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ORIGINAL PAPER

Tissue Injury and Astrocytic Reaction, But Not Cognitive Deficits, Are Dependent on Hypoxia Duration in Very Immature Rats Undergoing Neonatal Hypoxia–Ischemia

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Abstract

Preterm birth and hypoxia–ischemia (HI) are major causes of neonatal death and neurological disabilities in newborns. The widely used preclinical HI model combines carotid occlusion with hypoxia exposure; however, the relationship between different hypoxia exposure periods with brain tissue loss, astrocyte reactivity and behavioral impairments following HI is lacking. Present study evaluated HI-induced behavioral and morphological consequences in rats exposed to different periods of hypoxia at postnatal day 3. Wistar rats of both sexes were assigned into four groups: control group, HI-120 min, HI-180 min and HI-210 min. Neurodevelopmental reflexes, exploratory abilities and cognitive function were assessed. At adulthood, tissue damage and reactive astrogliosis were measured. Animals exposed to HI-180 and HI-210 min had delayed neurodevelopmental reflexes compared to control group. Histological assessment showed tissue loss that was restricted to the ipsilateral hemisphere in lower periods of hypoxia exposure (120 and 180 min) but affected both hemispheres when 210 min was used. Reactive astrogliosis was increased only after 210 min of hypoxia. Interestingly, cognitive deficits were induced regardless the duration of hypoxia and there were correlations between behavioral parameters and cortex, hippocampus and corpus callosum volumes. These results show the duration of hypoxia has a close relationship with astrocytic response and tissue damage progression. Furthermore, the long-lasting cognitive memory deficit and its association with brain structures beyond the hippocampus suggests that complex anatomical changes should be involved in functional alterations taking place as hypoxia duration is increased, even when the cognitive impairment limit is achieved.

Keywords Neonatal hypoxia–ischemia · Neurodevelopment · Reflexes · Cognitive function · Astrocytes · Injury severity

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Introduction

Preterm birth is a public health problem and a major cause of neonatal death and neurological disabilities in children [1, 2]. Previous reports have shown an increase in survival rates between newborns with 24 to 28 gestational weeks and weighing less than 1500 g [3–5]. Short-term complications such as periventricular hemorrhage and perinatal hypoxia–ischemia have been associated with long-term psychomotor delay; executive function impairments and attentional, socialization and learning deficits [1, 3, 5–8].

An adaptation of the Rice–Vannucci model is currently used to study the effects of hypoxia–ischemia injury in rodents [9–11]. This model combines the permanent carotid artery ligation followed by hypoxic exposure [12]. Most studies induce the injury at post-natal day (PND) 7 in rats as a HI model for term/late-preterm infants [13,

14]. Despite variations across research groups, the PND7 model is well described, with hypoxia exposure ranging from 90 to 120 min with 8% of oxygen and causing consistent cognitive deficits and unilateral tissue damage in which the hippocampus is the most affected brain structure [15–17]. The model used to mimic preterm HI uses PND3 rats, since its brain development stage resembles that of a very preterm infant [10, 18]. Nevertheless, studies that used the same HI protocol (i.e., hypoxia duration) in PND7 and PND3 showed that, while the first ones presented significant cognitive and tissue damage, the latter presented almost no detectable lesion [19, 20]. These results highlight the need for refining the protocols to produce a better experimental HI injury protocol to model preterm human brain lesion.

HI is associated with motor impairments and cognitive deficits, being the last one a main neurodevelopmental sequelae of immature cerebral injury [7, 21, 22]. The influence of the age and hypoxia duration in the severity of brain damage was already shown in previous papers [23, 24]. However, evidence of the relationship between duration of hypoxia and functional impairments as well as its histological consequences is scarce. Towfighi et al. (1997) exposing PND 2–3 rats to 5% of oxygen (O_2) during 30, 60, 90 and 120 min after common carotid artery ligation reported morphological damage to cerebral cortex, striatum, thalamus and hippocampus, from 24 to 72 h after HI. Similarly, Stadlin et al. (2003) following variable hypoxia exposure periods (10, 15, 20, 30 or 45 min) under 6–7% O_2 concentration and variable parameters of temperature and humidity showed that environmental conditions could cause an interplay among variables, thus altering HI brain damage and the biochemical responses 3 days after the insult [9]. However, there is only one study showing sensorimotor and muscular impairments of different periods of hypoxia produced with 8% oxygen in PND3 rats in [23], but cognitive effects and tissue damage have not been investigated.

Glial responses to injury are key factors in determining the prognosis of brain damage and astrocytes are among the brain cell types responding to injury [25]; in the presence of brain lesion these cells become reactive and secrete several molecules that modulate the neuroinflammatory process [25, 26]. In addition, astrocytes are found in great numbers in sensitive areas to HI, such as the corpus callosum (CC) [27]. CC is the major fiber tract responsible for connecting and integrating neural activity between hemispheres [28] and HI-induced white matter loss is associated with cognitive deficits [29], suggesting also a disruption of intercortical connections [30]. Therefore, the aim of present study is to evaluate the short and long-term behavioral, histological and astroglial responses to different periods of hypoxia in PND3 rats undergoing hypoxia–ischemia.

Materials and Methods

Animals

Wistar dam and pup rats were kept in standard conditions, at 22 ± 2 °C room temperature and 12 h light/dark cycle. Water and food were provided ad libitum. A total of six litters were used and three animals per litter were submitted to hypoxia–ischemia procedure for each experimental group. Two–three pups from the same litter were used as controls. The total number of animals employed in the present study was 48. All procedures were performed according to the guidelines of Care and Use of Laboratory Animals of the Brazilian Society for Neuroscience. The project was approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul - Protocol # 28641.

Hypoxia–Ischemia Procedure

At PDN3, pups of both sexes were randomly assigned to four groups: Control, HI-120 min, HI-180 min and HI-210 min (n = 10–12 animals per group). Animals assigned to the HI procedure were previously anesthetized with 4% halothane. Following the Rice-Vannucci protocol, a small incision was made through the medial longitudinal neckline, the right common carotid was exposed, the vagus nerve was isolated and the artery was permanently ligated with 4.0 surgical silk thread. Animals were then returned to standard cages with their dams. After 2 h of post-surgery recovery, animals were put in the hypoxia chamber, with an atmosphere of 8% of O_2 and 92% of N_2 at 37 °C, during the respective time of hypoxia of the experimental group: 120, 180 or 210 min.

For the control group (sham), pups received anesthesia and had the right carotid artery isolated, but not occluded; they were kept under atmospheric conditions [20]. Animals were maintained at 36–37 °C using a heat pad and were kept away from their dams during 210 min, i.e., the time of the longest hypoxia exposure; after that pups were returned to their respective dams. The mortality rate observed in the HI procedure was lower than 5% in the HI-120 min group, 10% in the HI-180 min group and 20% in the HI-210 min group.

Behavioral Assessment

Neurodevelopment testing assessed righting reflex, negative geotaxis and olfactory discrimination at PND 7, 14 and 21. Body weight was recorded before the tests. Pups were separated from their dams during the examination

and were immediately returned to their cages at the end of experiments. All tests were performed between 9:30 and 12:00 h by the same investigators. Exploratory abilities and cognitive function were evaluated from PND 35 to PND 44 in the open field and the Morris water maze test, respectively.

Righting Reflex Test

The test assesses motor and coordination function. Each animal was put in supine position with the whole body in surface contact. The latency required to return to the prone position, with its four paws on the floor, was recorded [31–33].

Negative Geotaxis Test

This test was used to evaluate vestibular and proprioceptive functions. The animal was put on a board inclined 45º with its head down. The time used to turn around 180º and to put on both its face up and its body in longitudinal axis was recorded. The maximum duration for this test was of 60 s [32, 34]. After each testing the board was cleaned with a 70% ethanol solution.

