Long-term Results of Enriched Environment and Erythropoietin after Hypobaric Hypoxia in Rats

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Summary

After global cerebral hypoxia, many patients are severely disabled even after intensive neurorehabilitation. Secondary mechanisms of brain injury as a result of biochemical and physiological events occur within a period of hours to months, and provide a window of opportunity for therapeutic intervention. Erythropoietin (EPO) has been shown to be neuroprotective in the brain subjected to a variety of injuries. Fifty-nine 3-month-old male Wistar rats were randomly distributed to experimental groups with respect to the housing (enriched environment – EE, standard housing – SH), to hypoxia exposure, and to EPO treatment. An acute mountain sickness model was used as a hypobaric hypoxia simulating an altitude of 8000 m. One half of the animals received erythropoietin injections, while the others were injected saline. Spatial memory was tested in a Morris water maze (MWM). The escape latency and the path length were measured. Better spatial learning in MWM was only seen in the group that received erythropoietin together with enriched environment. EPO administration itself had no influence on spatial memory. The results were very similar for both latencies and path lengths. These results support the idea that after brain injuries, the recovery can be potentiated by EPO administration combined with neurorehabilitation.

Key Words

Global cerebral hypoxia – Enriched environment – Erythropoietin – Morris water maze – Spatial memory

Introduction

Brain damage is manifested by functional deficiencies due to both primary and secondary mechanisms. For example, the primary injury represents the direct mechanical damage that cannot be changed. Secondary mechanisms are the result of biochemical and physiological events that lead to cell death. They occur within a period of hours to days or even months, and provide a window of opportunity for therapeutic interventions with a potential to prevent or reduce secondary damage and to improve the long-term outcome.

Global cerebral ischemia occurs when cerebral blood flow is reduced throughout most of the brain. Complete global ischemia can be caused by cardiac arrest, aortic occlusion, neck cuff etc. (Traystman 2003). The decrease of tissue oxygenation induced by hypobaric hypoxia alters many physiological and psychological processes in an altitude- and duration-dependent manner (Titus *et al.* 2007, Pokorný *et al.* 1989).

The physiological basis for neurorehabilitation is neuroplasticity that is responsible for functional restitution or recovery after secondary brain damage. There are several mechanisms of neuroplasticity after brain damage – vicariation, diaschisis, sprouting, long-term potentiation, neuronal reorganization, unmasking of neuronal functional pathways, neurogenesis, and others (Trojan and Pokorný 1989, Zhang *et al.* 2004). Neurorehabilitation can be enhanced by promising neuroprotective and neurorestorative approaches. Various substances (e.g. erythropoietin, nitric oxide and others) are used for such purposes after the brain injury (Stein 2007, Xiong *et al.* 2009).

Erythropoietin (EPO), a naturally occurring cytokine, is most widely recognized for its role in the stimulation of maturation, differentiation and survival of hematopoietic progenitor cells (Xiong *et al.* 2009). It is a glycoprotein which has emerged as a multifunctional growth factor that plays a significant role in the nervous system. EPO and its receptors are expressed throughout the brain in glial cells, neurons and endothelial cells. EPO is a key example of

gene expression that is regulated in an oxygen-dependent manner (Marti 2004). EPO has multiple protective effects in the CNS that are, at least partially, mediated through the upregulation of antiapoptotic molecules (Chong *et al.* 2002). EPO has been shown to be neuroprotective in the brain after exposure to a variety of injuries, including cerebral ischemia, head injury, seizures and experimental autoimmune encephalomyelitis (Marti 2004).

A milestone in the history of biological activities of EPO was the paper of Brines *et al.* (2000) who demonstrated that the cross-talking between peripheral and central EPO systems is possible. The most striking effect of these interacting systems is the ability of peripherally injected human recombinant EPO (r-Hu-EPO) to protect brain tissue from a variety of injuries including ischemia/hypoxia, as well as trauma, immune-mediated inflammation, and excessive neuronal excitation (Brines and Cerami 2008). It has not been elucidated how EPO mediates its effects across blood-brain barrier, but the observations are consistent with a specific receptor-mediated translocation of EPO into the brain (Brines et al. 2004). Although a rat EPO has 192 amino acids compared to human EPO with 165 amino acids, most of the experiments in rats were done with r-Hu-EPO (Brines *et al.* 2000, Marti 2004, Brines and Cerami 2008).

