VERSITA

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 02 April 2008
Revised form accepted: 28 May 2008
Published online: 25 July 2008

Volume 13 (2008) pp 649-655
DOI: 10.2478/s11658-008-0029-2

Short communication

ERYTHROPOIETIN AFFECTS GABAERGIC TRANSMISSION IN HIPPOCAMPAL NEURONS *in vitro*

TOMASZ WÓJTOWICZ* and JERZY W. MOZRZYMAS
Laboratory of Neuroscience, Department of Biophysics, Wrocław Medical University, ul. Chałubińskiego 3, 50-367 Wrocław, Poland

Abstract: Erythropoietin is a potent regulator of erythropoiesis. It acts via the specific membrane receptor (EpoR). Erythropoietin is also known to be present in the central nervous system, and its concentration and the expression of EpoR change during development, which raises the possibility that this modulator might be involved in the regulation of neuronal functions in the developing brain. The GABAergic system undergoes profound changes during development and is particularly susceptible to modulation by endogenous factors. Therefore, we decided to investigate the impact of Epo on GABAergic transmission in hippocampal neurons developing *in vitro*. An analysis of miniature IPSCs (mIPSCs) revealed that a long-term treatment with Epo (48 or 72 h) resulted in a major acceleration of the decaying phase of these currents while the amplitude and current frequency remained unchanged. Interestingly, this effect was restricted to the youngest considered age group (6-8 DIV), indicating that Epo-mediated modulation of mIPSCs depends on the developmental stage of the neurons. We conclude that Epo may exert a modulatory action on GABAergic transmission in developing neural networks.

Key words: $GABA_A$ receptors, Erythropoietin, Miniature post synaptic current mIPSC, Neuronal culture

INTRODUCTION

Erythropoietin (Epo) is a growth factor that regulates erythropoiesis by binding to a specific membrane receptor (EpoR). During erythropoiesis, it stimulates the

* Author for correspondence. E-mail: twojtow@biofiz.am.wroc.pl

Abbreviations used: Epo – erythropoietin; GABA – gamma aminobutyric acid; mIPSC – miniature inhibitory postsynaptic current

proliferation, differentiation and maturation of erythroid progenitor cells [1]. It was thought to be exclusively produced in the fetal liver and adult kidney. However, Epo and EpoR were recently found to be expressed in other tissues including the nervous system, implying a role of this haematopoietic cytokine in the function of the brain [2-4]. Erythropoietin is described as a neuroprotective factor during cerebral ischemia (see [1] for review), experimental brain injury [5], hypoxia [6] and brain haemorrage [7], which shows its importance in the functioning of the brain. Several lines of evidence indicate that the hippocampus is influenced by Epo. In particular, it is known that Epo is protective against neuronal death in the CA1 region of the rat hippocampus following global cerebral ischemia [8], and that Epo preconditioning suppressed neuronal apoptosis in the hippocampus of rats following *status epilepticus in vivo* [9]. It has been shown that systemically delivered recombinant Epo may transiently increase adult hippocampal neurogenesis [10]. Both Epo and EpoR are expressed in rodent and primate hippocampal neurons, and are present during development [2, 11, 12]. Additionally, the level of Epo is variable through development, reaching its highest concentration during gestation [13]. It is thus likely that Epo and EpoR play a role in neurodevelopment and/or brain homeostasis, but their impact on the functions of the developing brain has not been systematically studied. It is known that GABAergic transmission undergoes profound changes during development [14] and that its disfunction might be associated with brain disorders (e.g. epilepsy with ensuing neurodegeneration). It is thus interesting to check whether a long-term Epo treatment of neurons developing *in vitro* affects GABAergic transmission. Our analysis of GABAergic miniature inhibitory postsynaptic currents (mIPSCs) provides evidence that within a restricted window of time, Epo does induce significant changes in the mIPSC time course.

