as the decrease (in %) of the $GABA_A$-current peak amplitude after six 10s applications of GABA (500 µM) at 40 s intervals (Palma et al., 2007). In all experiments, the holding potential was −60 mV. Current decay was estimated as the time taken for the current to decay from its peak to 10% of its peak value (T0.1). Dose-current response relationships were estimated by fitting the data with a Hill equation.

Slices preparation and electrophysiological recordings from cerebellar Purkinje cells

The basic procedure for obtaining and maintaining rat cerebellar slices were described elsewhere (Giovannelli et al., 1998). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n.116, G.U., Suppl. 40, February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Unless otherwise specified, 25–40-day-old Sprague Dawley rats were used for experiments. Parasagittal 250-µm-thick cerebellar slices were cut in ice-cold oxygenated saline (ACSF, in mM: NaCl, 125; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1.25; $NaHCO_3$, 26; glucose, 10; equilibrated at pH 7.3 with 95% O_2 and 5% CO_2) using a DSK vibrating Microslicer (Dosaka, Japan), and then maintained at room temperature (22–25 °C). Purkinje cells (PCs) were visualized through a × 40 water-immersion objective by an Axioskope II FS microscope (Carl Zeiss, Germany). During measurements, slices were superfused with oxygenated saline (1.5–2.0 ml/min).

Whole-cell patch pipettes (2–4 MΩ) were prepared from borosilicate glass capillaries (Harvard Apparatus, Massachusetts, USA) and filled with the following intracellular solution (in mM): CsCl, 140; $MgCl_2$, 2; HEPES, 10; Na-ATP, 4; EGTA, 0.5; equilibrated at pH 7.3 with CsOH.

Membrane currents were recorded at 10 KHz with an Axopatch 200A (Axon, Applied Biosystem, CA, USA). The series resistances, ranging between 5 and 11 MΩ, as estimated from slow transient cancelation, were compensated by 75–80%. EPO (1 U/ml) was bath-applied with a gravity-driven perfusion system. Holding potentials were − 70 mV.

The experiment was performed applying a puff of GABA (10 µM) by a patch pipette close to the cell body of PCs using a Picospritzer II system (General Valve, USA), while superfusing the slice with ACSF supplemented with TTX 0.5 µM to reduce the spontaneous activity. GABA puffs (10–-50 msec) were applied every 30–60 s, and EPO was administered dissolved in ACSF only in the presence of stable and reproducible GABA currents, usually for 5 min or until its effect reached a plateau. During recordings, a step voltage pulse (10 mV, 10 msec) was used to monitor cell membrane resistance. All data in which cell membrane resistance changed more than 10% were discarded. A total number of 14 PCs from 5 rats were studied. At the end of each experimental session the slice was changed to avoid possible effects due to EPO-R desensitization. Four experiments were discarded because of instability of GABA currents or of cell membrane resistance. Data were acquired in episodic mode, sampled at 10 KHz and analyzed off line. Data acquisition and analysis were performed by Clampex and Clampfit software respectively (Axon, Applied Biosystem, CA, USA) and plotted by the Origin software (Microcal, OR, USA). Paired Student t-test was used to verify the statistical significance of the data.

Chemicals and Solutions

Oocyte Ringer had the following composition (in mM): NaCl 82.5; KCl 2.5; $CaCl_2$ 2.5; $MgCl_2$ 1; Hepes 5, adjusted to pH 7.4 with NaOH. All drugs were purchased from Sigma (Sigma Italia) at the exception of GABA (from Tocris, UK) and rHuEpo (recombinant human EPO, from R&D Systems). rHuEpo was dissolved in PBS + 0.2% BSA and stored as frozen stock solution (2000 U/ml).