The aim of our study was to reveal whether EPO given to rats after hypobaric hypoxia could influence the final outcome of cognitive functions, especially spatial memory measured by means of a Morris water maze. We were also interested to discover how important is the role of enriched environment in this model.

Materials and methods

Animals

This study was performed in accordance with the *Guide for Care and Use of Laboratory Animals of Central Commission for Animal Welfare (CCAW)* of the Charles University of Prague. All efforts were adopted to minimize animal pain or discomfort, and to reduce the total number of used experimental animals.

Fifty-nine 3-month-old male Wistar rats from our own breeding facility were used. Their initial body weight was approximately 400-500 g. They were maintained in a temperature-controlled room (20-23 °C), on a 12 h light/dark cycle, with commercial rat chow (Velas F1, Velas s.r.o., Lysá nad Labem, Czech Republic) and fresh water available *ad libitum*. The rats were always tested between 10 a.m. and 2 p.m. The animals were randomly distributed in experimental groups with respect to:

- housing – enriched (EE+) or standard (EE–) environment (EE is described further)
- hypoxia event – hypoxia (Hypo+) or sham-hypoxia (Hypo–)
- erythropoietin treatment – EPO+ or EPO–

Hypobaric hypoxia, sham-hypoxia

The acute mountain sickness model was used as a hypobaric hypoxia. The barometric pressure and atmospheric oxygen pressure were reduced. The experimental animals were exposed on the 8th day of the experiment to this hypoxia (Hypo+) for 60 min in an experimental chamber, simulating an altitude of 8000 m. This altitude was reached within 10 min as well as its reversal. Sham-hypoxia (stress situation) was performed by placing the animals in another experimental hypobaric chamber at the same time, but without the reduction of the barometric pressure.

Enriched environment

Environmental enrichment (EE+) began at the start of this study, i.e. in rats aged 3 months and continued throughout the whole experiment. Each group consisted of 8-9 rats that were kept in three large half-plastic and half-wire cages. Two of the cages measured 57 x 38 x 20 cm, and the last – the middle 57 x 38 x 40 cm supported by two wooden floors connected by circular port (12 cm in diameter). Moreover, these cages contained toys and various objects suitable for training. The standard housing (EE-) consisted of standard plastic cages (27 x 42 cm) without toys or other objects.

Erythropoietin

The animals received a single intraperitoneal injection on the 8th day of the experiment immediately after the exposure to hypoxia/sham-hypoxia. Half of the animals received erythropoietin injections (EPREX, epoetinum alfa, Janssen-Cilag, 400 or 1000 IU/0.1 ml) in the dosage of 5000 IU/kg body weight, ad the other half of the rats received saline injections (Natrium Chloratum, sol. isotonica, Hoechst-Biotika, Germany).

Morris water maze

Spatial memory was tested in a Morris water maze (MWM). The maze consisted of a circular pool 183 cm in diameter, filled with clear water at a temperature of 22-23 °C. The depth of the water was 23 cm, and a transparent platform 10 cm in diameter was submerged 2 cm below the surface in the northwest quadrant in a constant position throughout the whole experiment. The pool was divided in four equal sections (north-east, north-west, south-east and south-west) and had four points designed as start positions – north, west, south and east. The movements of rats were recorded with a video camera fixed on the ceiling over the maze and connected to a computer.

The trial began by gently placing a rat in the water, facing the wall at one of the four starting points. The rat was trained to find the platform within 60 s. When the rat did not reach the platform, it was placed on it and left there for 15 s after each unsuccessful trial. When the rat reached the platform, it was allowed to stay there for 15 s and then it was placed in the water again, from another starting point.

The *escape latency* (the time needed to find the hidden platform) was measured in each trial, and then the mean latency for every rat and every training day was calculated. In case the rat did not find the platform, the latency was evaluated as 60 s. The *path length* (the length from the start to reaching the platform) in each trial was measured, and then the mean length for every rat and every day was calculated. In the case that the rat was not successful in finding the platform, the length of the path within 60 s was recorded.

Timeline of experimental procedures

The rats were given four training periods. Each period took five consecutive days with eight trials in MWM each day. The periods started in the first, twenty-second, thirty-sixth and fifty-seventh day of the experiment, respectively. On the 8th day of the experiment, the hypoxia /sham hypoxia was performed.

