Early Postnatal Hypoxia Induces Behavioral Deficits but not Morphological Damage in the Hippocampus in Adolescent Rats

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Summary

Hypoxia is one of the major pathological factors affecting brain function. The aim of the present study was to describe the effect of neonatal hypobaric hypoxia on the behavior of rats and to analyze its effect on hippocampal neurodegeneration. Hypobaric hypoxia at a simulated altitude of 9000 m was induced for one hour in neonatal rat pups (PND7 and PND9) of both sexes. Subsequently, the rats underwent behavioral testing on PND25 and PND35 using a LABORAS apparatus to assess spontaneous behavior. Hypoxia did not cause any morphological damage in the hippocampus of rats. However, hypoxia on PND7 led to less horizontal locomotor activity both in males (on PND25) and females (on PND35). Hypoxia on PND9 led to higher rearing in females on PND25. Hypoxic males exhibited higher grooming activity, while females lower grooming activity on PND35 following hypoxia induced on PND7. In females, hypoxia on PND9 resulted in higher grooming activity on PND25. Sex differences in the effect of hypoxia were observed on PND35, when hypoxic males compared to hypoxic females displayed more locomotor, rearing and grooming activity. Our data suggest that hypoxia on PND7 versus PND9 differently affects locomotion and grooming later in adolescence and these effects are sex-dependent.

Key words

Behavior • Hippocampus • Short-term hypoxia • Open field test • Sex differences

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Introduction

Hypoxic and hypoxic-ischemic injuries *in utero* or during birth are major causes of newborn morbidity and mortality. They represent major health concerns in neonatal medicine (Sarnat *et al.* 1976, Berger *et al.* 2002, Zayachkivsky *et al.* 2015). Despite continuous advances in neonatology and intensive care, there is no effective treatment except hypothermia for hypoxia-ischemia (Riljak *et al.* 2016, Potter *et al.* 2018). As the immature neonatal brain is highly susceptible and responsive to external and internal stimuli, the timing of hypoxia exposure is an important factor in the severity of behavioral changes (Berger *et al.* 2002, Cuaycong *et al.* 2011, Potter *et al.* 2018). Various animal models of hypoxic and hypoxic-ischemic conditions have been developed to mimic neonatal ischemic and/or hypoxic brain damage (Vannucci 1990, Cuaycong *et al.* 2011). It has previously been shown that hypoxia-induced behavioral changes (e.g. impaired motor function and novelty recognition) are dependent not only on the timing of hypoxia exposure but also on the intensity of hypoxia (Bona *et al.* 1998, Carty *et al.* 2010). Hypoxia interferes with multiple biochemical pathways. These metabolic changes are reflected in brain synaptogenesis, the glial cell response, blood brain barrier permeability and the

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balance and clearance of neuromediators (Nyakas *et al.* 1996, Pappas *et al.* 2015, Potter *et al.* 2018). Considering these changes, it has been reported that hypoxic episodes during the perinatal period of life can trigger depression-like behavior (Bogdanova *et al.* 2014), spatial memory changes (Chen *et al.* 2018), impaired learning ability (Biswal *et al.* 2016), excitotoxicity (Ginet *et al.* 2014) and the induction of cell death in nervous tissue (Arumugam *et al.* 2018). Previous animal studies have reported that male and female rats are affected by hypoxia differently and show a different extent of behavioral deficits (Hill *et al.* 2011, Potter *et al.* 2018). Compared to their female counterparts, male infants exhibit an increased risk of hypoxic-ischemic events and also display greater behavioral and cognitive disruption (Raz *et al.* 1995, Costeloe *et al.* 2000, Lauterbach *et al.* 2001, Donders *et al.* 2002).

Brain development between postnatal days (PNDs) 7-12 in rats is approximately equivalent to the end of the third trimester of pregnancy in humans (Clancy *et al.* 2001, Semple *et al.* 2013). In the present study, we investigated the effect of hypobaric hypoxia at a simulated altitude of 9000 m on PND7 or PND9 on the spontaneous behavior of young rats during the post-weaning period (PND25) and periadolescent period (PND35). Due to the evidence of sex differences in the behavior of neonatal models of hypoxia, both male and female rats were used. In addition, the effect of exposure to early postnatal hypoxia on the severity of brain injury was evaluated, with a focus on morphological changes in the hippocampus using histochemical methods. We hypothesized that the impact of hypoxia would be more severe when applied on PND7 rather than PND9 and that males would display more pronounced behavioral impairments compared to those displayed by females.

Materials and Methods

Animals and housing conditions

In the current study, Wistar albino rats (n=96; males n=48, females n=48) were used. The dams were obtained from the Centre for Experimental Biomodels, First Faculty of Medicine, Charles University, Prague and were housed individually. Following the delivery dams were caged with their offspring until weaning on PND21, later on, the offspring was tested as described below.

All rats were kept in a controlled environment (temperature 22±2 °C, humidity 55±10 %, 12:12-hour light-dark cycle with lights on at 06:00 a.m. and lights off 06:00 p.m.) with *ad libitum* access to food and water. Behavioral testing took place between 08:00 and 12:00 in a slightly illuminated room with light intensity level 100-120 lux at the level of the cages. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and were in agreement with the Czech Government Requirements and Requirements of European Communities Council Directive (86/609/EEC). Maximal efforts were made to minimize the suffering of the animals during the experiment.

Simulation of hypobaric hypoxia

At the age of PND7 (males n=12, females n=12) or PND9 (males n=12, females n=12), rat pups of both sexes were exposed to hypobaric hypoxia (apart from their mothers) for 60 min at a simulated altitude of 9000 m (p=230 mm Hg). The hypobaric chamber was an airtight box (60x60x60 cm) equipped with a carbon dioxide sorbent. The time needed to reach the desired altitude was 20 min. The same time was needed to return back to room atmospheric pressure. Following hypoxia exposure, the rat pups were returned to their nest and their mothers until weaning. The number of control rat pups equals to the number of the pups exposed to hypobaric hypoxia. The control groups underwent the same procedure except for the change in atmospheric pressure.

Tissue processing

For histological analyses, in total 48 rat pups (female: n=24; male: n=24) were anaesthetized with an intraperitoneal injection of thiopental (40 mg/kg body weight) and were immediately perfused transcardially. The transcardial perfusions were performed 24 h or 5 days after hypoxia induction (i.e. PND8, PND12, PND10 or PND14, n=3 for each gender and time point, one animal served as a control). Another group of rat pups (female: n=3; male: n=3, PND7 and PND9) underwent the same procedure and the transcardial perfusions 24 h after the last behavioral testing (i.e. PND36). First, ice-cold saline (0.9 % NaCl) was used for transcardial perfusion, followed by fixation with ice-cold 4 % paraformaldehyde (dissolved in 0.1 M phosphate buffer, pH 7.4). Thereafter, the brain was removed carefully from the cranium of each animal and fixed in 4 % paraformaldehyde overnight. Following fixation, the brains were cryoprotected in 20 % sucrose solution for at least 1 day.