RNA extraction and quantitative real-time PCR

The EPO and EPO-R mRNA expression levels were analyzed in four surgically resected brain specimens from drug-resistant TLE patients [patients 1–4; Table 1] by quantitative real-time, qRT-PCR assay. Two RNA samples of healthy cerebral human cortex from autopsies [patients 8–9; Table 1] and three non-epileptic surgical samples [patients 10–12; Table 1] were included. Total RNA was extracted from resected brain specimens using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was removed by DNase I digestion in column during the RNA extraction (Qiagen) and in order to eliminate any residual of genomic DNA, a further DNA digestion was performed using DNase I enzyme (Ambion, Austin, TX, USA). RNA was quantified spectrophotometrically (UV = 260 nm). One microgram of total RNA was reverse transcribed using Omniscript RT (Qiagen) and random hexamers (Promega Corporation, Madison, WI, USA) in a final volume of 20 µl as described in manufacturer's protocol. TaqMan Probes for EPO and EPO-R, (Hs 00181092 and Hs 00171267 respectively; Applied Biosystems) were used to accurately quantify their expression in the TLE specimens and the Ciclophyllin A was used as internal reference (Hs 99,999,904, Applied Biosystems).

Measurements were made by an iCycler machine (Bio-Rad) and using the Universal TaqMan Master mix 2X (Applied Biosystems). The qRT-PCR was carried out in 96 well optical plate (Bio-Rad). For each well, the 25 µl reaction contained: 12.5 µl of Universal TaqMan Master mix 2X, 0.3 µM each forward and reverse primer, 8 µl of RNase-free H_2O and 2 µl cDNA template. The cycling conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Primers efficiency was checked and real-time PCR products were confirmed by gel electrophoresis and sequencing. Threshold cycle was defined as the fractional cycle number at which the fluorescence signal passed ten times value of ground fluorescence standard deviation. The amount of the target transcripts was related to that of the reference Ciclophyllin A gene as described by Pfaffl et al. (Pfaffl, 2001).

Statistics

Unless otherwise specified, data are presented as means ± S.E.M. The experiments and data analysis were performed by different researchers and each tissue specimen was used several times in different sets of experiments. The normality of data sets was tested according to the Shapiro–Wilk test. Statistical significance was assessed by Student's test and performed with Sigmaplot Systat (Systat Software GmbH, CA, USA) using raw data. Only differences considered significant ($P < .05$) are indicated in the text and figures legends.

RESULTS

To investigate EPO action on epileptic cortical tissues, we first micro-transplanted $GABA_A$Rs from 7 TLE patients into *Xenopus* oocytes (see Table 1).

Microinjection of cell membranes from the human brain tissues into *Xenopus* oocytes leads to the incorporation of $GABA_A$-Rs that, when activated by GABA (500 µM), evoke inward I_{GABA} of variable amplitudes (Miledi et al., 2006). In our study, oocytes injected with TLE cortical membranes exhibited I_{GABA} amplitudes ranging from −18 to −200 nA (n = 140; patients 1–7; Table 1); these currents were blocked, as expected, by the competitive $GABA_A$-Rs antagonist bicuculline (100 µM; n = 5/2, 5 oocytes/2 frogs; patients 1,2; Table 1; not shown).

In agreement with our previous studies (Roseti et al., 2013; Ruffolo et al., 2018), this variability in I_{GABA} amplitude was not dependent by which patient was used for membranes preparation, since each human sample was obtained from the same amount of tissue, but it was probably due to a different expression efficiency in the different oocytes.