Neurological evaluation (composite neuro-score)

Experimental animals were tested one hour before hypoxia/sham hypoxia, one hour after hypoxia/sham hypoxia, and the second day after hypoxia/sham hypoxia (the 9th day of the experiment). The last evaluation (i.e. on the 16th day) was cancelled because of the normal neurological status of all animals. Scoring for each animal ranged from 0 points (severely impaired) to 4 points (normal neuromotor function) for each of following modalities: forelimb flexion (left/right) during suspension by the tail, hindlimb flexion with the forelimbs

remaining on the flat surface, resistance to lateral propulsion (left/right), and the ability to keep balance on an inclined plane (left/right/vertical position). This test shows high inter-observer reliability and has been used in numerous studies (Faden *et al.* 1989, Lipert-Grüner *et al.* 2007). It reveals primarily neuromotor functions.

Statistics

Medians of path length and latencies were calculated for different groups during the experiment. Kruskal-Wallis analysis – a non-parametric (distribution-free) statistical test was chosen for evaluation of the differences between path length or differences between latencies in various groups. This non-parametric test uses the ranks of the data rather than their raw values to calculate the statistics. It is an alternative to the independent group ANOVA when the assumption of normality or equality of variance is not met. The normality of variance was tested with the Shapiro-Wilk test.

Results

Results are presented as medians of path lengths or latencies (Figs 1A-D) and the Mean Rank of the path lengths and latencies (Figs 2A-B and 3A-B) in groups 1, 2, 5 and 6. These groups are very important from the point of view of rehabilitation. We compared the effect of EPO in animals subjected to hypoxia with regard to the enriched environment. Groups 1 and 2 are the groups where rats were kept in enriched environment, were subjected to hypoxia and were treated with either EPO or saline. On the contrary, groups 5 and 6 consisted of rats kept in standard plastic cages, subjected to hypoxia and administered EPO or saline. The differences between the groups are relatively small but some very important significances were seen.

Figures 1A-B show the medians of path lengths and latencies in groups1 and 2. The group kept in EE with EPO administration after hypoxia (group 2) deals better than the group without it (group 1). These results are more evident when Kruskal-Wallis analysis is used (Figs 2A-B). The differences in path lengths are significant especially on the 22nd, 24th, 26th and 40th day ($p=0.008$, $p=0.037$, $p=0.049$ and $p=0.049$) (Fig. 2A). Similar results were obtained for latencies (Fig. 2B).

Figures 1C-D depict the medians of path lengths and latencies in groups 5 and 6. The rats of these groups were kept in standard housing, were subjected to hypoxia and were administered EPO or saline. Kruskal-Wallis analysis was also used for the same groups and situations (Figs 3A-B). Figure 3A concerning the path lengths shows that the group with EPO administration is even worse after hypoxia than before it, while the group with saline administration is slightly better after hypoxia although not significantly. The situation concerning latencies is very similar and there are no significant changes between the groups after hypoxia.

There were no changes in the neurological evaluation of the rats after hypobaric hypoxia or sham hypoxia measured with neuro-score. According to our results, there were no significant differences in spatial memory measured by MWM between the rats that received EPO and those who got saline after hypobaric hypoxia when we did not take into account the environment in which the animals were kept (data not presented). Significant differences after hypoxia were seen only between groups 1 and 2, i.e. only between those rats that were kept in enriched environment. The rats, which got EPO and were kept in enriched environment (group 2), were significantly better than those which were kept in enriched environment but got only saline (group 1).

Discussion

Long-term functional outcome of some patients after global cerebral hypoxia are very bad (especially cognitive functions), in spite of using different neurorehabilitation methods (Lippert Grüner *et al.* 2007, FitzGerald et al. 2010). This is why research is focused on neurorehabilitation combined with neuroprotection that could have much better results (Xiong *et al.* 2009). After a decade of research, there were only two perspective substances selected – progesterone which was studied in several clinical trials (Stein *et al.* 2008), and erythropoietin that has neuroprotective, neuroregenerative and antiinflammatory effects (Marti 2004, Ostedkar *et al.* 2010, Cherian *et al.* 2011).

Hypobaric hypoxia causes different morphological and functional changes in several parts of an adult and newborn brain especially in hippocampus (CA3 and CA1 areas, gyrus dentatus), thalamus, striatum and cortex depending on the duration and simulated altitude of hypoxia (Kirino 1982, Šimonová *et al.* 2003). Neurons in CA3 hippocampal area probably play a very important role in memory processing (Lisman 1999, Lorincz and Buzsaki 2000). Neurons in the CA1 area are destroyed very quickly because they have more ionotropic NMDA receptors (Cassina *et al.* 2002). On the contrary, the CA3 area has less ionotropic and more metabotropic glutamate receptors that are responsible for delayed neurotoxicity (Maiti *et al.* 2007).