Detection of neuronal degeneration and apoptosis

Neuronal degeneration was evaluated histologically in each group of rats using Fluoro-Jade (FJB) and bisbenzimide staining. Bisbenzimide (Hoechst 33342, Sigma-Aldrich, Czech Republic) and FJB (Histo-Chem Inc., USA) co-staining was performed as described previously (Riljak *et al.* 2007, Riljak *et al.* 2010). Briefly, the brains were sectioned at -20 °C using a cryostat (Leica CM 3050S, Leica Biosystems, Germany), and every third 40-µm thick section from each animal brain was collected to assess neuronal degeneration. The tissue sections were then mounted on gelatinized slides (Menzel-Gläser Superfrost Plus, Thermo Scientific, Germany) and allowed to dry at room temperature. Then, the sections were co-stained using FJB and the nuclear counterstain bisbenzimide. The sections were immersed in ethanol, distilled water and potassium permanganate ($KMnO_4$, Sigma-Aldrich, Czech Republic). The slides were immersed in a 0.001 % FJB solution for 30 min in a dim room with occasional gentle shaking. Thereafter, the sections were immersed in a 0.01 % bisbenzimide staining solution for 10 min, dehydrated, coverslipped using DPX neutral mounting medium (Neo-Mount, Merck, Germany) and allowed to dry. The sections were visualized using an epifluorescence microscope (Olympus AX 70 Provis, Olympus, Czech Republic) with appropriate filter settings for blue and green fluorescence. The following regions of brain tissue were analysed for neuronal degeneration or apoptosis: the CA1 and CA3 regions of the hippocampus, the hilus, and the dorsal and ventral blades of the dentate gyrus. The presence of FJB-positive cells was determined in each of the above listed regions of the hippocampus.

Nissl staining

Each fourth coronal section of the brain (obtained as described above in sections 2.3. and 2.4.) was stained with cresyl violet (Sigma-Aldrich, Czech Republic). The mounted brain tissue sections were dehydrated in a graded series of ethanol (70 %, 80 %, 96 %) for 2 min each and then stained with Nissl solution (1 % cresyl violet, 0.2 mol/l acetic acid, and 0.2 mol/l sodium acetate, 4:1, pH=3) for approximately 20 min. When the desired color intensity was reached, the slices were washed twice in distilled water and in a graded series of ethanol (96 %, 80 %, 70 %) for 2 min each. The slides were then immersed in xylene (Penta, Czech Republic) for 5 min. Subsequently, the slides were incubated in another xylene bath (for approximately 45 min) and were mounted with Roti-Histokitt II mounting medium (Roth, Germany) and coverslipped.

Behavioral testing

Open field test (LABORAS system)

For behavioral testing in total 48 rat pups were used (males n=24, females n=24). LABORAS is an automated system for the continuous tracking of small rodent behavior. On PND25 and PND35, the spontaneous behavior of the animals was analysed. The animals were weighed and placed in the LABORAS system, and the behavior of the animals was monitored for 1 h. The LABORAS system transforms the mechanical vibrations generated by the animal (during locomotion, rearing, grooming, etc.) into electrical signals. These signals are processed, classified and compared with predetermined characteristic patterns by LABORAS software (Van de Weerd *et al.* 2001). Spontaneous behavior was analysed over successive 10-min intervals (0-10 min, 10-20 min, 20-30 min, 30-40 min, 40-50 min, 50-60 min). Each recorded behavioral parameter, such as the time spent in locomotion, the time spent rearing, the time spent grooming and the distance travelled, was evaluated separately. Throughout the one-hour sessions, the animals were left undisturbed. Following the behavioral testing, the animals were returned to their home cages. On PND35, all animals were retested as described above.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA). To analyze the spontaneous behavior of the rats and detect sex differences, two-way analysis of variance (ANOVA) with Bonferroni-corrected *post hoc t*-test was used. $p<0.05$ were considered statistically significant. The data are presented as the mean ± standard error of the mean (SEM).

Results

Locomotor activity in rats exposed to hypoxia on PND7

Open field test performed on PND25

Regarding the distance travelled by the males (Fig. 1A), two-way ANOVA indicated a main effect of treatment [$F_{(1,67)}=14.7$, $p<0.001$] and time [$F_{(5,67)}=90.5$, $p<0.001$]. The interaction between these two factors was not significant [$F_{(5,67)}=1.52$, $p=0.2$]. Significant effects of

treatment [$F_{(1,72)}$=7.17, p<0.01] and time [$F_{(5,72)}$=71.4, p<0.001] on locomotion duration (Fig. 1C) were revealed using two-way ANOVA. The time x treatment interaction was not significant [$F_{(5,72)}$=1.27, p=0.29]. Compared to the controls, the hypoxic male rats travelled a shorter distance (by 24 %, $t_{(67)}$=3.34, p<0.01) and spent less time in locomotion (by 23 %, $t_{(72)}$=2.94, p<0.05) in the first 10 min of the test. A main effect of time [$F_{(5,72)}$=22.7, p<0.001] but not of treatment [$F_{(1,72)}$=3.17, p=0.08] on the rearing activity of the males (Fig. 1E) was found using two-way ANOVA. The interaction between these two factors was not significant [$F_{(5,72)}$=1.18, p=0.33].

Regarding the locomotor activity of the females (the distance travelled, Fig. 1B), two-way ANOVA showed a significant effect of time [$F_{(5,72)}$ = 37, p<0.001] but not of treatment [$F_{(1,72)}$=0.07, p=0.8]. The time x treatment interaction was not significant [$F_{(5,72)}$=0.47, p=0.8]. A main effect of time was observed on the locomotion duration of the females [$F_{(5,72)}$=33.3, p<0.001, Fig. 1D]. The effect of treatment [$F_{(1,72)}$=0.003, p=0.95] and the interaction between these two factors was insignificant [$F_{(5,72)}$=0.73, p=0.60]. In the case of rearing duration (Fig. 1F), two-way ANOVA showed a main effect of time [$F_{(5,72)}$=22.9, p<0.001] but not of treatment [$F_{(1,72)}$=0.12, p=0.73]. The time x treatment interaction was also not significant [$F_{(5,72)}$=1.48, p=0.2]. The Bonferroni-corrected *post hoc* *t*-test did not show significant differences in either horizontal (distance travelled and the duration of locomotion) or vertical (rearing duration) locomotor activity in the females.