Olfactory Discrimination Task

Using a clean standard cage for rats, fresh bedding was put on one side and home cage bedding on the other one. Each animal was positioned in the center of the cage with the head toward the wall. The latency required to enter on its home bedding was recorded. The maximum duration for this test was of 180 s; this latency was assigned when animals did not complete the activity [35]. After each testing the cage was cleaned with a 70% ethanol solution.

Open Field

Open field test was used to observe the animal´s exploratory abilities using a previously described protocol [36]. Each animal was put in the center of the chamber (Diameter: 100 cm. Height: 30 cm) for 5 min. Variables recorded were: latency to leave the center, number of crossings (both in periphery and in the center zone) and the number of standing on rear limbs (rearings).

Morris Water Maze Test

The Morris water maze test was run in order to evaluate spatial memory as previously described. A black pool (Diameter of 200 cm) was filled into a depth of 40 cm with room-temperature water and a platform was put 2 cm below the water level. Four virtual quadrants, nominated as N, S, W, E, were defined to determine training sequence. In a period of 5 days, animals were put into the water for 60 s to find the platform. In case that the task was not completed (i.e., the rat did not find the platform), it animal was gently guided to the platform and left to remain during 15 to 20 s, helping to identify the wall cues. Latency required to find the platform was registered and the average trial latency was calculated. Probe trial was performed on the sixth day, with the platform removed and the animal placed far away from the platform position. The latency for first crossing the platform region, the time spent in the target quadrant and the distance travelled were recorded using the ANY-Maze software (Cechetti et al. 2012).

Histological Analysis

Six to seven rats per group were used for histological analysis. Animals were anesthetized with isoflurane (5%) after completing behavioral evaluation. Perfusion was run using 9% saline solution followed by 4% formaldehyde solution through the left cardiac ventricle. The brain was removed from the skull and kept in fixative solution and cryoprotected with sucrose (30%) solution. Serial 30 µm slices were cut on a cryostat (Leyca) and one slice in every 10th section was put on gelatin-coated slides and stained with hematoxylin and eosin (Sigma-Aldrich. St Louis. MO. USA). The regions of interest (cortex, hippocampus and corpus callosum) were identified using the rat brain atlas [37]. All measurements were calculated using the software NIH-ImageJ and, the Cavalieri method of hemispheric and structural volume measurements was used. The values are expressed as mean volume ± SEM (mm^3) [20] and the volume ratio was calculated using the equation (Ipsilateral volume (mm^3))/(Contralateral volume (mm^3)). The injury severity was classified as previously reported: mild ≤ 25% of surface damage, moderate 25% to 50% and severe ≥ 50% [38, 39].

Immunofluorescence

Coronal brain sections (30 µm) were washed in tris buffered saline (TBS), followed by a solution of TBS plus 0.05% tween-20 (T-TBS) in order to permeabilize cell membranes. Sections were then blocked with 1% albumin overnight. Primary antibody against glial fibrillary protein (anti-GFAP) (rabbit IgG, 1:100, Sigma-Aldrich) for identifying astrocytes was used. This procedure was carried out in 1% albumin in T-TBS at 4 °C for 24 h. Following TBS washings, sections were incubated with secondary antibody anti-mouse Alexa 488 (1:500, Molecular Probes, Invitrogen, USA) for 1 hour. Slices were covered in aqueous mounting medium (FluorSave®, Calbiochem, Darmstadt, Germany) and coverslipped.

Morphological Astrocyte Analysis

Astrocytes in corpus callosum close to the inter-hemispheric line (between − 2.30 and − 4.30 mm from the bregma) were selected to evaluate morphological characteristics following HI. A total of 19 animals were used for the analysis (control group = 4, HI-120 min = 5, HI-180 min = 6 and HI-180 min = 4). Three cells by each coronal brain section (n = 4) were analyzed, totaling 12 astrocytes per animal [26]. Images were captured using a Leica SP8 confocal microscope with the following parameters: Objective: 63 ×, Resolution 1024 × 1024 pixels and means: 4 and XYZ-axis. Lighting conditions and magnifications were kept during the process of capture. As for the morphological analysis, images were collected in 20 µm plane and Sholl's concentric circle method was used [26]. The soma of each astrocyte was identified and the number of intersections of circles of increasing radii from the center were quantified using the Image Pro-plus software (Version 6.1, Media Cybernetics, Silver Spring, EUA) [40].

Statistical Analysis

The software SPSS, version 21 was used for all analysis and the Shapiro–Wilk test was run for determination of normal distribution. Non-parametric data (neurobehavioral tests) are expressed as median and interquartile range. Parametric data are expressed as mean ± SEM. The statistical differences of variables between contra and ipsilateral sides were assessed by Student's *t* test. For assessing weight gain over time, as well as water maze data during training days, repeated measured ANOVA was used. To analyze differences between groups one-way ANOVA and Duncan's post hoc were used; p values < 0.05 were considered significant. Multiple regression analysis was run to determine whether several independent variables of interest could significantly improve the R^2, predicting the outcome of a dependent variable. Such analysis was performed using neurobehavioral and Morris water maze performance, as dependent variables, combined with morphological data (percentage of tissue loss, ipsilateral and contralateral volume) of cerebral cortex, corpus callosum and hippocampus.

Results

Body Weight

As observed in Fig. 1a, a progressive increase in the body weight was found in all groups. However, HI rats showed small gains when compared to control group (CG) at PND 7, PND 14 and PND 21 ($F_{(3,48)} = 14.637$, $p = 0.000$; $F_{(3,48)} = 7.637$ $p = 0.000$ and $F_{(3,48)} = 4.926$, $p = 0.005$, respectively). At PND 35, animals of the HI-180 min and HI-210 min groups showed a significant decrease of body weight as compared to CG ($F_{(3,48)} = 6.344$, $p = 0.001$).

Neurobehavioral Assessment

There was a significant increase in the latency to assume the prone position in HI-180 min and HI-210 min groups at PND 14 compared to the CG in the righting reflex testing. Such difference was also observed in the HI-210 min group at PND 21 ($X^2_{(3)} = 10.624$, $p = 0.014$ and $X^2_{(3)} = 12.600$, $p = 0.006$, Fig. 1b). In addition, at PND 7, animals of the HI-180 min and HI-210 min groups had worse performance in negative geotaxis (Fig. 1c) and olfactory discrimination tests (Fig. 1d) when compared to CG ($X^2_{(3)} = 8.583$, $p = 0.035$ and $X^2_{(3)} = 9.780$, $p = 0.021$, respectively). No significant differences were observed in other testing days ($P > 0.05$). However, HI-120 min animals presented lower latency as compared to HI-180 min for olfactory discriminatory test at PND21 ($X^2_{(3)} = 8.403$, $p = 0.038$).

Open Field

No significant differences were observed among HI groups compared to CG in central and peripheral crossings, in latencies to leave the 1st circle nor in the number of rearings in the open field test (Table 1), indicating no HI-induced hyperactivity.

Morris Water Maze Test

All animals improved their performance during the training period in the Morris water maze test over days. As depicted in the Fig. 2a, there were no differences between HI groups and CG in the latencies to find the platform as from the first to the third training day ($F_{(3,43)} = 0.836$, $p = 0.481$). However, on the fourth and fifth days, all HI groups showed higher latencies compared to CG ($F_{(3,39)} = 6.377$, $p = 0.001$ and $F_{(3,39)} = 4.688$, $p = 0.007$, respectively), evidencing HI-induced reference memory impairments, independent on the hypoxia duration. On the probe trial, HI groups had significantly higher latencies to cross the platform region ($F_{(3,39)} = 3.327$, $p = 0.029$; Fig. 2b) as well as showed a reduction of the time spent in the target quadrant ($F_{(3,39)} = 3.025$, $p = 0.041$; Fig. 2c), thus confirming spatial learning and memory deficits following the HI insult.