MATERIALS AND METHODS

Hippocampal cell culture

The primary cell culture was prepared as described in detail in [15]. Briefly, postnatal day 1-3 Wistar rats were decapitated. This procedure was performed in accordance with the regulations of the Polish Animal Welfare Act. The hippocampi were dissected, sliced, treated with trypsin, mechanically dissociated and centrifuged twice at 40 g, plated in Petri dishes, and cultured. The presence of glia was diminished using Ara-C (cytosine-D-arabinofuranoside) applied 2 days after preparation at a concentration of 1 μM. Electrophysiological recordings were made from pyramidal cells (judging from their shape) that were in culture for between 6 and 18 days. The stock of the recombinant human Epo was prepared by dissolving it in phosphate buffer saline. The cultured neurons were supplemented with Epo in the culture medium at a final concentration of 20 U/ml for 0, 48 and 72 h before the recordings. The culture medium was exchanged (ca. 50% of volume) two or three times a week.

Developmental model

As extensively explained in [16], GABAergic transmission in neuronal cultures *in vitro* undergoes key developmental changes related to the IPSC characteristics observed *in vivo*. Taking this into account, we examined the kinetics of mIPSCs during *in vitro* development in the model of cultured hippocampal neurons. To consider the developmental aspects, the cultures were arbitrarily divided into three age groups: 6-8, 9-11 and over 12 days *in vitro* (DIV).

Electrophysiological recordings

Currents were recorded in the whole-cell mode of the patch-clamp technique using a Multiclamp 700B (Molecular Instruments, Sunnyvale, CA, USA) at a holding potential (V_h) of -70 mV. The intrapipette solution contained 137 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM 1,2-bis(2-aminophenoxy) ethane-N,N,N0-tetraacetic acid (BAPTA), 2 mM ATP, and 10 mM HEPES (pH 7.2 with CsOH). The composition of the standard external solution was 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES (pH 7.2 with NaOH). Miniature IPSCs were recorded in the presence of tetrodotoxin (1 μM) and kynurenic acid (1 mM). For the whole-cell recordings, the patch pipettes had 3-4.5 MΩ when filled with internal solution. The whole-cell recordings were considered for analysis when the access resistance was below 20 MΩ. Typically, the access resistance was in the range 8-15 MΩ. Occasionally, the series resistance compensation procedure was applied at 20-80%. The cells in which the series resistance showed instability during recordings were not considered in the analysis. Any recordings in the whole-cell mode were started at least 3 min after the patch rupture. This time was sufficient to stabilize the recording conditions. For acquisition and analysis, the pClamp 9.2 software (Molecular Instruments, Sunnyvale, CA, USA) was used. For the analysis of the synaptic currents, the current signals were low-pass filtered at 3 kHz with a Butterworth filter and sampled at 10 kHz using the analog-to-digital converter Digidata 1322 (Molecular Instruments, Sunnyvale, CA, USA).
All the chemicals used were from Sigma-Aldrich (Steinheim, Germany), except the BAPTA (Merck, Warsaw, Poland), tetrodotoxin (Latoxan, Valence, France), and HEPES and NaOH (Karl Roth, Karlsruhe, Germany).

Data analysis

The mIPSC parameters such as amplitude, frequency and area were determined with the pClamp 9.2 software (Molecular Instruments, Sunnyvale, CA, USA). The deactivation current was fitted with a sum of two exponential functions: $y(t) = A_1 \exp(-t/\tau_{fast}) + A_2 \exp(-t/\tau_{slow})$ where A_1 and A_2 are the percentages and τ_{fast} and τ_{slow} are the time constants. For normalized currents, $A_1 + A_2 = 1$. The mean time constant was calculated as $\tau_{mean} = A_1\tau_{fast} + A_2\tau_{slow}$. The data is expressed as ± SEM, and Student's unpaired *t*-test was used to compare the data. All the experiments were performed at room temperature (22-24ºC).