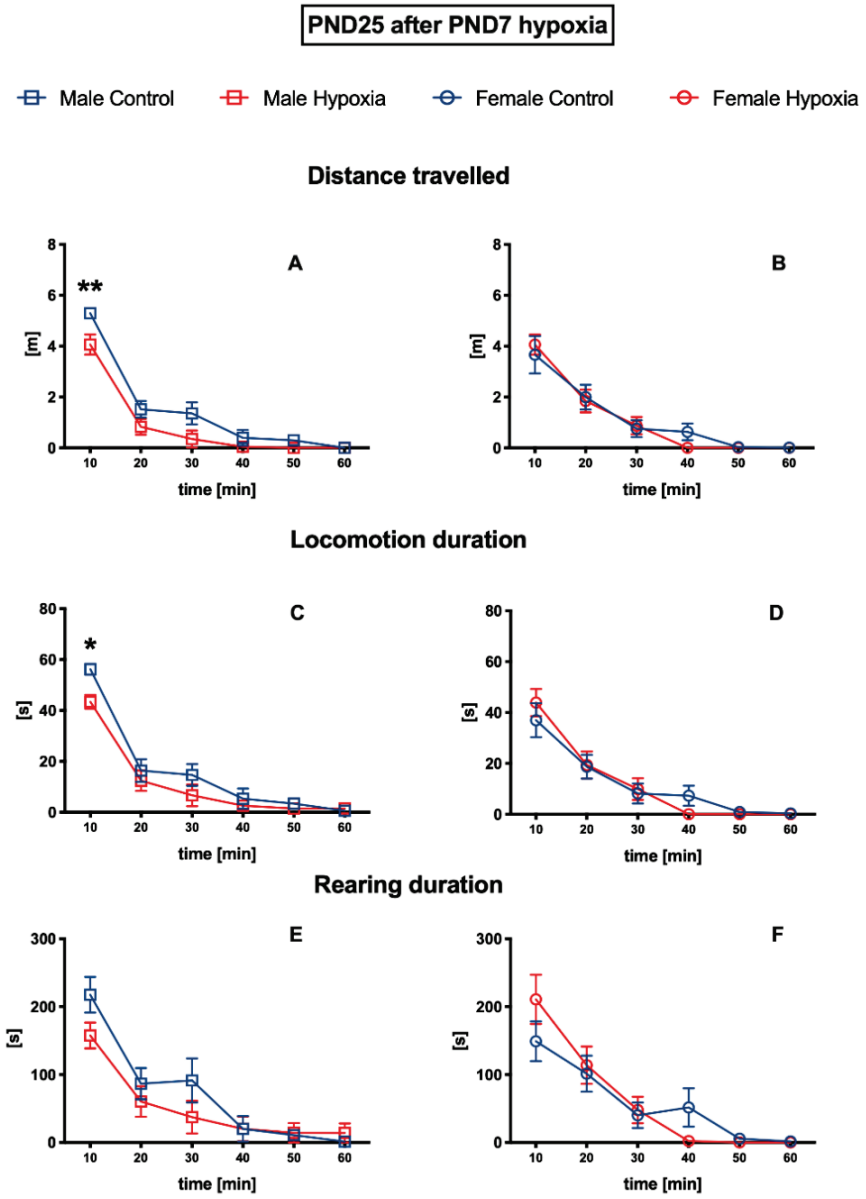

Fig. 1. Locomotor activity of male (n=12) (**A, C, E**) and female (n=12) (**B, D, F**) pups on PND25 following hypoxia exposure on PND7. Panels A and B show the distance travelled, C and D show the locomotion duration and E and F show the rearing duration (verticalisation) in a particular 10-minute interval. PND – postnatal day, * p<0.05, ** p<0.01. The data are presented as the mean ± SEM.

Open field test performed on PND35

In the males, two-way ANOVA showed a significant effect of time on every observed parameter during the testing period [distance travelled: $F_{(5,72)}$=27.6, p<0.001, Fig. 2A; the duration of locomotion: $F_{(5,72)}$=30, p<0.001, Fig. 2C; rearing duration: $F_{(5,72)}$=22.9, p<0.001, Fig. 2E]. However, no effect of treatment on the distance travelled [$F_{(1,72)}$=0.14, p=0.7, Fig. 2A], duration of locomotion [$F_{(1,72)}$=0.15, p=0.7, Fig. 2C] or rearing [$F_{(1,72)}$=0.008, p=0.92, Fig. 2E] was found. The treatment x time interaction was not significant for any of the observed parameters [distance travelled: $F_{(5,72)}$=0.4, p=0.85, Fig. 2A; duration of locomotion: $F_{(5,72)}$=0.43, p=0.83, Fig. 2C; rearing duration: $F_{(5,72)}$=0.24, p=0.94, Fig. 2E]. The Bonferroni-corrected *post hoc t*-test showed no significant differences in these behavioral parameters. In the females, a main effect of both treatment [$F_{(1,72)}$=4.97, p<0.05] and time [$F_{(5,72)}$=25.94, p<0.001] on the distance travelled was shown by two-way ANOVA. The treatment x time interaction was significant [$F_{(5,72)}$=4.43, p<0.01, Fig. 2B]. The hypoxic females moved less than the control females during the first 20 min of the testing period (1st 10 min session: by 37 %, $t_{(72)}$=2.96, p<0.05; 2nd 10 min session: by 65 %, $t_{(72)}$=3.91, p<0.01, Fig. 2B). Two-way ANOVA revealed a significant effect of time but not of treatment on both the duration of locomotion [time: $F_{(5,72)}$=26.3, p<0.001; treatment: $F_{(1,72)}$=1.18, p=0.18, Fig. 2D] and rearing [time: $F_{(5,72)}$=16.34, p<0.001; treatment: $F_{(1,72)}$=0.87, p=0.36, Fig. 2F]. The hypoxic females spent less time in locomotion (by 43 %) than control females, an effect seen only during the 2nd 10 min session of open field testing ($t_{(72)}$=2.8, p<0.05, Fig. 2D). The time x treatment interaction was not significant for either the duration of locomotion [$F_{(5,72)}$=3.14, p=0.01, Fig. 2D] or rearing [$F_{(5,72)}$=2.13, p=0.07, Fig. 2F].

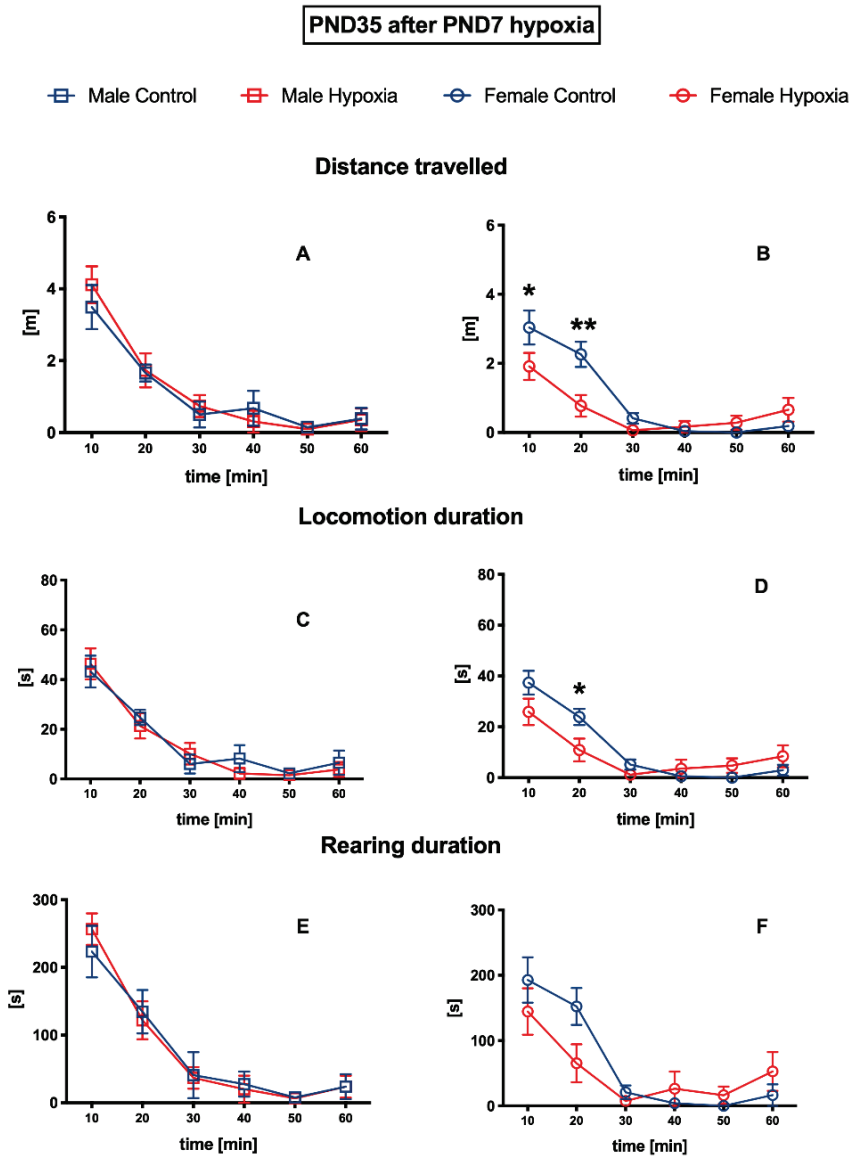

Fig. 2. Locomotor activity of male (n=12) (**A, C, E**) and female (n=12) (**B, D, F**) pups on PND35 following hypoxia exposure on PND7. Panels A and B illustrate the distance travelled, C and D illustrate the locomotion duration and E and F illustrate the rearing duration (verticalisation) in a particular 10-minute interval. PND – postnatal day, *p<0.05, **p<0.01. The data are presented as the mean ± SEM.