GFAP Immunofluorescence

GFAP immunofluorescence was performed at PND 45 in the corpus callosum, near to the inter-hemispheric line, in order to investigate astrocyte survival and reactive gliosis. Fluorescence intensity was evaluated and revealed an increase in

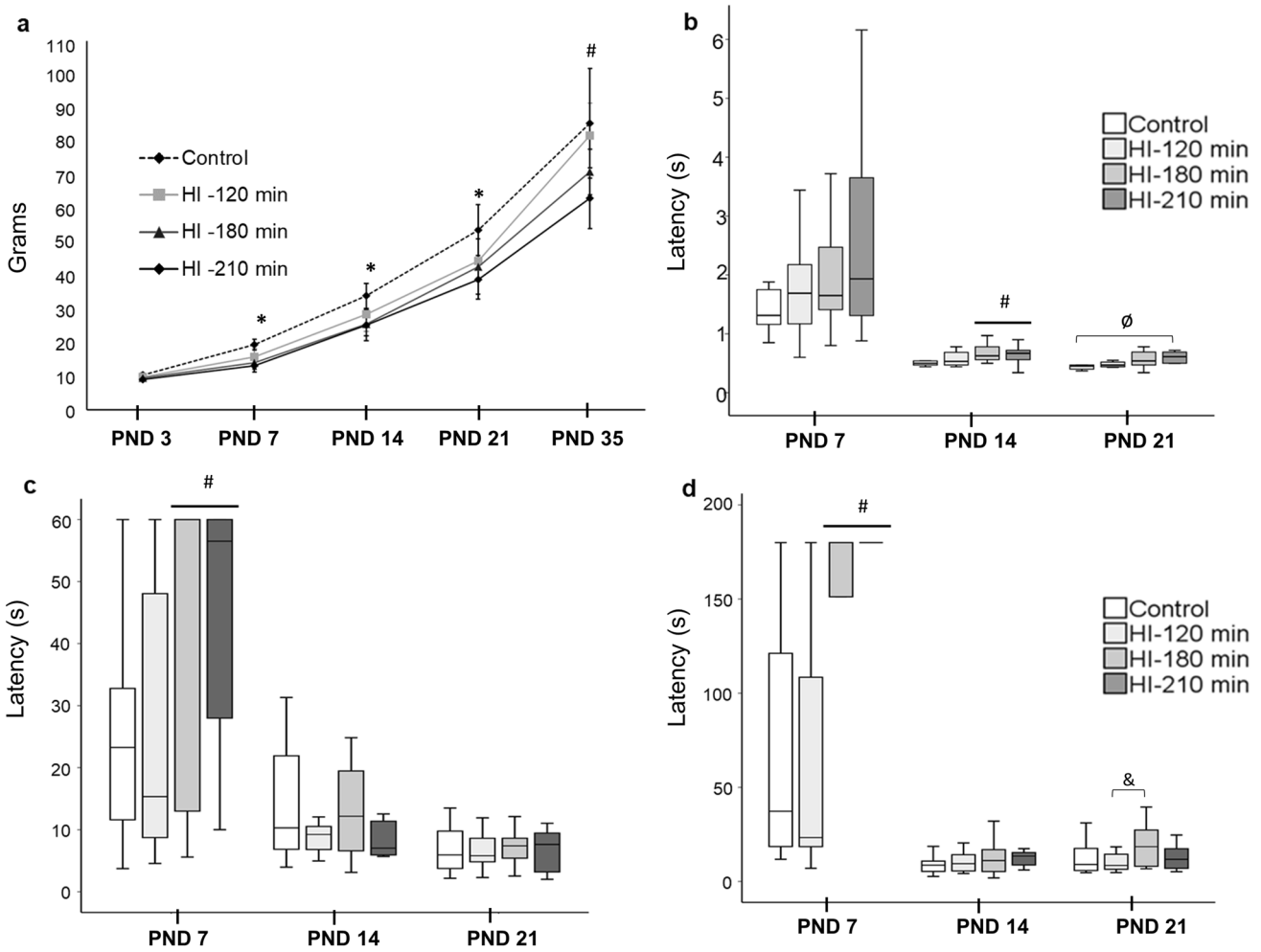

Fig. 1 Pups' body weight and neurobehavioral assessment: **a** Pups' weight during development. Data are expressed as mean ± SEM. Results were analyzed by Repeated Measures ANOVA. **b** Righting reflex. **c** Negative geotaxis. **d** Olfactory discrimination. Data are expressed as median and interquartile ranges of animals' latencies to perform the test. Results were analyzed by Kruskal–Wallis ANOVA and its corresponding pairwise test. (n = 10–12 per group) *Difference between control group versus all HI groups, [#]Difference between HI-180 and HI-210 groups when compared to control. [Ø]Difference of HI-210 min versus control group. [&]Difference between HI-120 min and HI-180 min. Significance was accepted whenever $p < 0.05$

Table 1 Open field test performance

Open field	Control Mean ± SEM	HI-120 min Mean ± SEM	HI-180 min Mean ± SEM	HI-210 min Mean ± SEM	p value
Total crossings	70 ± 5.42	72.91 ± 5.50	77.29 ± 4.35	82.9 ± 9.37	0.492
Central crossings	28.64 ± 4.85	28.45 ± 3.65	29.86 ± 3.99	23.5 ± 3.99	0.765
Peripheral crossings	98.64 ± 8.54	101.36 ± 6.96	107.14 ± 7.23	106.4 ± 12.82	0.878
Total rearing	31.21 ± 3.63	42.18 ± 2.69	39.14 ± 2.86	41.8 ± 5.01	0.106
Latency to leave to 1st circle	3.66 ± 0.68	2.58 ± 0.65	3.95 ± 0.93	2.53 ± 0.51	0.439

GFAP expression only in the HI-210 min group, in comparison to the CG ($F_{(3,14)} = 3.65$, $p = 0.039$) (Fig. 3a, b).

Sholl's morphological analysis was also performed. Neither the number of crossings of each concentric circle nor the total number of crossings ($F_{(3,18)} = 0.47$, $p > 0.05$) showed any difference when assessed in adulthood (Fig. 3c, d). These results suggest that despite the significant increase in reactive astrogliosis by the increasing time of hypoxia exposure, there is no effect on the number or length of astrocyte projections.

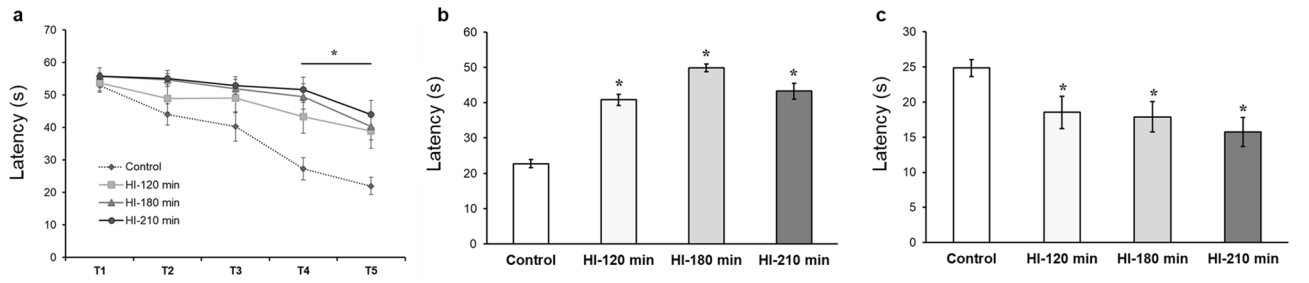

Fig. 2 Morris Water Maze. **a** Reference memory protocol during training sessions were analyzed by repeated measures one-way ANOVA. **b** Latencies for the first crossing platform region in the Morris water maze. **c** Time spent in the target quadrant during the probe trial. Results were analyzed using one-way ANOVA. Data are expressed as mean ± SEM (n = 10–12 per group) *Difference of all HI groups when compared to control group. Significance was accepted whenever $p < 0.05$