RESULTS

To investigate the effect of Epo on GABAergic transmission, mIPSCs were measured in the whole-cell mode at a holding voltage of -70 mV. The presence of TTX (1 μM, a blocker of voltage-gated sodium channels) blocked the action potential-driven synaptic transmission revealing the mIPSCs resulting from a spontaneous transmitter release. Glutamatergic currents were eliminated by the application of an aspecific blocker of ionotropic glutamate receptors (1 mM kynurenic acid). Under these conditions, GABAergic mIPSCs were revealed as inward currents (Fig. 1 A). Acute application of Epo (20 U/ml) to the culture medium during the recordings (0 h) did not affect the characteristics of the mIPSCs in the investigated groups of neurons (n = 5, data not shown). The presence of Epo and its receptor in the developing brain and their persistence in the mature brain [11, 13] suggest a time-dependent action on neurons. Therefore, we considered a long-term treatment with Epo by supplementing the culture medium with this drug for 48 and 72 h before recordings. Between 6 and 8 DIV, the amplitude of the mIPSCs was -48.55 ± 2.02 pA (n = 25), and this parameter was not significantly changed either by the time *in vitro* or by treatment with Epo (n equals at least 17 in each group, data not shown). As described in detail in [16], the frequency of mIPSCs clearly increased with age, but this parameter was not affected by the addition of Epo (n equals at least 17 in each group, data not shown). The time course of the IPSC is characterized by a rapid onset and considerably slower decaying phase (Fig. 1 B). Since the synaptic agonist is present very briefly in the synaptic cleft (for less than 1 ms, see [17, 18]), the decaying phase of the mIPSCs is commonly believed to reflect the kinetics of the deactivation process (current time course following an abrupt removal of the agonist). For all the considered experimental groups, the deactivation kinetics could be well described by a sum of two exponentials, and the mean deactivation time constant (τ_{mean}) was calculated as described in the Materials and Methods section. Under control conditions at 6 to 8 DIV, the average decaying time constant τ_{mean} was 46.3 ± 1.77 ms (n = 25), and as expected, it became shorter with age (44.4 ± 1.9 ms, n = 17 and 39.4 ± 1.3 ms, n = 20 for 9-11 and 12+ DIV respectively, $p < 0.05$, Fig. 1 C). As already discussed in [16], such an acceleration of mIPSCs reflects a developmental regulation of the GABAergic system that is correlated with increased expression of the α1 subunit. Interestingly, a detailed analysis of the time course of mIPSCs revealed that administering Epo modulated the deactivation kinetics, but this effect critically depended on the developmental stage. In this model, 48 and 72 h treatment with Epo significantly reduced the τ_{mean} of the recorded mIPSCs at 6-8 DIV, by 18 and 13% respectively ($p < 0.05$, Fig. 1 B, C). This change was a consequence of a decrease in the percentage of the slower component of the decaying phase from 0.56, respectively to 0.45 (n = 17) and 0.46 (n = 20; $p < 0.05$). The effect of Epo treatment was not observed in the 9-11 and 12+ DIV age groups, indicating that long-term treatment with this drug only alters mIPSC kinetics at early developmental stages.