Locomotor activity in rats exposed to hypoxia on PND9

Open field test performed on PND25

Regarding the distance travelled by the males (Fig. 3A), two-way ANOVA indicated a main effect of time [$F_{(5,48)}$=4.59, p<0.01] but not of treatment [$F_{(1,48)}$=1.4, p=0.24]. The interaction between these two factors was also not significant [$F_{(5,48)}$=0.76, p=0.58]. Two-way ANOVA confirmed a significant effect of time [$F_{(5,48)}$=3.87, p<0.01] and an insignificant effect of treatment [$F_{(1,48)}$=2.37, p=0.13] on locomotion duration (Fig. 3C) in the males. The time x treatment interaction was not significant [$F_{(5,48)}$=0.49, p=0.79]. No effect of time [$F_{(5,48)}$=1.27, p=0.29], treatment [$F_{(1,48)}$=2.67, p=0.1] or the interaction between them [$F_{(5,48)}$=0.87, p=0.51] on rearing activity in the male offspring was found (Fig. 3E).

Regarding the locomotor activity of the females (distance travelled, Fig. 3B), two-way ANOVA showed a significant effect of both time [$F_{(5,48)}$=14.85, p<0.001] and treatment [$F_{(1,48)}$=5.17, p<0.05]. The time x treatment interaction was not significant [$F_{(5,48)}$=0.88, p=0.5]. The Bonferroni-corrected *post hoc t*-test did not show any significant differences. A main effect of treatment and time on the locomotion duration of the females [treatment: $F_{(1,48)}$=5.79, p<0.05; time: $F_{(5,48)}$=18.29, p<0.001, Fig. 3D] was observed. The interaction of these two factors was insignificant [$F_{(5,48)}$=0.87, p=0.5]. The Bonferroni-corrected *post hoc t*-test did not show any significant differences. In the case of rearing duration (Fig. 3F), two-way ANOVA showed a main effect of time [$F_{(5,48)}$=9.66, p<0.001] and treatment [$F_{(1,48)}$=9.3, p<0.01]. The time x treatment interaction was not

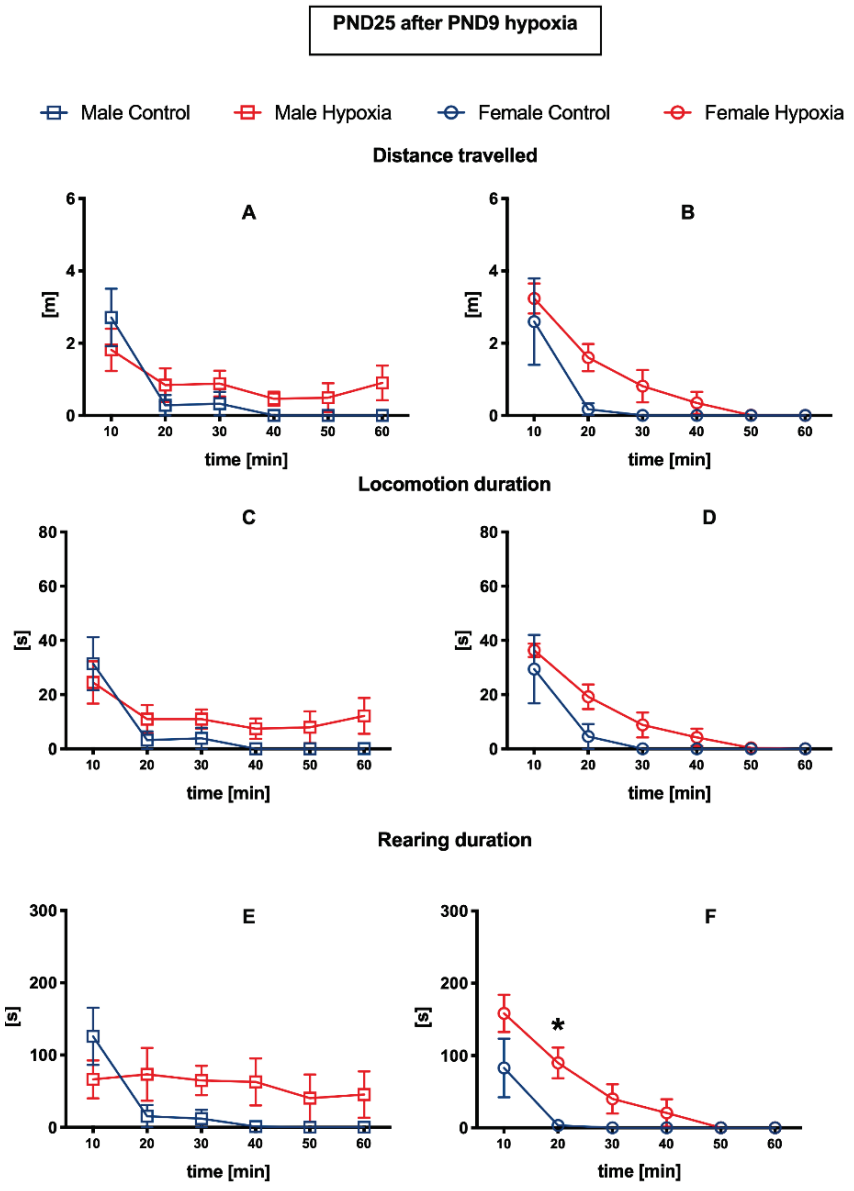

Fig. 3. Locomotor activity of male (n=12) (**A, C, E**) and female (n=12) (**B, D, F**) pups on PND25 following hypoxia exposure on PND9. Panels A and B illustrate the distance travelled, C and D illustrate the locomotion duration and E and F illustrate the rearing duration (verticalisation) in a particular 10-minute interval. PND – postnatal day, * p<0.05. The data are presented as the mean ± SEM.

significant [$F_{(5,48)}$=1.56, p=0.19]. The Bonferroni-corrected *post hoc t*-test showed significant differences in vertical locomotor activity (a more than 20-times longer rearing duration in the hypoxic females) in the females in the 20th minute [$t_{(48)}$=2.9, p<0.05].