EPO mediates its effects through the binding to its cognate receptors. Thus, an EPO receptor must be expressed at the site of action in the CNS to enable EPO to elicit its biological functions. Indeed, the expression of the EPO receptor mRNA and protein was demonstrated in the brain of a mouse, rat, monkey, and humans (Digicaylioglu *et al.* 1995). Both EPO mRNA and protein are found in the brain of numerous mammals including humans. The EPO receptor is widely expressed in most cerebral cell types, including neurons, endothelial cells, microglial cells and astrocytes (Marti 2004). EPO seems to be a part of an

endogenous defense system enabling the brain to counteract detrimental effects of hypoxia and ischemia (Marti 2004).

The aim of the study was to reveal long-term effects of EPO and EE on the spatial memory and neuromotor functions of rats. There were no changes in neuro-score, i.e. no neuromotor problems in any studied group, but we have seen the differences between studied groups in spatial memory tested in MWM.

Environmental enrichment is comparable with multidisciplinary rehabilitation in patients (Pereira et al. 2008). Its positive effect on neuromotor and cognitive functions was described in several studies (Grabovski *et al.* 1995, Gobbo and O´Mara 2004) as well as on neuroanatomic and neurophysiologic functions (Zhao *et al.* 2001, Pereira *et al.* 2008).

We have seen the best results in the group where EE and EPO were applied together. EPO itself without EE had no significant effect on the results. The stimulation of brain endogenous protective mechanisms may be the key to future successful approaches to neuroprotection, as the activation of endogenous mechanisms can be efficient and well tolerated (Siren *et al.* 2001). EPO acts in the central nervous system primarily as a direct protective factor in neurons *via* the activation of antiapoptotic pathways. The protective effect on neurons might be supported by the action of EPO and other growth factors on endothelial cells, resulting in the cell survival and the stimulation of new vessel growth, as well as on glial cells, leading to a modulation of inflammatory responses (Marti 2004). EE plays a very important role as it increases hippocampal brain-derived neurotrophic factor, enhances cell survival, increases neurogenesis, dendritic branching and dendritic spines, as well as synaptogenesis (Van Praag *et al.* 2002). This takes some time so that we have seen the results after 40 and mainly after 60 days).

It was highly interesting that better results were also found in the group with EE and EPO without hypoxia. It means that EE plays a dominant role in the restoration of cerebral functions and EPO itself has no effect on the functional outcome.

EPO binding to erythropoietin receptor (EpoR) mediates neuroprotection by endogenous EPO or by exogenous EPO (e.g. r-Hu-EPO). The level of EpoR expression in brain tissue has been proposed to determine the cytoprotective effects of EPO (Chen *et al.* 2006, Yu *et al.* 2002). The number of EpoR is different in various parts of brain. It would be desirable to find new ways to enhance their expression in those brain parts where they are insufficient. It could be done e.g. by combining repeated mild hypoxia with EE (Sanchez *et al.* 2009).

EPO, which is a molecule induced by hypoxia, is considered to have a key role in the enhancement of brain robustness by hypoxia (Sharp and Bernaudin 2004). Thus, recombinant human erythropoietin (rhEpo)can be considered as an "enviro-mimetic", defined as any exogenous molecule that mimics the beneficial effects of environmental changes (Nithianantharajan and Hannan 2006). There is a concept that the optimization of the effect of a neuroprotective agent may require the preliminary induction of its targeted receptor (Lipton 2007). Concerning rhEpo, future studies should elucidate the mechanisms promoting the movement of EpoR towards the cell surface (Ravid *et al.* 2007), and the mechanisms selectively involved in the induction of EpoR after environmental manipulations, to develop drugs capable of inducing EpoR and to influence the final functional outcome of people after brain injuries.