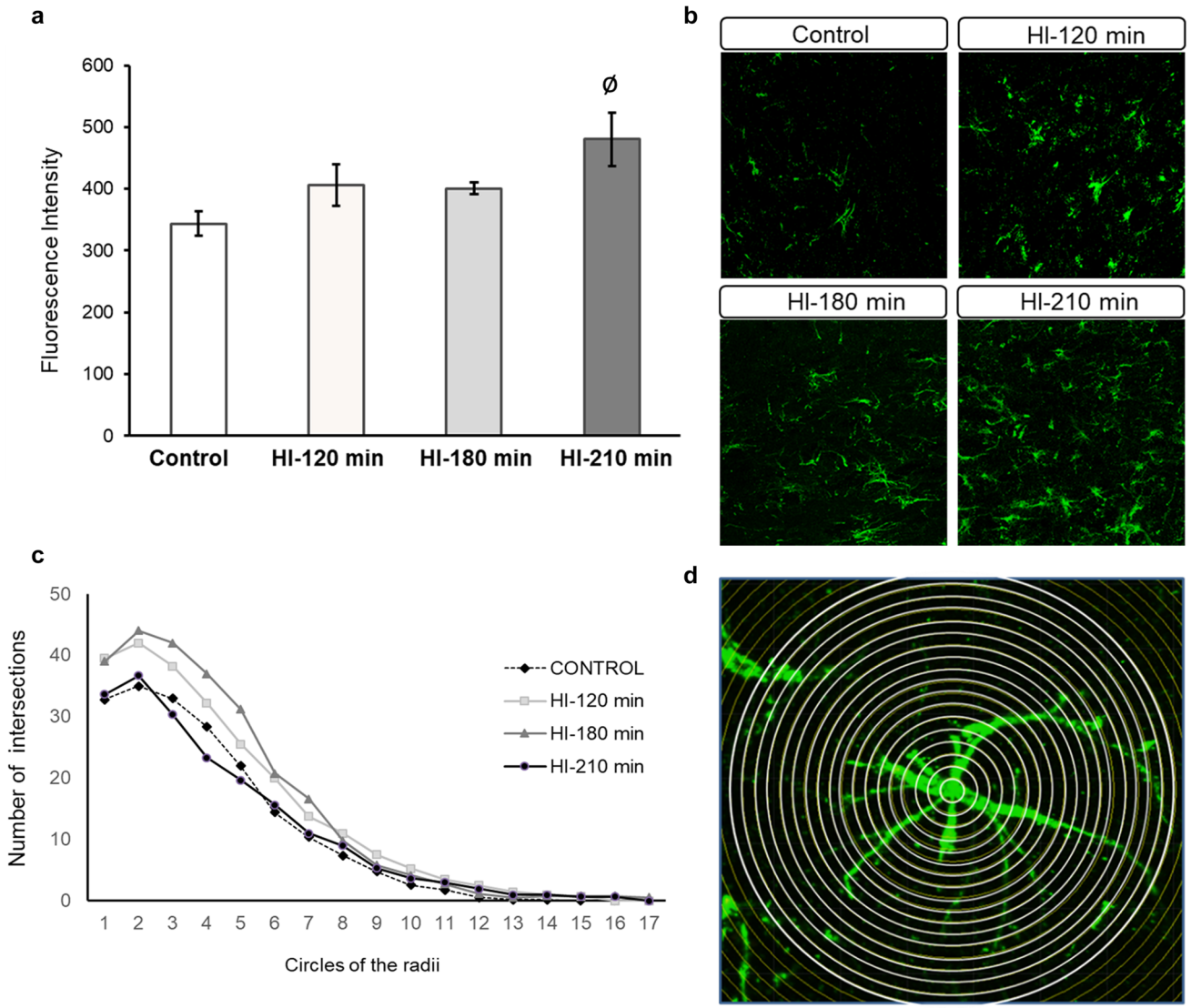

Fig. 3 Sholl's analysis: **a** analysis of fluorescence intensity; **b** Representative images of the immunofluorescence in the corpus callosum, near to the inter-hemispheric line **c** number of astrocyte intersections at circles of increasing radii from the center and **d** Representative image of Sholl's concentric circle method. (n = 5–6 per group) Data analyzed by one-way ANOVA. ^{Ø}Difference between HI-210 min versus control group. Significance was accepted whenever $p < 0.05$

Histological Analysis

As shown in the Fig. 4, all HI groups showed a significant reduction in the volume of the injured cerebral hemisphere, cortex, corpus callosum and hippocampus compared to CG ($F_{(3,25)} = 6.761$, $p = 0.002$; $F_{(3,25)} = 8.074$, $p = 0.001$; $F_{(3,25)} = 5.605$, $p = 0.005$; $F_{(3,25)} = 5.861$, $p = 0.004$; $F_{(3,25)} = 3.616$, $p = 0.028$ respectively). A progressive increase in percentage of tissue loss was observed in the right hemisphere (ipsilateral to the carotid occlusion) of HI animals, as compared controls, as follows: right hemisphere (HI-120 min: 40.3%, HI-180 min: 57.1% and HI-210 min: 61.6% decrease); right cerebral cortex (HI-120 min: 51.9%, HI-180 min: 64.7% and HI-210 min: 71.9% decrease); right corpus callosum (HI-120 min: 55.5%, HI-180 min: 81.3% and HI-210 min: 88.5% decrease) and right hippocampus (HI-120 min: 44.2%, HI-180 min: 57.8% and HI-210 min: 65.9% decrease).

Animals undergoing HI-180 and HI-210 min also showed a significant decrease in the contralateral corpus callosum ($F_{(3,25)} = 3.186$, $p = 0.044$) compared to CG. Furthermore, HI-210 min rats showed a decrease in the contralateral hippocampus ($F_{(3,25)} = 2.999$, $p = 0.05$), evidencing that prolonged hypoxia exposure cause both ipsilateral and contralateral tissue loss. It is also highlighted the vulnerability of specific brain regions to neonatal HI insult. Table 2 shows the ratio between ipsilateral and contralateral hemispheres, confirming the tissue loss in the hemispheres,