Open field test performed on PND35

Two-way ANOVA revealed a main effect of time [$F_{(5,54)}$=22.2, p<0.001, Fig. 4A] but not of treatment [$F_{(1,54)}$=0.29, p=0.59] on horizontal locomotor activity in the males. The interaction between these two factors was also not significant [$F_{(5,54)}$=0.46, p=0.8]. Similarly, regarding locomotor activity duration in the males, a main effect of time [$F_{(5,54)}$=30.9, p<0.001], but not of treatment, was observed [$F_{(1,54)}$=0.61, p=0.44]. The treatment x time interaction was insignificant [$F_{(5,54)}$=0.73, p=0.61, Fig. 4C]. There was a main effect of time [$F_{(5,54)}$=15.53, p<0.001] but not of treatment [$F_{(1,54)}$=1.08, p=0.30] on the rearing activity of males. The treatment x time interaction was not significant [$F_{(5,54)}$=0.47, p=0.80, Fig. 4E]. The females behaved in a similar way (Fig. 4B, 4D, 4F); for all tested parameters, the only significant effect observed was the effect of time [distance travelled: $F_{(5,42)}$=22.7, p<0.001; locomotion duration: $F_{(5,42)}$=15.14, p<0.001; rearing duration: $F_{(5,54)}$=18.9, p<0.001]. No significant effect of treatment was detected on the distance travelled [$F_{(1,42)}$=0.001, p=0.97], the locomotion duration [$F_{(1,42)}$=0.74, p=0.39] or the rearing duration [$F_{(1,42)}$=0.44, p=0.51]. The time x treatment interaction was not significant for any of the

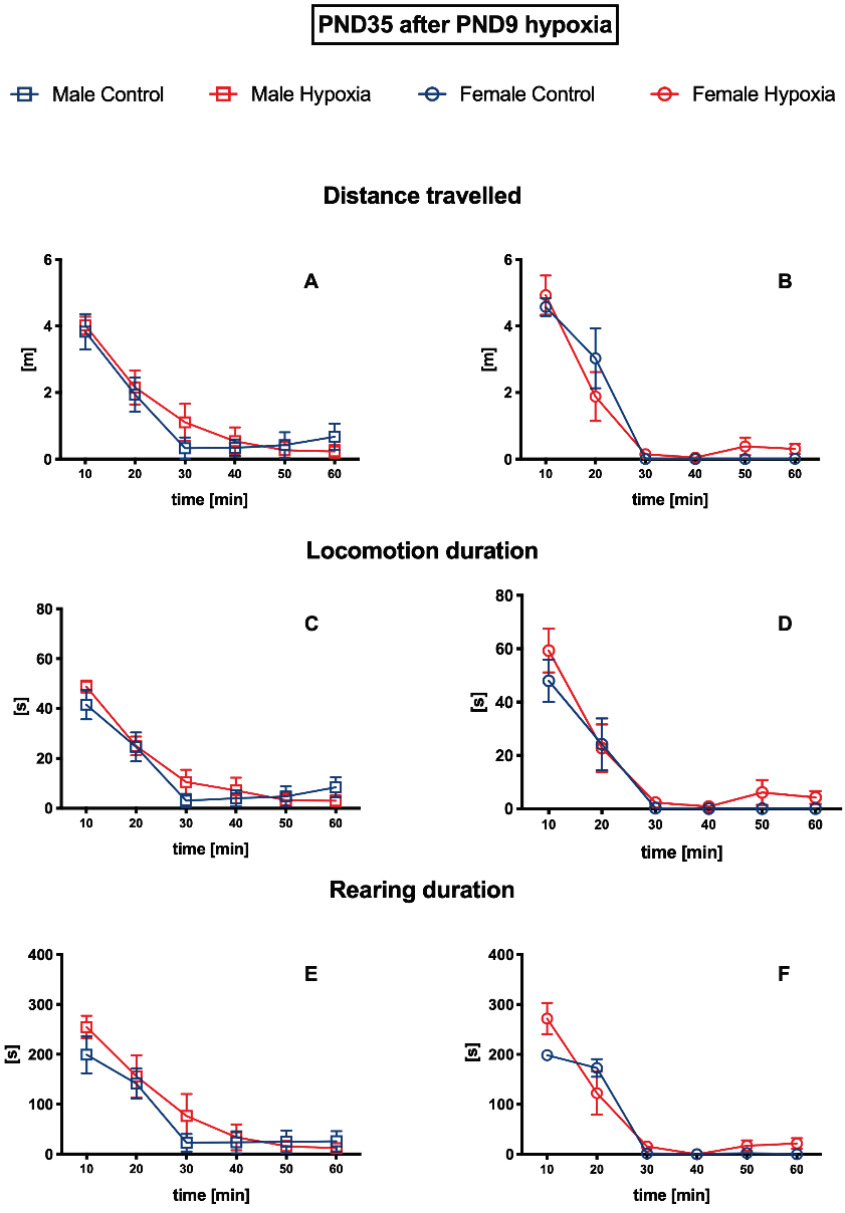

Fig. 4. Locomotor activity of male (n=12) (**A, C, E**) and female (n=12) (**B, D, F**) pups on PND35 following hypoxia exposure on PND9. Panels A and B show the distance travelled, C and D illustrate the duration of locomotion and E and F show the duration of rearing (verticalisation) in a particular 10-minute interval. PND – postnatal day. The data are presented as the mean ± SEM.

observed parameters [distance travelled: $F_{(5,42)}$=0.5, p=0.77; locomotion duration: $F_{(5,42)}$=0.17, p=0.97; rearing duration: $F_{(5,42)}$=0.76, p=0.58].

Effect of early postnatal hypoxia on grooming activity in rats in the open field test

Grooming activity of the rats exposed to hypoxia on PND7

In the open field test performed on PND25 (Fig. 5A, 5B), two-way ANOVA indicated the main effect of time on grooming activity in both sexes [male: $F_{(5,71)}$=5.41, p<0.01, Fig. 5A; female: $F_{(5,72)}$=7.14, p<0.001, Fig. 5B]. The effect of treatment was not significant either in females [$F_{(1,72)}$=0.68, p=0.41] or males [$F_{(1,71)}$=1.07, p=0.3]. Similarly, the treatment x time interaction was insignificant [males: $F_{(5,71)}$=0.46, p=0.8; females $F_{(5,72)}$=1.47, p=0.21].

When the males were retested on PND35 (Fig. 5C), significant effects of all factors were observed [time: $F_{(5,72)}$=5.26, p<0.001; treatment: $F_{(1,72)}$=4.98, p<0.05; time x treatment interaction: $F_{(5,72)}$=2.87, p<0.05]. The Bonferroni-corrected *post hoc* *t*-test showed that the hypoxic males spent significantly more time grooming (a five-fold increase relative to the controls) [$t_{(72)}$=3.99, p<0.001] between the 20th and 30th minute. A main effect