Our results support the hypothesis that EPO combined with an enriched environment can influence the final outcome of spatial memory and learning of rats after hypobaric hypoxia. It is very important for medical practice in brain injuries to search for new strategies which can reduce final disabilities of patients. Taking into account our results, we can expect

that EPO given after brain injury to patients, who have multidisciplinary neurorehabilitation can influence the neurorestoration process, and helps to achieve better functional outcomes for these patients,

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Table 1. Distribution of rats in groups and their characteristics

Group No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Number of Rats**
Number of rats	7	8	7	7	8	8	7	7	59
Enriched environment (EE)	+	+	+	+	–	–	–	–	29
Hypobaric hypoxia	+	+	–	–	+	+	–	–	31
Erythropoietin (EPO)	–	+	–	+	–	+	–	+	30

Legends to figures

Fig. 1. Median of path length (**A, C**) and latencies (**B, D**) in the groups 1 and 2 (EE+ and Hypo+, EPO– or EPO+) (**A, B**) as well as in the groups 5 and 6 (EE– and Hypo+, EPO– or EPO+) (**C, D**). Open symbols with broken line represent EPO– animals, whereas full symbols with full line depict EPO+ animals. Significance of the differences between EPO– and EPO+ rats:* $p<0.10$, ** $p<0.05$.

Fig. 2. Mean Rank of path length (**A**) and latencies (**B**) in the groups 1 and 2 (EE+ and Hypo+, EPO– or EPO+). Significance of the differences between EPO– and EPO+ rats according to Kruskal-Wallis analysis:* $p<0.10$, ** $p<0.05$.

Fig. 3. Mean Rank of path length (**A**) and latencies (**B**) in the groups 5 and 6 (EE– and Hypo+, EPO– or EPO+). Significance of the differences between EPO– and EPO+ rats according to Kruskal-Wallis analysis:* $p<0.10$, ** $p<0.05$.