Co-application of EPO (10 U/ml) (Viviani et al., 2005) with GABA 500 µM, did not alter the I_{GABA} amplitude (GABA, 73.4 ± 17.4 nA; +EPO, 69.4 ± 14.7 nA; n = 8/2; patients 1–3; Table 1) as well as when EPO incubation was prolonged to 4 or 10 min before GABA application (not shown). However, using EPO longer exposure (from 30 min to 5 h), the I_{GABA} amplitude increased progressively (30 min EPO: 20.5 ± 2.2%; 60 min EPO: 28.7 ± 4.7%) of increase respectively (n = 8/2; patients 1,3,6; Table 1), reaching a maximal plateau of increase (42.8 ± 6.7%) after 3 h of incubation (GABA, 67.4 ± 10.9 nA; +EPO, 90.2 ± 13.5 nA; n = 65/8; $P < .001$; patients 1–7, Fig. 1, Table 1). In another set of experiments, we injected oocytes with membranes from autopsies (patients 8–9, Table 1) and from surgical samples (patients 10–12, Table 1) of non-epileptic cortical tissues. Also in this case, the treatment with EPO (10 U/ml) induced an increase of I_{GABA} amplitude (GABA, 111.3 ± 14.7 nA; +EPO, 149.8 ± 17.6 nA; n = 34/5; $P < .001$; patients 8–12; Fig. 1, Table 1), that was comparable to that obtained in TLE injected oocytes (180 min, 36.2 ± 4.0 $P > .05$).

This effect was reversible after a 3 h wash-out, and it was not accompanied by changes in the current decay (GABA: T0.1 = 2.4 ± 0.2 s; + 3 h EPO: T0.1 = 2.7 ± 0.3 s; n = 15/3; $P > .05$ patients 1–3, Table 1). Noteworthy, we obtained no changes of I_{GABA} amplitude in oocytes incubated for 30 min to 3 h either with oocytes Ringer (OR) alone (GABA, 63.2 ± 14.5 nA; +OR, 64.9 ± 14.6 nA; n = 10/2; patients 1,3; Table 1), or plus vehicle (GABA, 79.2 ± 10.5 nA; +PBS and 0.2% BSA, 77.4 ± 9.2 nA; n = 10/2; patients 1,3; Table 1), indicating that the reported EPO effect was genuine.

Then, additional experiments were performed to verify whether EPO could induce a shift in GABA reversal potential (E_{GABA}). It was found that the increase in I_{GABA} amplitude was not associated with a change in E_{GABA} (GABA,