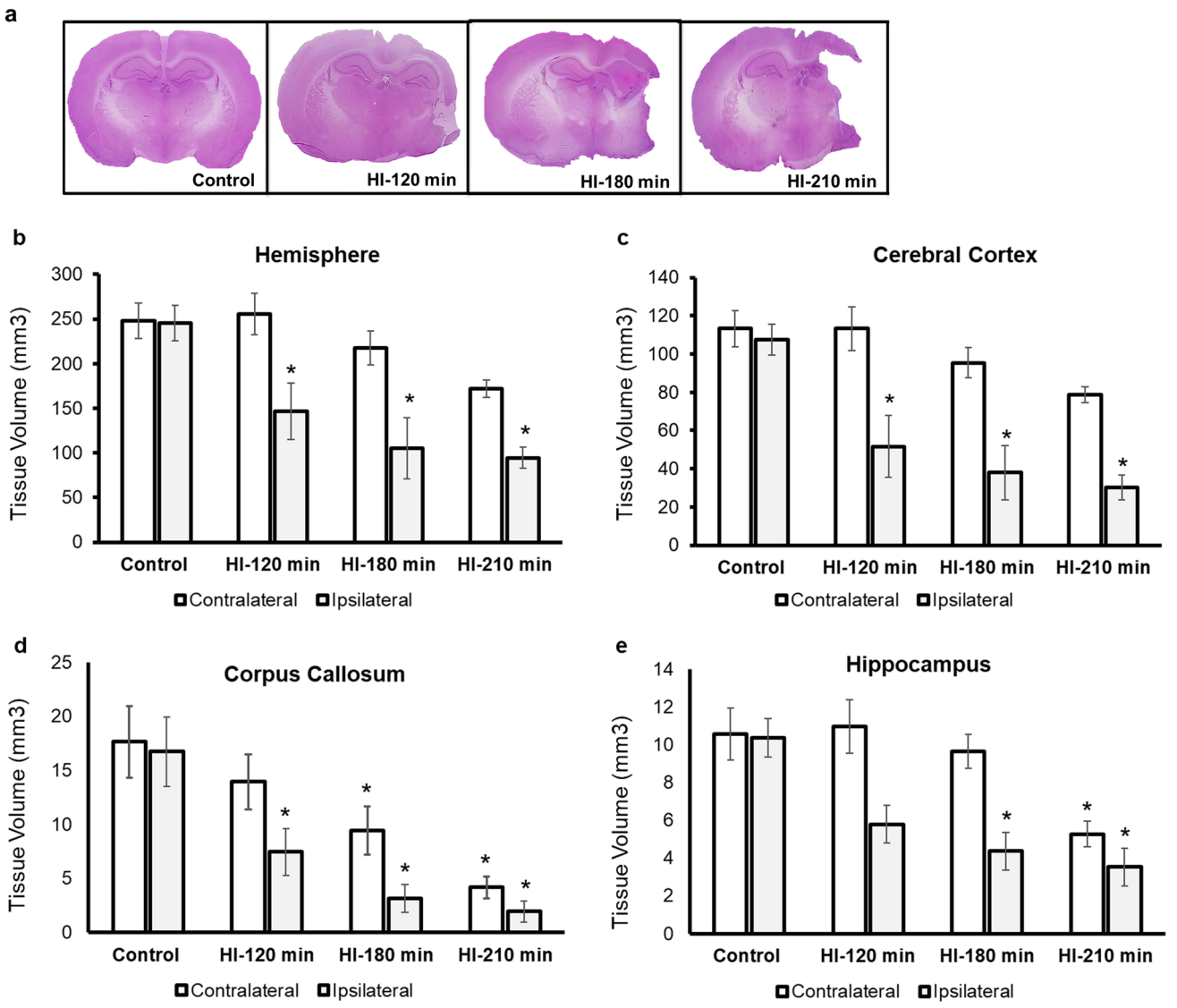

Fig. 4 Histological analysis: **a** Representative images of brain damage severity. Tissue volume of the ipsilateral and the contralateral side to carotid occlusion on: **b** Brain hemisphere, **c** Cerebral cortex, **d** Corpus Callosum and **e** Hippocampus (n = 5–6 per group). Data were analyzed by one-way ANOVA. *Difference of HI versus control groups. Significance was accepted whenever $p < 0.05$

Table 2 Hemispheric and structural volume ratio

	Hemisphere	Cerebral cortex	Corpus callosum	Hippocampus
Control	0.99 ± (0.00)	0.96 ± (0.02)	1.00 ± (0.01)**	0.99 ± (0.03)
HI-120 min	0.57 ± (0.11)*	0.47 ± (0.14)*	0.63 ± (0.10)*	0.55 ± (0.18)*
HI-180 min	0.55 ± (0.14)*	0.44 ± (0.16)*	0.44 ± (0.15)*	0.51 ± (0.16)*
HI-210 min	0.53 ± (0.06)*	0.39 ± (0.07)*	0.50 ± (0.07)*	0.67 ± (0.09)

Values are expressed as mean volume ± SEM (n = 5–6 per group)*HI vs. control $p < 0.05$

cerebral cortex, corpus callosum, and hippocampus for all HI groups compared to CG ($F_{(3,25)} = 6.283$, $p = 0.003$; $F_{(3,25)} = 6.379$, $p = 0.003$; $F_{(3,25)} = 4.964$, $p = 0.009$ and $F_{(3,25)} = 3.844$, $p = 0.022$, respectively). Thereby, animals of HI-120 min showed moderate ipsilateral tissue damage, HI-180 min oscillate among moderate and severe damage and HI-210 min exhibited severe damage (see Fig. 4a).

Multiple regression analysis was run to associate neurobehavioral and Morris water maze performance with histological parameters (percentage of tissue loss, ipsilateral and contralateral volume) of cerebral cortex, corpus callosum and hippocampus, assessed in adulthood. It was observed that the latency to perform the negative geotaxis task at PND 7 correlated with the ipsilateral volume of cortex and hippocampus plus contralateral volume of hippocampus and corpus callosum (adjusted $R^2 = 0.759$, $p = 0.001$; Fig. 5a, b). There was also a correlation of the latency to find the platform on the last day of training in the water maze with the percentage of cortical tissue loss and the contralateral volume of the hippocampus (adjusted $R^2 = 0.770$, $p = 0.001$; Fig. 5c, d). These results confirm that functional alterations following HI are related with the greater of the tissue loss in more than one brain structure,