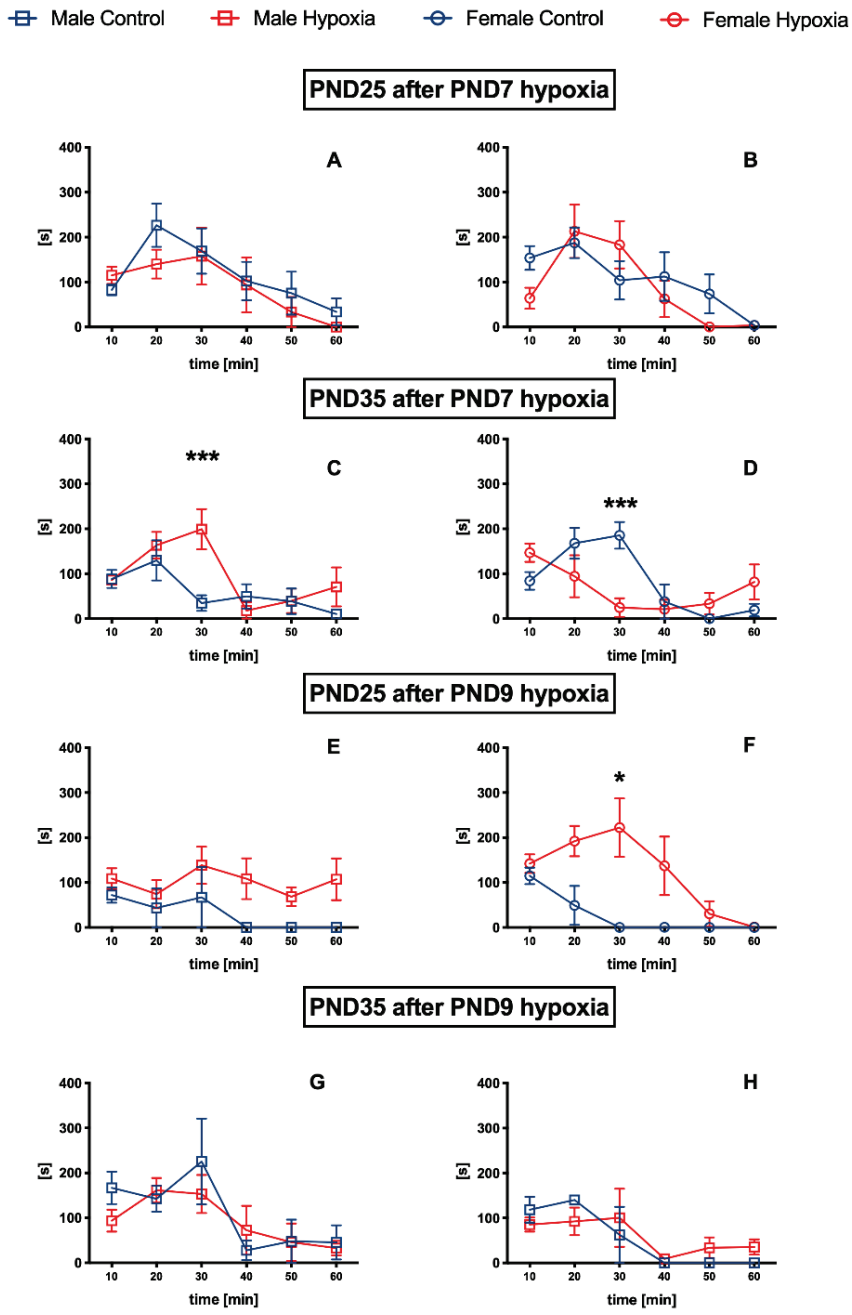

Fig. 5. Grooming activity of male (n=12) (**A, C, E, G**) and female (n=12) (**B, D, F, H**) pups exposed to hypoxia on PND7 or PND9. Panels A and B show the grooming activity on PND25 of the pups exposed to hypoxia on PND7; C and D show the grooming duration on PND35 of the pups exposed to hypoxia on PND7; E and F illustrate the grooming duration on PND25 of the pups exposed to hypoxia on PND9; and G and H show the grooming duration on PND35 of the pups exposed to hypoxia on PND9. PND – postnatal day, * p<0.05, *** p<0.001. The data are presented as the mean ± SEM.

of time [$F_{(5,72)}$=5.8, p<0.001] but not of treatment [$F_{(1,72)}$=0.88, p=0.35] and a significant interaction of these two factors [$F_{(5,72)}$=4.83, p<0.001] on grooming activity were observed on PND35 in the females (Fig. 5D). The Bonferroni-corrected *post hoc t*-test revealed significantly lower grooming activity in the hypoxic females (by 87 %) than in the controls between the 20th and 30th minute of the open field test [$t_{(72)}$=4.0, p<0.001].

Grooming activity of the rats exposed to hypoxia on PND9

On PND25, two-way ANOVA yielded the following results for the males (Fig. 5E): the effect of treatment was significant [$F_{(1,48)}$=8.08, p<0.01], and the effect of time [$F_{(5,48)}$=0.72, p=0.61] and the interaction between these factors [$F_{(5,48)}$=0.3, p=0.91] were not significant. In contrast, in the females (Fig. 5F), the effect of time [$F_{(5,48)}$=2.66, p<0.05] and the effect of treatment [$F_{(5,48)}$=11.29, p<0.01] were both significant, and the interaction between these factors was not significant [$F_{(5,48)}$=1.63, p=0.17]. The Bonferroni-corrected *post hoc t*-test showed that the hypoxic females engaged in grooming significantly more than the control females between the 20th and 30th min [$t_{(48)}$=3.26, p<0.05]. On PND35, in the males, the main effect of time [$F_{(5,54)}$=4.29, p<0.01] but not of treatment [$F_{(1,54)}$=0.41, p=0.52] was found, and the time x treatment interaction [$F_{(5,54)}$=0.6, p=0.7] was not significant (Fig. 5G). The Bonferroni-corrected *post hoc t*-test did not show any significant differences between the hypoxic and control males. According to the ANOVA results, the hypoxic females did not significantly differ from the controls [Fig. 5H; time: $F_{(5,42)}$=2.3, p=0.06; treatment: $F_{(1,42)}$=0.05, p=0.82; time x treatment interaction: $F_{(5,42)}$=0.34, p=0.89].

Sexually dimorphic behavioral outcomes of early postnatal hypoxia

An additional aim of the present study was to describe the sex differences in the effects of early postnatal hypoxia on the behavior of offspring. To detect these sex differences in the hypoxic animals, two-way ANOVA with sex (male/female) and time (0-10, 10-20, 30-40, 40-50, 50-60 min) as the factors was performed.

Sex differences in the behavior of rats following early postnatal hypoxia on PND7

In the open field test conducted on PND25 (data not shown, see supplementary material), the comparison of the recorded behavioral parameters of the hypoxic males and females confirmed only the main effect of time on all observed outcomes [distance travelled: $F_{(5,67)}$=71.1, p<0.001; locomotion duration: $F_{(5,72)}$=53.2, p<0.001; rearing duration: $F_{(5,72)}$=25.2, p<0.001; grooming activity: $F_{(5,71)}$=6.70, p<0.001]. The effect of sex was not significant [distance travelled: $F_{(1,67)}$=2.50, p=0.12; locomotion duration: $F_{(1,72)}$=0.22, p=0.64; rearing duration: $F_{(1,72)}$=1.08, p=0.30; grooming: $F_{(1,71)}$=0.008, p=0.93]. The sex x time interaction was not significant for any of the observed parameters [distance travelled: $F_{(5,67)}$=1.24, p=0.30; locomotion duration: $F_{(5,72)}$=0.63, p=0.68; rearing duration: $F_{(5,72)}$=1.47, p=0.21; grooming activity: $F_{(5,71)}$=0.67, p=0.65].