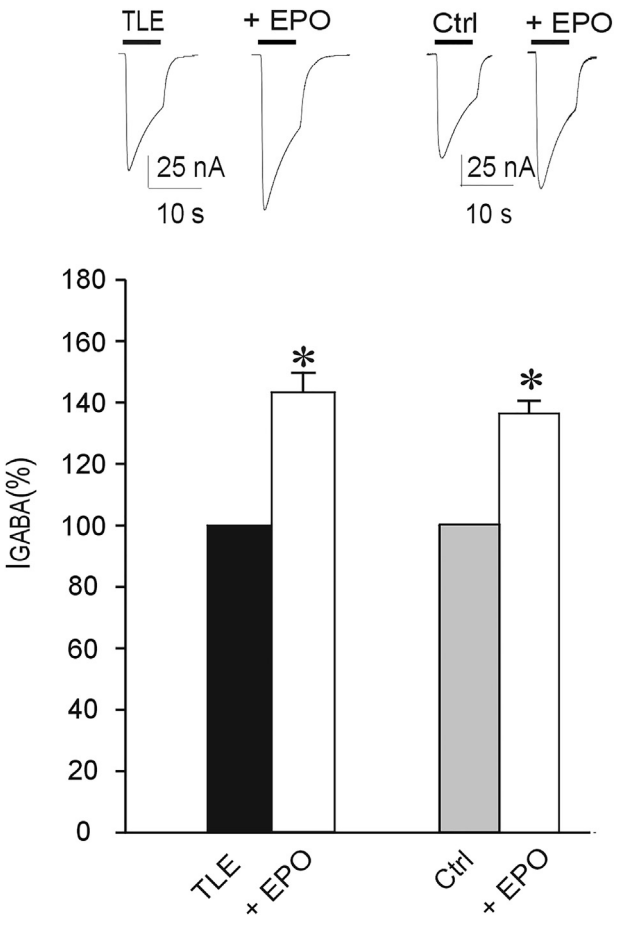

Fig. 1. Increase of GABA currents by EPO in oocytes injected with TLE and non-epileptic cortex membranes. The bar-chart represents the I_{GABA} amplitude (mean ± S.E.M., expressed as percentage of control peak amplitude) after 3 h treatment with EPO (10 U/ml) in specimens from patients with TLE (n = 65, **P* < .001 *vs* GABA alone, Wilcoxon Signed Rank Test; patients 1–7, Table 1) and control tissues from patients without epilepsy (n = 34, **P* < .001 *vs* GABA alone, Paired t-test; patients 8–12, Table 1). The holding potential was −60 mV. Horizontal black bars indicate GABA (500 μM) applications. (*Inset*) Sample currents elicited by GABA application before and after EPO treatment in oocytes injected with TLE cortical membranes (*left*) or non-epileptic cortical membranes (*right*).

E_{GABA} = -23.1 ± 0.5 mV; +EPO, E_{GABA} = -23.7 ± 0.6 mV; n = 9/2; patients 1–2, Table 1). In addition, to better understand the mechanism by which EPO influences I_{GABA} in human brain tissues, GABA dose-current response relations were obtained in TLE injected oocytes. We found that the EC_{50} value was not modified after EPO treatment (Fig. 2) indicating that the EPO-induced increase of I_{GABA} is not related to a change in the $GABA_A$Rs sensitivity.

We previously reported the I_{GABA} rundown in epileptic brain tissues (Palma et al., 2004, 2005b) may be considered a hallmark of GABAergic impairment in TLE (Ragozzino et al., 2005). Therefore, we performed further experiments to investigate whether EPO could affect the I_{GABA} rundown in these patients.

In four TLE patients, the I_{GABA} fell to 51.4 ± 3% at the sixth GABA application (range, 42–61%; n = 16/4; patients 4–7; Fig. 3, Table 1). A pre-treatment of three hours with EPO on the same oocytes, did not affect the amount (as

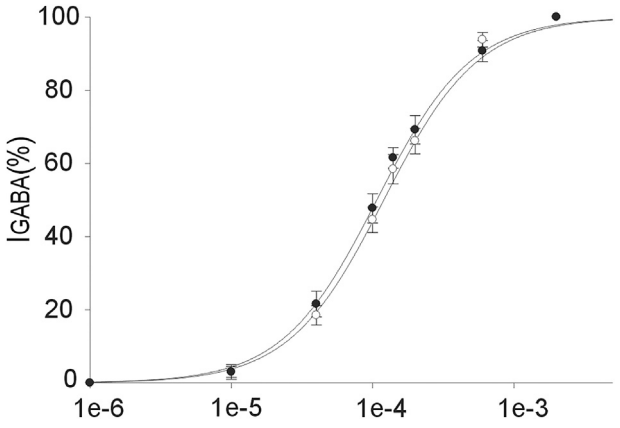

Fig. 2. GABA dose-current response relationships in oocytes injected with epileptic membranes before and after EPO treatment. GABA dose-current response relationships in control (●) and EPO-treated oocytes (○). EPO refers to 3 h treatment. EC_{50} = 120 ± 4 μM; n_H = 1.3 ± 0.07 I_{max} = 145 ± 15 nA (○); and EC_{50} = 107 ± 3.7 μM; n_H = 1.3 ± 0.06. I_{max} = 152 ± 12 nA (●). Points represent mean ± S.E.M. from 7 oocytes injected with membranes from TLE patients (patients 6 and 7, Table 1).

percentage) of I_{GABA} rundown (54 ± 4%; range, 39–63%; Fig. 3, Table 1), while it was still able to increase I_{GABA} amplitude (GABA, 103.3 ± 12 nA; +EPO, 144.4 ± 15.8 nA; n = 16/4; patients 4–7; Fig. 3, Table 1).