Fig. 1

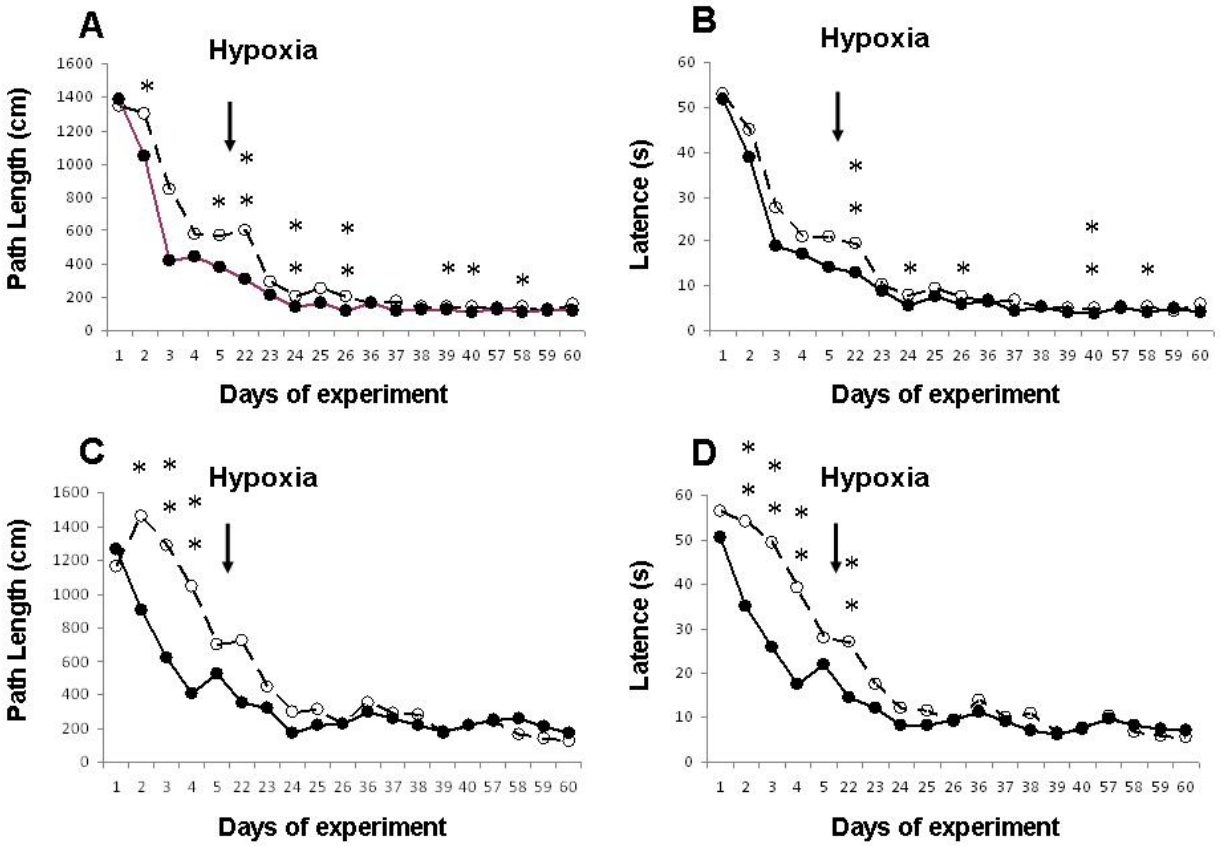

A
Hypoxia
Path Length (cm)
Days of experiment
B
Hypoxia
Latence (s)
Days of experiment
C
Hypoxia
Path Length (cm)
Days of experiment
D
Hypoxia
Latence (s)
Days of experiment

Fig. 2

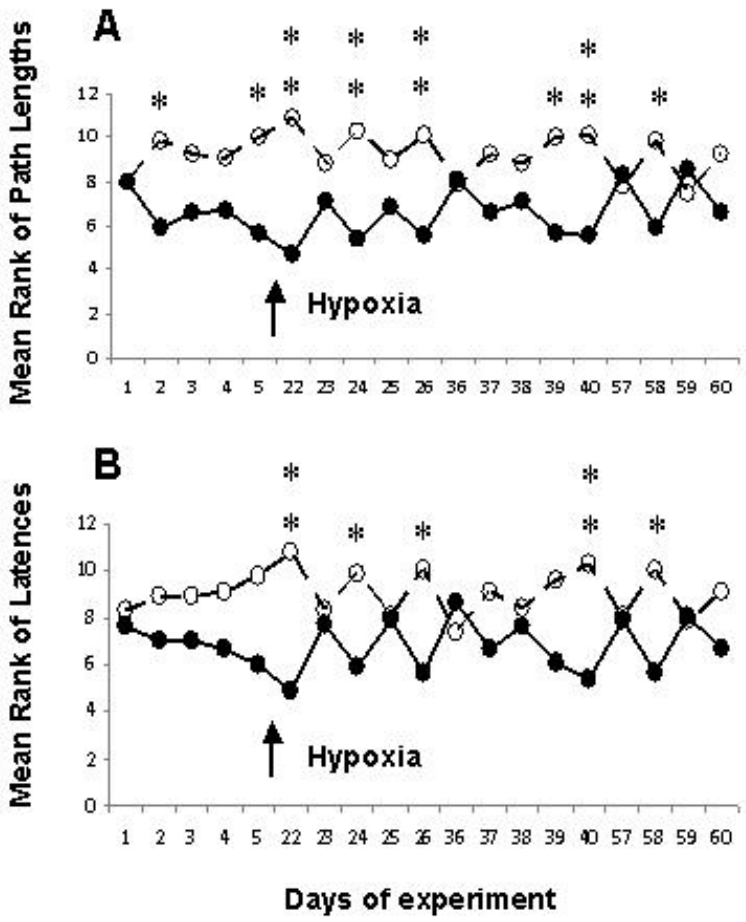

A
Mean Rank of Path Lengths
Hypoxia
B
Mean Rank of Latences
Hypoxia
Days of experiment