On PND35 (data not shown, see supplementary material), two-way ANOVA confirmed significant effects of sex [$F_{(1,72)}$=9.99, p<0.01] and time [$F_{(5,72)}$=23.3, p<0.001] and a significant sex x time interaction [$F_{(5,72)}$=4.28, p<0.01] on the distance travelled in the open field test. Similarly, a main effect of both sex and time [sex: $F_{(1,72)}$=5.05, p<0.05; time: $F_{(5,72)}$=21.45, p<0.001] on locomotion duration was revealed. The interaction of these two factors was also significant [$F_{(5,72)}$=3.12, p<0.05]. In the first 10-minute interval, compared to the hypoxic female, the hypoxic males travelled longer distances [by 79 %, $t_{(72)}$=4.89, p<0.001] and spent longer time in locomotion [by 95 %, $t_{(72)}$=3.67, p<0.01]. The main effect of time on rearing duration [$F_{(5,72)}$=20.5, p<0.001] was confirmed. The effect of sex was close to being significant [$F_{(1,72)}$=3.78, p=0.06], and the sex x time interaction was significant [$F_{(5,72)}$=2.66, p<0.05]. The hypoxic males reared more (by 77 %) than the hypoxic females in the first 10-minute interval [$t_{(72)}$=3.49, p<0.01]. Regarding the grooming duration, a significant effect of time [$F_{(5,72)}$=4.30, p<0.01] and a insignificant effect of sex [$F_{(1,72)}$=2.67, p=0.11] was observed. Two-way ANOVA showed a significant interaction between these two factors [$F_{(5,72)}$=3.55, p<0.01]. The Bonferroni-corrected *post hoc t*-test revealed significantly lower grooming activity in the hypoxic females (by more than 8 times) than the hypoxic males between the 20th and 30th minute of the open field test [$t_{(72)}$=4.0, p<0.001].

Sex differences in the behavior of rats following early postnatal hypoxia on PND9

Regarding sex differences in the distance travelled by the rats tested by the open field test on PND25, a significant effect of time [$F_{(5,72)}$=10.1, p<0.001] and a significant time x sex interaction [$F_{(5,72)}$=2.50, p<0.05] were found. However, the effect of sex was not significant [$F_{(1,72)}$=0.21, p=0.65]. Regarding the duration of

locomotion, two-way ANOVA showed a significant effect of time [$F_{(5,72)}$=9.28, p<0.001], but the effect of sex [$F_{(1,72)}$=0.09, p=0.75] and the time x sex interaction [$F_{(1,72)}$=2.02, p=0.09] were not significant.

Similar results were found regarding the duration of rearing and grooming in the rats. The main effect of time was found in both parameters [rearing: $F_{(5,72)}$=4.12, p<0.05; grooming: $F_{(5,72)}$=3.25, p<0.05]. Two-way ANOVA did not reveal a significant effect of either sex [rearing: $F_{(1,72)}$=0.26, p=0.61; grooming: $F_{(1,72)}$=0.76, p=0.39] or the sex x time interaction [rearing: $F_{(5,72)}$=2.32, p=0.05; grooming: $F_{(5,72)}$=2.13, p=0.07]. However, there was a tendency to reach significance, as seen in the p values.

The retesting of the animals on PND35 yielded the following results: only the factor of time was significant for each tested behavioral parameter [distance travelled: $F_{(5,72)}$=35.6, p<0.001; duration of locomotion: $F_{(5,72)}$=37.3, p<0.001; duration of rearing: $F_{(5,72)}$=29, p<0.001; and duration of grooming: $F_{(5,72)}$=3.22, p<0.05]. The effect of sex and the sex x time interaction were not statistically significant for any of the observed behavioral outcomes [distance travelled (sex: $F_{(1,72)}$=0.21, p=0.65; interaction: $F_{(5,72)}$=1.28, p=0.28); duration of locomotion (sex: $F_{(1,72)}$=0.22, p=0.88; interaction: $F_{(5,72)}$=1.07, p=0.39); duration of rearing (sex: $F_{(1,72)}$=1.18, p=0.28; interaction: $F_{(5,72)}$=0.68, p=0.64) and duration of grooming (sex: $F_{(1,72)}$=2.84, p=0.096; interaction: $F_{(5,72)}$=0.41, p=0.84)].

Histochemistry did not detect obvious morphological damage

Histological evaluation was performed one and five days after hypoxia induction, and it did not demonstrate any obvious signs of neuronal degeneration or apoptosis. The same results were obtained by microscope analysis during adolescence (after the last open field test on PND35, Fig. 6).

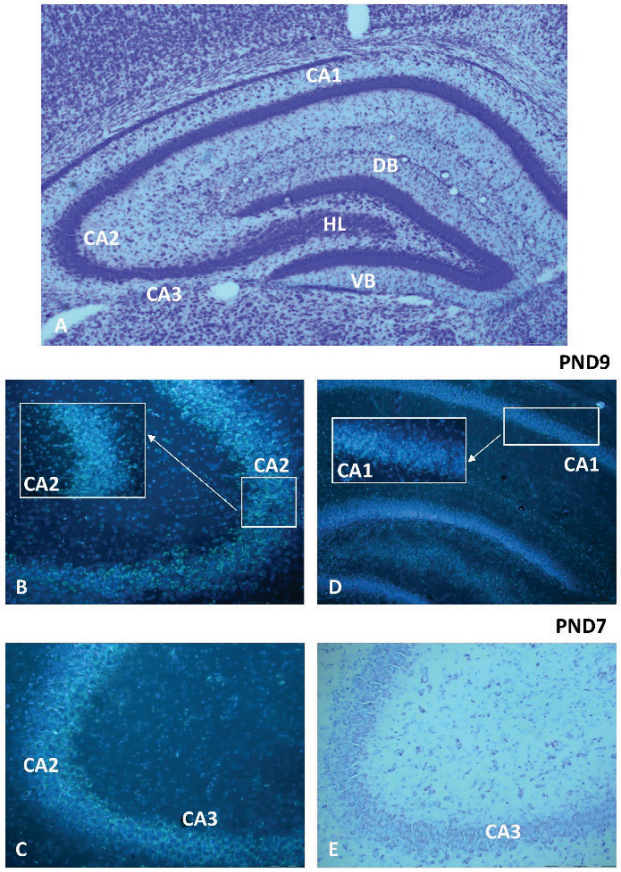

Fig. 6. Histological analyses performed on rat pups one and five days after hypoxia exposure. Hypoxia did not produce any visible morphological damage to the hippocampus. Panel A is a representative picture of the hippocampus of a control male rat perfused on PND12; Nissl staining; scale bar: 500 μm. Panel B is a representative picture of the CA2 region of the hippocampus of a rat exposed to hypoxia on PND9 and perfused on PND14; bisbenzimide staining; scale bar: 200 μm. Panel C is a representative picture of the CA2 and CA3 regions of the hippocampus in a rat exposed to hypoxia on PND7 and perfused on PND12; bisbenzimide staining; scale bar: 200 μm. Panel D is a representative picture of the CA1 region of the hippocampus and the hilus of the dentate gyrus in a rat exposed to hypoxia on PND9 and perfused on PND14; bisbenzimide staining; scale bar: 200 μm. Panel E is a representative picture of the CA3 region of the hippocampus in a rat exposed to hypoxia on PND7 and perfused on PND12; Nissl staining; scale bar: 500 μm.