Since there are studies pointing out that the signaling cascade induced by the interaction of EPO with its own EPO-R is

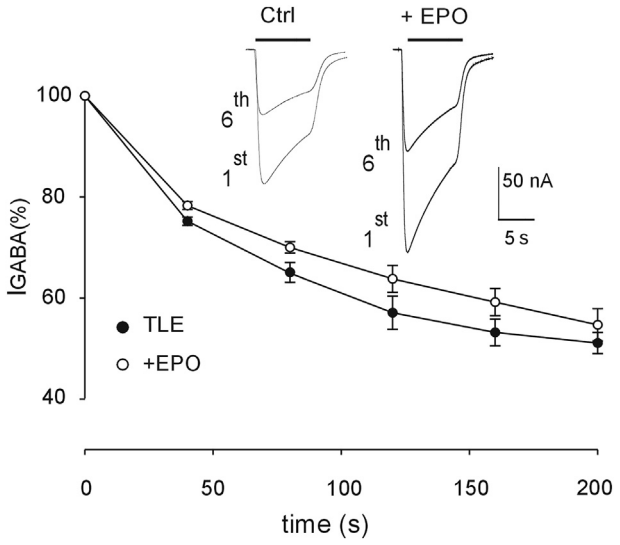

Fig. 3. EPO does not alter GABA-current *rundown* in oocytes injected with epileptic human membranes.Time course of I_{GABA} *rundown* in oocytes injected with membranes from cortical tissue of TLE patients before (●) and after 3 h treatment with EPO (○; n = 16; patients 4–7, Table 1). Points represent mean current amplitudes ± S.E.M. of six 10 s applications of GABA (500 μM) at 40 s intervals of 16 oocytes collected from four frogs. Data were normalized to I_{max} = 103.3 ± 12 nA (●); I_{max} = 144.4 ± 15.8 nA (○). Holding potential, −60 mV. (*Inset*) Superimposed currents elicited by the first and sixth GABA (500 μM; horizontal bar) application before and after treatment with EPO in oocytes injected with TLE cortical membranes. Samples from one oocyte representative of 16 (patients 4–7, Table 1).

followed by a phosphorylation cascade of several downstream targets (Chamorro et al., 2015; Beneduce et al., 2018), we performed another set of experiments using staurosporine 5 μM, a nonspecific kinases inhibitor (Rüegg and Burgess, 1989).

We found that 60 min pre-treatment with 5 μM staurosporine which *per se* did not affect I_{GABA} amplitude (Palma et al., 2005b), was able to prevent the EPO increase of I_{GABA} amplitude in oocytes injected with cortical membranes from TLE patients (GABA, 58.6 ± 4.5 nA; staurosporine + EPO, 53 ± 4.4 nA; n = 15/3; patients 1,6,7; Table 1), suggesting that the EPO effect is not related to a direct interaction on $GABA_A$-Rs, but rather than to an activation of intracellular kinase pathways.

A further investigation of this signaling pathway has been performed using 1 μM GF-198203X, a PKC inhibitor selective for α, β1,δ, ζ isoforms (Roseti et al., 2015) and surprisingly, we found that this agent was not capable of preventing EPO effect (GABA, 44.0 ± 5.1 nA; GF + EPO, 62.4 ± 3.5 nA; n = 8/2; patients 1,3; Table 1).

In order to investigate the regulation of endogen EPO/EPO-R system in patients with TLE and healthy subjects, we performed a qRT-PCR assay to quantify the relative gene expression levels. (Fig. 4A and B).

We found no statistically significant differences in the expression of both EPO and EPO-R in TLE patients (1–7, Table 1) *versus* controls (8–12, Table 1). This result suggests that EPO effect in TLE is not due to an increase of EPO/EPO-R system, at least in human cortex, but it is consistent with our observation that exogenous EPO is able to affect I_{GABA} in both epileptic and control tissues. Finally, to completely exclude that I_{GABA} increase induced by EPO was not related to an artifact of oocytes' expression system, we performed whole-cell patch-clamp recordings on Purkinje cells obtained from rat cerebellar slices. This preparation was chosen because it is well known that Purkinje cells receives high amount of inhibitory input and express different patterns of