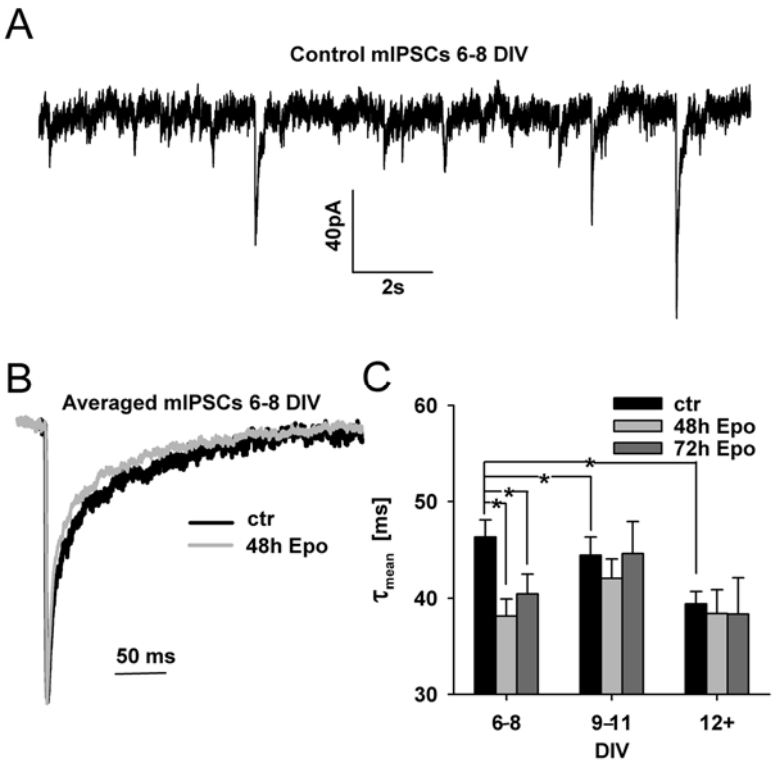

Fig. 1. The impact of Epo on the kinetics of mIPSCs recorded in a hippocampal neuronal culture. A – Examples of mIPSCs recorded at -70 mV under control conditions. B – Typical averaged and superimposed mIPSCs recorded under control conditions (black line) and in the 6-8 DIV culture treated for 48 h with Epo (grey line). Note the shortening of the decaying phase. C – Statistics of the effects of Epo treatment on the mean decay time constant (τ_{mean}) for different age groups. The asterisk indicates a significant difference between the compared values.

DISCUSSION

In this study, to the best of our knowledge, we provide the first evidence that GABAergic transmission is regulated by Epo during development *in vitro*. Importantly, the modulatory effect of Epo reported on here is restricted to the early phases of development. This observation indicates that there is a restricted time-window within which GABAergic synaptic currents are particularly sensitive to modulation by Epo. Such a clear Epo effect limited to the earliest developmental stage considered here is particularly interesting in the light of previous findings that the Epo level changes with development, being highest at early stages of gestation [1]. On the other hand, Epo treatment did not affect the mIPSC amplitude or frequency, indicating that this compound does not affect the number of GABAergic synapses or the number of $GABA_A$ receptors in the individual synapses. The impact of such Epo modulation of mIPSCs at the

neuronal network level is not clear. IPSC shortening reduces the charge transfer and therefore the impact of IPSC on the membrane voltage. However, since GABA is known to induce depolarization rather than hyperpolarization at early developmental stages [19], such an effect on phasic GABAergic currents is expected to reduce the network excitability. Miniature IPSC shortening is a hallmark of neural network maturation and it is believed that such a process is associated with network refinement, giving rise to an improved coincidence detection. However, our data is insufficient to assess the Epo effect on neuronal network performance, and thus the specific role of Epo-mediated mIPSC acceleration remains to be elucidated.

The major question is whether the observed modulation of GABAergic currents by Epo is reproduced *in vivo*. A definite answer to such a question will require performing slice recordings on Epo-treated animals, but it is worth emphasizing that the model used in this study, although clearly distant from the *in vivo* conditions, maintains key developmental features (see [16]). Another question that cannot be answered based on this data is the mechanism whereby Epo affects mIPSCs in the 6-8 DIV group. The lack of any effect of Epo on mIPSCs when acutely applied to the fluids surrounding neurons indicates an indirect action of this modulation, but elucidating its nature will require additional studies. We conclude that Epo may affect GABAergic transmission and that this modulation occurs at an early stage of development *in vitro*.

Acknowledgements. We would like to thank Mr. Piotr Brzeźnicki for his help with the experimental part of this work. This study was supported by the Polish Ministry of Education and Science grants PBZ-MIN-001/P05/28 and N401 028 32/0664.