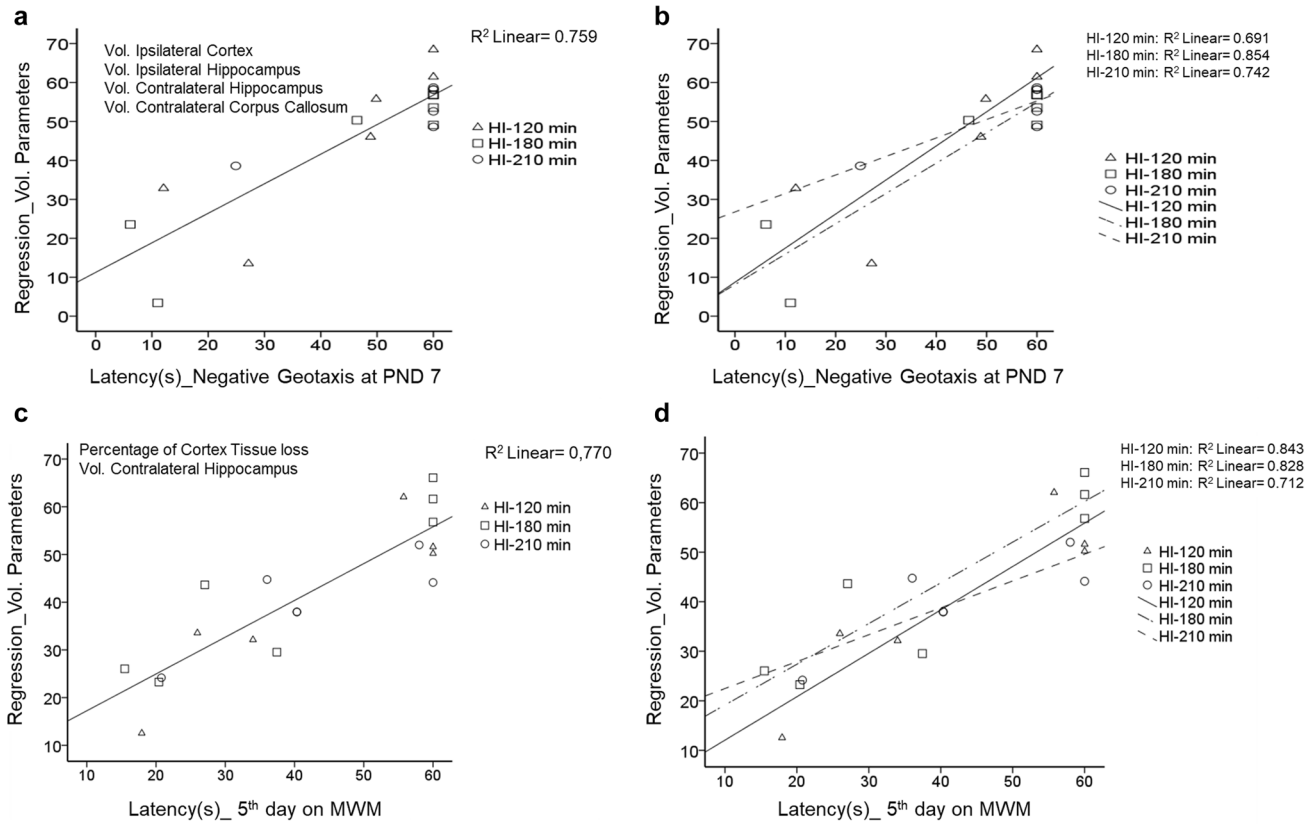

Fig. 5 Hierarchical multiple regression between volume ratio of brain structures and behavioral parameter: **a** correlation between latency during negative geotaxis at PND 7 and histological parameters (volume of the cortex and the hippocampus as well as the contralateral volume of the corpus callosum and hippocampus), **b** correlation expressed by subgroups; **c** correlation between escape latency on the fifth day on Morris Water Maze test and histological parameters (percentage of cortical tissue loss and the contralateral volume of the hippocampus); **d** correlation expressed by subgroups. Significance was accepted whenever $p < 0.05$

either ipsi- and contralateral to the lesion, evidencing a complex regional vulnerability after HI at PND3.

Discussion

Present study aimed to evaluate the influence of different periods of hypoxia on morphological and functional effects in very immature rat brain, undergoing hypoxia–ischemia at PND 3. As previously described, the severity of tissue damage induced by HI is associated with the duration of hypoxia [23, 24]. The novelty of the present study is that HI injury performed in PND 3 rats using hypoxia periods longer than 180 min increased astrocyte reactivity at adulthood and caused contralateral, along with ipsilateral damage in brain regions more susceptible to HI. Interestingly, prolonging the duration of hypoxia exposure did not affect spatial memory deficits, however histological changes in the cerebral cortex, corpus callosum and hippocampus were able to predict animal's performance in negative geotaxis and in the Morris water maze task.

Asphyxiated preterm infants often have significant body weight decrease and reduced weight gain over time when compared to healthy children [1]. This characteristic is also observed in HI animals [41]. Experimental studies show that even when an adequate nutritional supply is guaranteed, pups exposed to chronic hypoxia presented lower weight compared to controls, suggesting that the weight loss is secondary to the hypoxia itself [42, 43]. This is consistent with data here presented, in which animals exposed to longer periods of hypoxia (HI-180 and HI-210 min, as in Fig. 1) have decreased body weight as compared to controls. The weight loss following HI has been associated with worse performance in neurodevelopmental reflexes testing, without a significant impact in animal's mortality [31].

Motor coordination tests, such as righting reflex and negative geotaxis, are useful tools for early assessment of sensorimotor deficits, considering that very immature rats exposed to longer hypoxia periods (180 min) display sensorial and motor impairments at adulthood, as previously reported [23]. In agreement, at later stages of neurodevelopment, animals did not exhibit significant differences in reflex responses as compared to control groups. This was expected, since functional recovery has been described as animals become older, increasing their voluntary movements often without obvious gross motor deficits [31].

Reflexes maturation is considered an index of nervous system development that can be influenced by excitotoxicity, perinatal stress and systemic hypoxia [44]. Moderate and severe damage following HI, as observed in HI-180 min and HI-210 min, caused a delay in neurodevelopmental reflexes compared to the control group (Fig. 1). These results are similar to the ones reported in animals submitted to HI at PND 7 [31, 45]. Huang and co-workers observed delayed righting reflex 72 h in HI animals exposed to 150 min of hypoxia when compared to control group. However, the oxygen concentration used was 6%; that could help us to explain the differences observed in this behavioral task [46]. Allied to injury severity due to hypoxia exposure, animals' age contributes to the injury degree. Literature shows that very immature rats have increased anaerobic tolerance [9]. This entails in distinct patterns of HI injury at different stages of development [9, 24, 47], showing that shorter periods of hypoxia are enough to cause functional deficits in older animals [45, 48].

Cognitive deficits are observed in hypoxic-ischemic subjects at any neurodevelopmental period [22, 49]. Presented data shows that, although HI animals could acquire a spatial task, they improve their escape latency by no more than 50% in the Morris water maze test; interestingly the increase of hypoxia exposure did not result in greater memory impairment (Fig. 2). Towfighi et al. stated that hippocampus pyramidal cells are more resistant than cortical neurons in animals exposed to HI at PND 2–3 during 30–90 min using 5% FIO_2 [24]. Previous reports using HI at PND 3 report that short hypoxia exposure periods (90 min) caused significant spatial memory deficits when compared to control group, despite of mild histological tissue damage [20, 50]. Similarly, Nunn and colleagues (1994) using the 4-vessel occlusion model in adult rats reported that a small hippocampal loss (from 10 to 30%) causes impairment in the Morris water maze test [51]. Thus, spatial memory deficits can be induced both by mild and severe injuries. This could occur due to the higher vulnerability of hippocampal neurons during active neural proliferation at the postnatal period [42, 52, 53]. The fact that the long-lasting spatial learning and memory deficit was independent on the duration of hypoxia suggests that, in moderate/severe hippocampal cell loss, differences in brain remodeling are very subtle and may stabilize in adulthood, resulting in a cognitive impairment limit.

The progressive tissue loss consequent to the increasing hypoxia exposure suggest an exacerbation of the cascade of events responsible for tissue and cellular damage following HI insult, such as excitotoxicity, neuroinflammation and oxidative stress, which are well described in the literature [54–57], causing a progressive increase in the overall cerebral lesion. Furthermore, prolonged periods of hypoxia could interfere in neuroplastic responses to HI, both in the remaining tissue and in the contralateral brain structures to the carotid occlusion [58, 59]. In the experimental model of HI the combination of permanent carotid occlusion with the exposure to a hypoxic environment is important to mimic neonatal HI effects, since focal ischemia or hypoxia alone do not cause brain damage at early ages [24].

Animals exposed to HI-210 min did not show greater hippocampal damage when the ratio between injured (right)

and non-injured (left) hemispheres was assessed, indicating that longer hypoxia periods cause histological alterations also in non-ischemic structures of the contralateral hemisphere, which could interfere in further compensatory brain mechanisms and in the functional recovery [58]. It is well-known that neonatal HI causes a significant reduction in the ipsilateral hemisphere to carotid occlusion as well as in brain structures such as cerebral cortex, corpus callosum and hippocampus [42, 50], allowing the observation of focal effects. However, the exposure to 210 min of hypoxia showed a high prevalence of global hypoxia damage, overriding the focal ischemia injury. The reduction observed in brain structures of the contralateral hemisphere in animals exposed to the longer hypoxia period should be considered in future research, since assessments that require asymmetry evaluation between contralateral and ipsilateral hemispheres would not be useful. This may be the case for motor tests, such as the cylinder test, and for histological evaluation using ipsi/contralateral ratios.