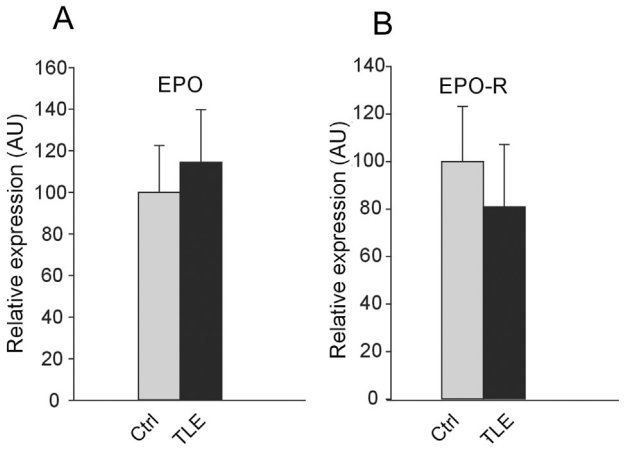

Fig. 4. qRT-PCR assay in human cortex samplesmRNA expression analysis of the EPO/EPO-R transcriptional system in patients afflicted with TLE. Total RNA was extracted from CNS of patients afflicted by TLE or non-epileptic controls. EPO (A) EPO-R (B) mRNA expression levels were normalized *versus* Ciclophyllin A. Results are represented as mean +/− SD; n = 11.

$GABA_A$-Rs (Konnerth et al., 1990; Llano et al., 1991). Moreover, EPO-R is clearly expressed in cerebellar neurons (Brines and Cerami, 2005). As in the experiments performed on oocytes, EPO application induced a washable increase of the I_{GABA} amplitude (GABA, 116.6 ± 16.6 pA; +EPO, 178.8 ± 24.5 pA; P = .002, n = 10, Fig. 5). Even if these last data need further investigation, they clearly show that EPO, also in cerebellar Purkinje cells, has a modulatory effect on I_{GABA} validating the micro-transplantation technique in oocytes.

DISCUSSION

EPO is the main haematopoietic hormone synthesized in mammalians with a broad spectrum of actions on both haematopoietic and non-haematopoietic cells. Indeed, EPO and its receptor EPO-R are expressed in different tissues, including CNS (Masuda et al., 1994; Marti et al., 1996; Brines and Cerami, 2005).

Many studies, both *in vitro* and *vivo*, reported the EPO's neuroprotective potential in traumatic brain or spinal cord lesions (Brines et al., 2000; Shingo et al., 2001; Bartesaghi et al., 2005; Kadota et al., 2009) and ischemic events (Ruscher et al., 2002; Joyeux-Faure, 2007).

EPO effects were also confirmed on animal models of TLE, reducing cells death in hippocampal areas, decreasing seizure severity and prolonging seizure latencies (Uzum et al., 2006; Nadam et al., 2007).

Although numerous papers have been published on the protective role of EPO in epilepsy, this is the first study that investigates whether EPO action may be related to $GABA_A$ receptors on “human tissue”.

Here, we found that EPO increases I_{GABA} amplitude in cortical tissues from patients with TLE and non-epileptic specimens. This effect was found by recording $GABA_A$ evoked currents in oocytes micro-transplanted with membranes (Miledi et al., 2006) from both surgical and human cortical samples from autopsies.

Although aware that the precise neuronal or glial origin of the microtransplanted membrane patches is unknown, we can state that the possibility of studying “real” human control tissues (autopsies) with no evidence of neurological disease or inflammation is a significant advantage of this technique.

In addition, we previously showed that, at least in term of qualitative information, we did not find differences between autoptic and our surgical samples (Roseti et al., 2013).

Notably, due to the limited quantity of tissue available, control specimens are usually unsuitable for obtaining viable brain slices or isolated neurons for extensive investigations with alternative techniques.

In detail, EPO incubation can significantly increase I_{GABA} amplitude up to 40% when co-applied with GABA in TLE cortex. This effect was time-dependent reaching its maximal effect after 3 h of incubation and completely reversible after a long-term washout. The same effect was obtained also in oocytes micro-transplanted with control non-epileptic membranes.