Discussion

Severe and rapid hypobaric hypoxia is an extreme external condition that activates various physiological and pathophysiological homeostatic pathways (Virues-Ortega *et al.* 2004, Jayalakshmi *et al.* 2007, Zhang *et al.* 2018). It alters the neurochemistry of the brain, disrupts myelination during development and triggers both caspase-dependent and -independent pathways of cell death (Potter *et al.* 2018). The following four major questions were addressed in this study: 1) what is the effect of hypoxia exposure on PND7 and PND9; 2) is this two-day shift in hypoxia exposure capable of causing different behavioral consequences in later life?; 3) what part of these behavioral changes can be ascribed to sex-related differences?; and 4) is hypobaric hypoxia alone able to induce structural damage to the hippocampus? Our model of hypoxia simulating an altitude of 9000 m did not induce any detectable morphological changes in the rat hippocampus. Ipsilateral damage to the brain, as described by the so-called Rice-

Vannucci model, requires the surgical ligation of the carotid artery i.e. ischemia, followed by normobaric hypoxia to induce structural damage (Vannucci 1990). As our experimental model included only hypoxia, we did not confirm any neurodegeneration within the examined areas of the brain. On the other hand, the same hypoxic insult was capable of influencing the spontaneous behavior of the rats in later life. Such morphological and functional discrepancies are interesting for two reasons. First, subtle changes in spontaneous behavior, which are not accompanied by structural alterations, highlight a more realistic clinical picture of mild perinatal hypoxic damage. However, it is not excluded that one of the perinatal hypoxia consequence is sex specific modification of some behavioral domains i.e. willingness to explore or immobility, without any obvious changes in the morphology of the brain. Second, the model indicates the importance of behavioral testing related to hypoxia exposure. Exposure to one-hour hypoxia (9000 m) is lethal to adult rats but not to immature PND7 and PND9 pups of both sexes. Immature brain tissue, unlike adult brain tissue, can withstand a significant period of oxygen deprivation because it requires less metabolic fuel (glucose plays only a minor role) (Nehlig *et al.* 1993). On the other hand, if a critical point of energy deprivation is reached, immature nervous tissue is more prone to excitotoxic damage (Doble 1999, Riljak *et al.* 2016). PND7 rats most closely approximate 30-32 weeks of gestation, while PND9-PND12 is representative of gestational weeks 40-42. There are substantial differences in brain development between PND7 and PND9. Myelination along with synaptogenesis occur in a relatively conserved pattern (Volpe 2000). In addition, white matter is more vulnerable as compared to grey matter and oligodendrocyte maturation is not yet presented in the PND7 (Volpe 2003, Hagberg *et al.* 2002). Another very important difference is presented in GABA signalling. The activation of the $GABA_A$ receptor in immature neurons triggers excessive excitation and thus might determine or even limit the neuronal differentiation (Ben-Ari *et al.* 2012). Hypoxia induced on PND7 was also recognized as a significant factor triggering the process of epileptogenesis. Moreover, there is dramatic decrease in postsynaptic densities of glutamate receptor associated with increased vulnerability to white matter injury in the developing brain (Shen *et al.* 2012). Hypoxia exposed rats suffer from synaptic transmission disturbances and changes in dendritic spine physiology and morphology. Such synapse-related changes are framed by the changed postsynaptic glutamate sensitivity and probably by different amount of released glutamate (Shen *et al.* 2012, Zhuravin *et al.* 2019).

Rats exposed to hypoxia on PND7 mainly exhibit changes in horizontal (locomotion) and vertical (rearing) activity, while only grooming is affected in rats exposed to hypoxia on PND9 (Volpe 2001, Hill *et al.* 2011). Hypoxia-related motor impairments may influence the ability to execute a specific task and to explore the environment of the experimental arena in a gender-dependent manner (Arteni *et al.* 2010, Peterson *et al.* 2015, Sanches *et al.* 2015, Huang *et al.* 2016). Newborn rodents express the same brain development characteristics (partially completed neurogenesis of specific brain areas and the initialization of myelination) as preterm infants (Romijn *et al.* 1991, Towfighi *et al.* 1997, Zhu *et al.* 2005, Cuaycong *et al.* 2011). Hypoxia interferes with neural proliferation in a sex-dependent manner (Mayoral *et al.* 2009). Male sex is a significant risk factor for worse neurocognitive outcomes of preterm delivery. Furthermore, neuroprotective treatments are less effective in males (Lan *et al.* 2011). The mechanisms of such gender-dependent differences in brain vulnerability are not known yet. There are a number of mechanisms to play a key role in hypoxia-ischemia brain injury due to their different impacts on mitochondrial function and their different antioxidant capacities, inflammatory responses and microglial activation abilities in males and females (Mayoral *et al.* 2009). The hypoxia-ischemia-induced loss of volume in specific areas of the brain and changes in the progression of myelinization are likely hormonally related (Netto *et al.* 2017). Estrogen and testosterone interfere with the susceptibility of the brain to hypoxia-induced damage in an age-dependent manner (Hill *et al.* 2012). In our study, male rats exposed to hypoxia on PND7 exhibited a continuous decline in moved distance, locomotion and rearing duration throughout the one-hour period, suggesting a normal profile of the habituation when exposed to the new environment. However, rats exposed to hypoxia on PND9 did not show the same decline in the observed behavioral parameters during the one-hour period, suggesting a potential inability to cope with a novel environment. The difference between the time spent rearing and the time in locomotion on PND25 and PND35 may be explained by the different levels of cortical vs. subcortical structural maturity. In contrast, the lower intensity of locomotion and rearing of the females was first

noticeable on PND35. This may be ascribed to all of the abovementioned hormonal and non-hormonal mechanisms. All the effects mentioned above are strictly functional, as we did not prove neurodegeneration.

What is surprising, however, is the disproportionate effect of exposure to higher-severity hypoxia on PND7 compared to PND9. The rat pups were more vulnerable, and the effect of hypoxia at this stage of development was long-lasting. Males in general showed higher sensitivity to this insult. Both, males and females postnatally exposed to hypoxia exhibited higher rearing activity. However, in females it reached statistical significance, while in males not. Moreover, the process of habituation was clearly disrupted in both, males and females. Males never decreased the rearing activity, while the females did (but clearly much later than control counterparts). It seems that hypoxia interferes with the capacity of unconditional learning processes. The interpretation of self-grooming data is not easy, as it might represent many behavioral aspects of the particular animal (Estanislau *et al.* 2013, Estanislau *et al.* 2019). The findings of the present study confirm complexity of the self-grooming behavior, since hypoxia induced on PND7 resulted in alteration of the grooming pattern not sooner than PND35 and this change was sex-dependent. If animals were exposed to hypoxia on PND9, the grooming profile changed already at PND25, however, it returned back to the profile of control animals on PND35. The present study suggests that the relationship between grooming and hypoxia deserves more attention in future.

In conclusion, hypobaric hypoxia is capable of inducing significant behavioral changes in rat pups when applied on PND7 and PND9. The injury was more severe on PND7 and predominantly affected the males. Hypoxia was able to modify the behavioral profile in terms of habituation and the ability of rats to cope with novelty. No structural changes were observed in the hippocampus. The observed changes in the spontaneous behavior of the rats were not accompanied by even subtle morphological changes. This study highlights the necessity of including both genders in future hypoxia studies. However, our study has some limitations. Hypoxia exposition took place for one hour only, so was relatively short-term. Secondly, other than 9000 m simulated altitude might bring another behavioral outcome (so might do various mutual combinations altitude vs. time of exposure). Future studies may aim to elucidate the different sensitivities and reactivities of the neuronal tissue of males and females to hypoxia exposure and may allow us to formulate specific therapeutic strategies for ameliorating or even preventing the effects of hypoxia.

Conflict of Interest

There is no conflict of interest.

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