Astrocytes are major glial cells acting in the interhemispheric remodeling during neurodevelopmental period and play a major role in modulating neurons and oligodendrocyte function, synapses formation, plasticity and sculpting the developing neuronal circuits [60, 61]. Moreover, astrocytes are important mediators of pathogenesis after brain injuries such as HI [62]. Reactive astrocytes can reduce or exacerbate the damage, depending on the injury severity [63], and have been associated with a diffuse glial reaction following white matter injury in preterm newborns [62]. Previous reports showed that astrocyte morphology is related to pathophysiological responses to injury and to recovery patterns following ischemic or hemorrhagic stroke [40, 64, 65]. In the present study, immunofluorescence analysis revealed that only the HI-210 group presented an increase in reactive astrogliosis. Nevertheless, Sholl's analysis did not evidence differences in astrocyte complexity in the corpus callosum, a well described affected structure following HI at PND3. In agreement, a previous study reported that animals submitted to HI at PND 7 did not exhibit astrocyte morphological changes, even in the presence of reactive astrogliosis [26]. Segovia and colleagues (2008) reported similar results using a model of chronic perinatal injury, suggesting that despite white matter areas appear to be more vulnerable to damage, this susceptibility was not associated with changes of astrocytes morphology [66]. These results indicate that astrocytes show greater resistance to increased periods of hypoxia following perinatal HI and that astrocyte reactivity is not followed by structural long-term changes as observed after brain insults in adult animals [40, 67]. Hence, differences between neonatal and adult models may be dependent on the number of astrocytes in the corpus callosum (greater in older animals) and on their stage of neurodevelopment at time of injury [27].

Summarizing, longitudinal assessment of PND3 rats undergoing neonatal hypoxia–ischemia indicates that astrocytic response and progressive brain tissue damage are determined by the duration of hypoxia. Regional susceptibility of the immature rat brain to HI can involve both hemispheres; with the longer hypoxia duration the contralateral hemisphere is also affected. Furthermore, the association of sensorimotor and spatial memory performance with brain structures beyond the hippocampus suggests that complex anatomical changes should be involved in functional alterations taking place as hypoxia duration is increased, even when the cognitive impairment limit is achieved. Therefore, a better understanding of the HI model and its variables is needed in order to predict tissue injury and the functional deficits, which are important outcomes for developing and evaluating experimental therapeutic strategies.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest.

References

1. Blencowe H, Cousens S, Chou D et al (2013) Born too soon: the global epidemiology of 15 million preterm births. Reprod Health 10:1–14. https://doi.org/10.1186/1742-4755-10-S1-S2
2. Liu L, Johnson HL, Cousens S et al (2012) Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet 379:2151–2161. https://doi.org/10.1016/S0140-6736(12)60560-1
3. Volpe JJ (2009) Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. Lancet Neurol 8:110–124. https://doi.org/10.1016/S1474-4422(08)70294-1
4. Xiong F, Zhang L (2013) Role of the hypothalamic-pituitary-adrenal axis in developmental programming of health and disease. Front Neuroendocrinol 34:27–46
5. Clowry GJ, Basuodan R, Chan F (2014) What are the best animal models for testing early intervention in Cerebral Palsy? Front Neurol 5:1–17. https://doi.org/10.3389/fneur.2014.00258
6. Jantzie LL, Corbett CJ, Berglass J et al (2014) Complex pattern of interaction between in utero hypoxia-ischemia and intra-amniotic inflammation disrupts brain development and motor function. J Neuroinflammation 11:131. https://doi.org/10.1186/1742-2094-11-131
7. Teune MJ, Bakhuizen S, Bannerman CG et al (2011) A systematic review of severe morbidity in infants born late preterm. Am J Obstet Gynecol 205:374.e1–374.e9. https://doi.org/10.1016/j.ajog.2011.07.015
8. Zhu LH, Bai X, Zhang N et al (2014) Improvement of human umbilical cord mesenchymal stem cell transplantation on glial

cell and behavioral function in a neonatal model of periventricular white matter damage. Brain Res 1563:13–21. https://doi.org/10.1016/j.brainres.2014.03.030
9. Stadlin A, James A, Fiscus R et al (2003) Development of a postnatal 3-day-old rat model of mild hypoxic-ischemic brain injury. Brain Res 993:101–110. https://doi.org/10.1016/j.brainres.2003.08.058
10. Sizonenko SV, Sirimanne E, Mayall Y et al (2003) Selective cortical alteration after hypoxic-ischemic injury in the very immature rat brain. Pediatr Res 54:263–269. https://doi.org/10.1203/01.PDR.0000072517.01207.87
11. Tang G, Gudsnuk K, Kuo SH et al (2014) Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. Neuron 83:1131–1143. https://doi.org/10.1016/j.neuron.2014.07.040
12. Rice JE, Vannucci RC, Brierley JB (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 9(2):131–141
13. Patel SD, Pierce L, Ciardiello A et al (2015) Therapeutic hypothermia and hypoxia-ischemia in the term-equivalent neonatal rat: characterization of a translational preclinical model. Pediatr Res 78:264–271. https://doi.org/10.1038/pr.2015.100
14. Patel SD, Pierce L, Ciardiello AJ, Vannucci SJ (2014) Neonatal encephalopathy: pre-clinical studies in neuroprotection. Biochem Soc Trans 42:564–568. https://doi.org/10.1042/BST20130247
15. Arteni NS, Salgueiro J, Torres I et al (2003) Neonatal cerebral hypoxia-ischemia causes lateralized memory impairments in the adult rat. Brain Res 973:171–178. https://doi.org/10.1016/S0006-8993(03)02436-3
16. Pereira LO, Nabinger PM, Strapasson ACP et al (2009) Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. Brain Res 1247:188–195. https://doi.org/10.1016/j.brainres.2008.10.017
17. Wang LS, Zhou J, Shao XM, Tang XC (2002) Huperzine A attenuates cognitive deficits and brain injury in neonatal rats after hypoxia-ischemia. Brain Res 949:162–170. https://doi.org/10.1016/S0006-8993(02)02977-3
18. Semple BD, Blomgren K, Gimlin K et al (2013) Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol 106–107:1–16. https://doi.org/10.1016/j.pneurobio.2013.04.001
19. Alexander M, Garbus H, Smith AL et al (2014) Behavioral and histological outcomes following neonatal HI injury in a preterm (P3) and term (P7) rodent model. Behav Brain Res 259:85–96. https://doi.org/10.1016/j.bbr.2013.10.038
20. Sanches EF, Arteni N, Nicola F et al (2015) Sexual dimorphism and brain lateralization impact behavioral and histological outcomes following hypoxia–ischemia in P3 and P7 rats. Neuroscience 290:581–593. https://doi.org/10.1016/j.neuroscience.2014.12.074
21. McCullough N, Parkes J, Kerr C, McDowell BC (2011) The health of children and young people with cerebral palsy: a longitudinal, population-based study. Int J Nurs Stud 50:747–756. https://doi.org/10.1016/j.ijnurstu.2011.01.011
22. Volpe JJ (2009) The encephalopathy of prematurity-brain injury and impaired brain development inextricably intertwined. Semin Pediatr Neurol 16:167–178. https://doi.org/10.1016/j.spen.2009.09.005
23. Durán-Carabali L, Sanches E, Marques M et al (2017) Longer hypoxia–ischemia periods to neonatal rats causes motor impairments and muscular changes. Neuroscience 340:291–298. https://doi.org/10.1016/j.neuroscience.2016.10.068
24. Towfighi J, Mauger D, Vannucci RC, Vannucci SJ (1997) Influence of age on the cerebral lesions in an immature rat model of cerebral hypoxia-ischemia: a light microscopic study. Brain Res Dev Brain Res 100:149–160
25. Burda JE, Sofroniew MV (2014) Reactive gliosis and the multicellular response to CNS damage and disease. Neuron 81:229–248. https://doi.org/10.1016/j.neuron.2013.12.034
26. Odorcyk FK, Nicola F, Duran-Carabali LE et al (2017) Galantamine administration reduces reactive astrogliosis and upregulates the anti-oxidant enzyme catalase in rats submitted to neonatal hypoxia ischemia. Int J Dev Neurosci 62:15–24. https://doi.org/10.1016/j.ijdevneu.2017.07.006
27. Ling E-A, Leblond CP (1973) Investigation of glial cells in semithin sections. II. Variation with age in the numbers of the various glial cell types in rat cortex and corpus callosum. J Comp Neurol 149:73–81. https://doi.org/10.1002/cne.901490105
28. Epelman M, Daneman A, Halliday W et al (2012) Abnormal corpus callosum in neonates after hypoxic-ischemic injury. Pediatr Radiol 42:321–330. https://doi.org/10.1007/s00247-011-2238-5
29. Sanches EF, Durán-Carabali LE, Tosta A et al (2017) Pregnancy swimming causes short- and long-term neuroprotection against hypoxia-ischemia in very immature rats. Pediatr Res 82:544–553. https://doi.org/10.1038/pr.2017.110
30. Moses P, Courchesne E, Stiles J et al (2000) Regional size reduction in the human corpus callosum following pre- and perinatal brain injury. Cereb Cortex 10:1200–1210. https://doi.org/10.1093/cercor/10.12.1200
31. Lubics A, Reglodi D, Tamás A et al (2005) Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. Behav Brain Res 157:157–165. https://doi.org/10.1016/j.bbr.2004.06.019
32. Cárdenas L, García-García F, Santiago-Roque I et al (2015) Enriched environment restricted to gestation accelerates the development of sensory and motor circuits in the rat pup. Int J Dev Neurosci 41:68–73. https://doi.org/10.1016/j.ijdevneu.2014.11.008
33. Fox WM (1965) Reflex-ontogeny and behavioural development of the mouse. Anim Behav 13:234–241. https://doi.org/10.1016/0003-3472(65)90041-2
34. Sanches EF, Arteni NS, Spindler C et al (2012) Effects of pre- and postnatal protein malnutrition in hypoxic-ischemic rats. Brain Res 1438:85–92. https://doi.org/10.1016/j.brainres.2011.12.024
35. Favero AM, Weis SN, Zeni G et al (2006) Diphenyl diselenide changes behavior in female pups. Neurotoxicol Teratol 28:607–616. https://doi.org/10.1016/j.ntt.2006.08.003
36. Arteni NS, Pereira LO, Rodrigues AL et al (2010) Lateralized and sex-dependent behavioral and morphological effects of unilateral neonatal cerebral hypoxia-ischemia in the rat. Behav Brain Res 210:92–98. https://doi.org/10.1016/j.bbr.2010.02.015
37. Paxinos G, Watson C (1998) The rat brain—in stereotaxic coordinates, Fourth
38. Furukawa S, Sameshima H, Yang L, Ikenoue T (2011) Acetylcholine receptor agonist reduces brain damage induced by hypoxia-ischemia in newborn rats. Reprod Sci 18:172–179. https://doi.org/10.1177/1933719110385129
39. Ota A, Ikeda T, Ikenoue T, Toshimori K (1997) Sequence of neuronal responses assessed by immunohistochemistry in the newborn rat brain after hypoxia-ischemia. Am J Obstet Gynecol 177:519–526. https://doi.org/10.1016/S0002-9378(97)70139-X
40. Mestriner RG, Saur L, Bagatini PB et al (2015) Astrocyte morphology after ischemic and hemorrhagic experimental stroke has no influence on the different recovery patterns. Behav Brain Res 278:257–261. https://doi.org/10.1016/j.bbr.2014.10.005
41. Tai W, Burke KA, Dominguez JF et al (2009) Growth deficits in a postnatal day 3 rat model of hypoxic-ischemic brain injury. Behav Brain Res 202:40–49. https://doi.org/10.1016/j.bbr.2009.03.043
42. Mayoral SR, Omar G, Penn AA (2009) Sex differences in a hypoxia model of preterm brain damage. Pediatr Res 66:248–253. https://doi.org/10.1203/PDR.0b013e3181b1bc34