To better clarify the possible mechanisms involved in the EPO mediated I_{GABA} amplitude increase, we investigated

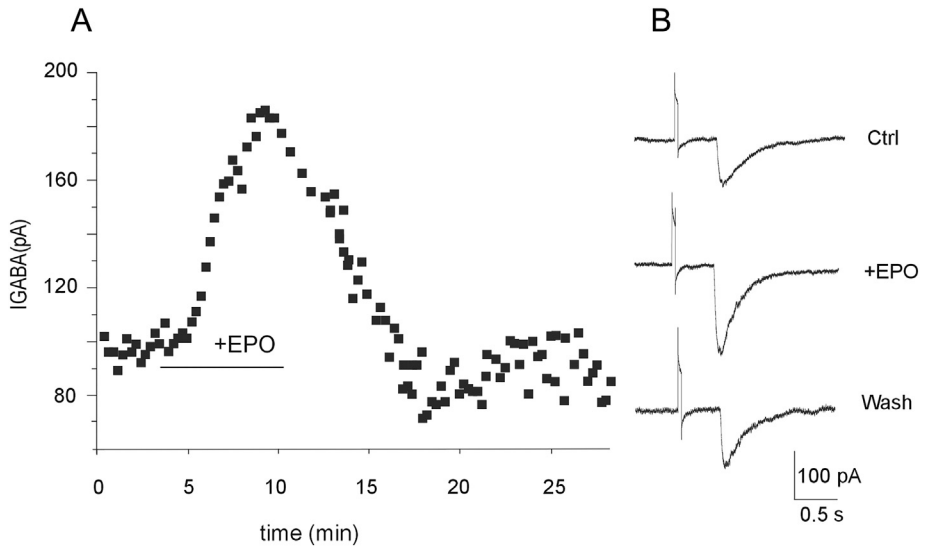

Fig. 5. EPO mediated I_{GABA} increase recorded in cerebellar Purkinje cells. The effect of EPO on I_{GABA} induced by a GABA puff (10 μM) on cerebellar Purkinje cells. Data are representative of the results obtained in 10 different trials (5 rats). EPO induced an increase of I_{GABA} amplitude of about 55% that recovers following the washout. Panel B: Representative I_{GABA} obtained by GABA puff relative to the data shown in panel A. The GABA induced current is preceded by a voltage step pulse (10 mV, 10 msec) to monitor cell membrane resistance.

whether EPO application could modify the $GABA_A$-Rs sensitivity. Our data demonstrated that this latter is unlikely.

We have previously shown that I_{GABA} *rundown* caused by repetitive $GABA_A$-Rs stimulation is stringently linked to occurrence of chronic seizures in TLE patients (Ragozzino et al., 2005) and in epileptic rats (Mazzuferi et al., 2010). Comparable conclusions were drawn studying hypothalamic hamartomas in epileptic patients since I_{GABA} *rundown* was found both in dissociated neurons and oocytes microtransplanted with these tissues (Li et al., 2011), thus confirming its pivotal role in seizure physiopathology (Janigro, 2006; Jansen et al., 2008). The GABAergic *rundown* is prevented by increasing the phosphorylation state of $GABA_A$-Rs or associated proteins using different agents, and can be modulated by cytokines and chemokines (Saliba et al., 2012; Cifelli et al., 2013; Roseti et al., 2015).

Here, we found that a 3 h incubation with EPO, did not change the I_{GABA} *rundown* in oocytes micro-transplanted with epileptic membranes, demonstrating that EPO neuroprotective action in epilepsy (Uzum et al., 2006; Nadam et al., 2007), at least in our expression system, is not related to the recovery of this phenomenon. Additionally, we did not observe modifications of E_{GABA} after EPO incubation and this information suggests that its effect does not involve a modulation of chloride homeostasis, which could depend on the phosphorylation of chloride cotransporters (Palma et al., 2005a).