REFERENCES

1. Genc, S., Koroglu, T.F. and Genc, K. Erythropoietin and the nervous system. **Brain Res.** 1000 (2004) 19-31.
2. Digicaylioglu, M., Bichet, S., Marti, H.H., Wenger, R.H., Rivas, L.A., Bauer, C. and Gassmann, M. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. **Proc. Natl. Acad. Sci. USA** 92 (1995) 3717-3720.
3. Masuda, S., Nagao, M., Takahata, K., Konishi, Y., Gallyas, F. Jr., Tabira, T. and Sasaki, R. Functional erythropoietin receptor of the cells with neural characteristics. Comparison with receptor properties of erythroid cells. **J. Biol. Chem.** 268 (1993) 11208-11216.
4. Tan, C.C., Eckardt, K.U., Firth, J.D. and Ratcliffe, P.J. Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. **Am. J. Physiol.** 263 (1992) F474-481.
5. Brines, M.L., Ghezzi, P., Keenan, S., Agnello, D., de Lanerolle, N.C., Cerami, C., Itri, L.M. and Cerami, A. Erythropoietin crosses the blood-brain

barrier to protect against experimental brain injury. **Proc. Natl. Acad. Sci. USA** 97 (2000) 10526-10531.
6. Kumral, A., Ozer, E., Yilmaz, O., Akhisaroglu, M., Gokmen, N., Duman, N., Ulukus, C., Genc, S. and Ozkan, H. Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. **Biol. Neonat.** 83 (2003) 224-228.
7. Buemi, M., Cavallaro, E., Floccari, F., Sturiale, A., Aloisi, C., Trimarchi, M., Grasso, G., Corica, F. and Frisina, N. Erythropoietin and the brain: from neurodevelopment to neuroprotection. **Clin. Sci. (Lond)** 103 (2002) 275-282.
8. Zhang, F., Signore, A.P., Zhou, Z., Wang, S., Cao, G. and Chen, J. Erythropoietin protects CA1 neurons against global cerebral ischemia in rat: potential signaling mechanisms. **J. Neurosci. Res.** 83 (2006) 1241-1251.
9. Yang, J., Huang, Y., Yu, X., Sun, H., Li, Y. and Deng, Y. Erythropoietin preconditioning suppresses neuronal death following status epilepticus in rats. **Acta Neurobiol. Exp. (Wars)** 67 (2007) 141-148.
10. Ransome, M.I. and Turnley, A.M. Systemically delivered Erythropoietin transiently enhances adult hippocampal neurogenesis. **J. Neurochem.** 102 (2007) 1953-1965.
11. Dame, C., Bartmann, P., Wolber, E., Fahnenstich, H., Hofmann, D. and Fandrey, J. Erythropoietin gene expression in different areas of the developing human central nervous system. **Brain Res. Dev. Brain Res.** 125 (2000) 69-74.
12. Juul, S. Erythropoietin in the central nervous system, and its use to prevent hypoxic-ischemic brain damage. **Acta Paediatr. Suppl.** 91 (2002) 36-42.
13. Juul, S.E., Yachnis, A.T., Rojiani, A.M. and Christensen, R.D. Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. **Pediatr. Dev. Pathol.** 2 (1999) 148-158.
14. Hollrigel, G.S. and Soltesz, I. Slow kinetics of miniature IPSCs during early postnatal development in granule cells of the dentate gyrus. **J. Neurosci.** 17 (1997) 5119-5128.
15. Andjus, P.R., Stevic-Marinkovic, Z. and Cherubini, E. Immunoglobulins from motoneurone disease patients enhance glutamate release from rat hippocampal neurones in culture. **J. Physiol.** 504 (1997) 103-112.
16. Pytel, M., Wojtowicz, T., Mercik, K., Sarto-Jackson, I., Sieghart, W., Ikonomidou, C. and Mozrzymas, J.W. 17 beta-estradiol modulates GABAergic synaptic transmission and tonic currents during development *in vitro*. **Neuropharmacology** 52 (2007) 1342-1353.
17. Clements, J.D. Transmitter timecourse in the synaptic cleft: its role in central synaptic function. **Trends. Neurosci.** 19 (1996) 163-171.
18. Mozrzymas, J.W. Dynamism of GABA(A) receptor activation shapes the "personality" of inhibitory synapses. **Neuropharmacology** 47 (2004) 945-960.
19. Cherubini, E., Gaiarsa, J.L. and Ben-Ari, Y. GABA: an excitatory transmitter in early postnatal life. **Trends. Neurosci.** 14 (1991) 515-519.