Fig. 3

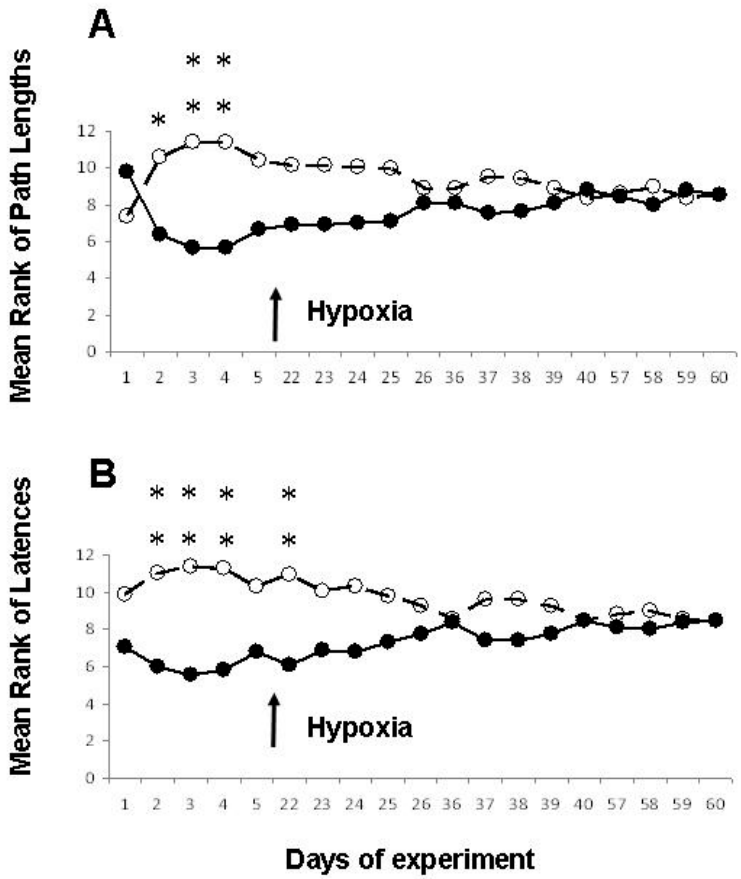
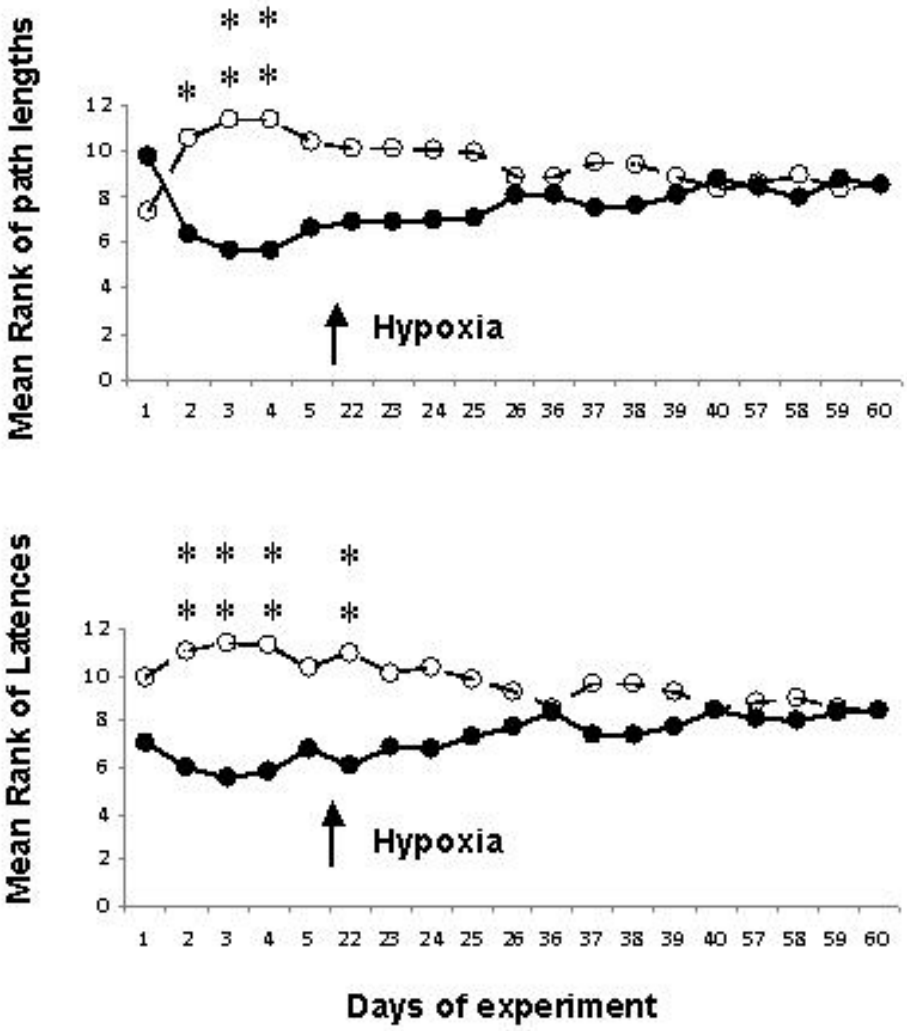

A
Mean Rank of Path Lengths
Hypoxia
B
Mean Rank of Latences
Hypoxia
1 2 3 4 5 22 23 24 25 26 36 37 38 39 40 57 58 59 60
Days of experiment

Fig. 3.


Mean Rank of path lengths
Hypoxia
Mean Rank of Latences
Hypoxia
Days of experiment