EPO and EPO-R are upregulated in different CNS physiological and pathological conditions, indicating thus an important role in response to different stimuli. Previous studies showed an increased expression of EPO-R in hippocampal formation (Ott et al., 2015) and in hippocampal blood vessels (Eid et al., 2004) of TLE patients. However, in our cortical samples from epileptic and control patients (the same used for electrophysiological experiments), we did not find significant differences in the expression levels of both EPO and EPO-R mRNAs suggesting that the increase of EPO/EPO-R system may vary in different brain areas and/or cell types. Our qRT-PCR results on human cortex demonstrate, at least in part, that EPO can increase I_{GABA} amplitude on both epileptic and control patients independently of the expression levels in these tissues.

Notably, the EPO increase of I_{GABA} amplitude in oocytes, was also confirmed by means of patch-clamp recordings from Purkinje cells in rat cerebellar slices preparation confirming the genuine effect of EPO on transplanted $GABA_A$-Rs.

Previous reports demonstrated that the binding of EPO to its receptor (EPO-R) triggers phosphorylation of JAK2, resulting in the activation of different downstream targets as STAT5, PI3 kinase, MAPK, and other kinase cascades (Myklebust et al., 2002; Palma et al., 2005b; Tao et al., 2009; Zheng et al., 2013; Chamorro et al., 2015).

We then investigated the possible mechanism of action of EPO on GABAergic transmission, using staurosporine, a kinase blocker that binds to many kinases with a very low selectivity (Rüegg and Burgess, 1989). Our results clearly showed that EPO effect is indeed linked to a phosphorylation, but likely not due to the isoforms of PKC that are blocked by GF (PKC α, β1,δ, ζ) (Morano et al., 2016). Further experiments will better elucidate this point.

Despite growing evidence for the clinical potential of EPO in treating several human diseases (from anemia to kidney diseases and renal failures), (Locatelli et al., 2017) most of the studies present in literature were performed using animal models, whereas only few evidences described EPO effects on human subjects.

However, while proper use of EPO seems to have important therapeutic applications, its misuse can lead to serious health risks. While EPO is one of the most used doping agent (Salamin et al., 2018) to improve sports performance (by increasing oxygen transportation) in healthy subjects, its

chronic use may be a risk factor for the onset of pathological conditions. These different aspects regarding EPO and its possible beneficial (neuroprotection, neurogenesis) or disagreeable effects (as thromboembolic events and tumor growth promotion) makes the use of EPO in clinic difficult, thus requiring further studies about its efficacy and safety doses (Yasuda et al., 2003; Jelkmann et al., 2008). However, we could hypothesize to develop new EPO analogues able to maintain its CNS protective action but without haematopoietic effects.

Our data suggest that EPO effects on $GABA_A$-Rs could represent one of the mechanisms through which EPO exerts its neuroprotective role in animal model of TLE (Uzum et al., 2006; Nadam et al., 2007).

The events that induce seizures in a normal brain are multifactorial, and each one has a specific time course during the epileptogenic processes. The result of these events leads to a brain hyperexcitability (with onset of epileptic seizures) linked to an imbalance between inhibitory and excitatory input. Therefore, the EPO mediated enhancement of I_{GABA} could represent an attempt to balance the changes induced by epileptic insult, as shown previously for other mechanisms (Grabenstatter et al., 2012).

In this scenario, $GABA_A$-Rs play a pivotal role, and the pharmacological modulation of their function represent an important target for the treatment of epileptic conditions, even if further studies are needed to better clarify the long-term effects of EPO on CNS and on others peripheral organs.

We can speculate that the EPO increase of I_{GABA} amplitude, even if different in terms of pharmacokinetic, strongly recalls the benzodiazepines (BDZs) action on $GABA_A$-Rs.

On the other hand, EPO can perturb the cognitive functions, the state of attention and the haematopoiesis in healthy individuals. These last points should be taken in serious consideration, both for subjects that use EPO as a doping agent to improve their sport performances and for individuals affected by neurological diseases.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

We declare that the funding sources had no involvement in study design project, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication.

DECLARATIONS OF INTEREST

None.

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