43. Ment LR, Schwartz M, Makuch RW, Stewart WB (1998) Association of chronic sublethal hypoxia with ventriculomegaly in the developing rat brain. Dev Brain Res. https://doi.org/10.1016/S0165-3806(98)00139-4
44. Horvath G, Reglődi Dora, Farkas J et al (2015) Perinatal positive and negative influences on the early neurobehavioral reflex and motor development. In: Antonelli Marta C (ed) Perinatal programming of neurodevelopment. Springer, New York, pp 149–167
45. Ashwal S, Ghosh N, Turenius CI et al (2014) The reparative effects of neural stem cells in neonatal hypoxic-schemic injury are not influenced by host gender. Pediatr Res 75:603–611. https://doi.org/10.1038/pr.2014.7.The
46. Huang Z, Song L, Wang C et al (2012) Hypoxia-ischemia upregulates TRAIL and TRAIL receptors in the immature rat brain. Dev Neurosci 33:519–530. https://doi.org/10.1159/000334475
47. Rice JE, Vannucci RC, Brierley JB (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 9:131–141. https://doi.org/10.1002/ana.410090206
48. Schuch CP, Diaz R, Deckmann I et al (2016) Early environmental enrichment affects neurobehavioral development and prevents brain damage in rats submitted to neonatal hypoxia-ischemia. Neurosci Lett 617:101–107. https://doi.org/10.1016/j.neulet.2016.02.015
49. Söderström I, Strand M, Ingridsson AC et al (2009) 17B-Estradiol and enriched environment accelerate cognitive recovery after focal brain ischemia. Eur J Neurosci 29:1215–1224. https://doi.org/10.1111/j.1460-9568.2009.06662.x
50. Sanches E, Arteni N, Scherer E et al (2013) Are the consequences of neonatal hypoxia-ischemia dependent on animals' sex and brain lateralization? Brain Res 1507:105–114. https://doi.org/10.1016/j.brainres.2013.02.040
51. Nunn JA, LePeillet E, Netto CA et al (1994) Global ischaemia: hippocampal pathology and spatial deficits in the water maze. Behav Brain Res 62:41–54. https://doi.org/10.1016/0166-4328(94)90036-1
52. Bothe HW, Bosma HJ, Hofer H et al (1986) Selective vulnerability of hippocampus and disturbances of memory storage after mild unilateral ischemia of gerbil brain. Stroke 17:1160–1163. https://doi.org/10.1161/01.STR.17.6.1160
53. Zanelli AS, Rajasekaran KK, Grosenbaugh KD, Kapur J (2015) Increased excitability and excitatory synaptic transmission during in vitro ischemia in the neonatal mouse hippocampus. Neuroscience 3:279–289. https://doi.org/10.1016/j.neuroscience.2015.09.046
54. McLean C, Ferriero D (2004) Mechanisms of hypoxic-ischemic injury in the term infant. Semin Perinatol 28:425–432. https://doi.org/10.1053/j.semperi.2004.10.005
55. Zhu C, Wang X, Xu F et al (2005) The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. Cell Death Differ 12:162–176. https://doi.org/10.1038/sj.cdd.4401545
56. Ferriero DM (2001) Oxidant mechanisms in neonatal hypoxia-ischemia. Dev Neurosci 23:198–202. https://doi.org/10.1159/000046143
57. Volpe JJ (2001) Neurobiology of periventricular leukomalacia in the premature infant. Pediatr Res 50:553–562. https://doi.org/10.1203/00006450-200111000-00003
58. Rocha-Ferreira E, Hristova M, Rocha-Ferreira E, Hristova M (2016) Plasticity in the neonatal brain following hypoxic-ischaemic injury. Neural Plast 2016:1–16. https://doi.org/10.1155/2016/4901014
59. Johnston MV (2009) Plasticity in the developing brain: implications for rehabilitation. Dev Disabil Res Rev 15:94–101. https://doi.org/10.1002/ddrr.64
60. Freeman MR (2010) Specification and morphogenesis of astrocytes. Science 330:774–778. https://doi.org/10.1126/science.1190928
61. Gobius I, Morcom L, Suárez R et al (2016) Astroglial-mediated remodeling of the interhemispheric midline is required for the formation of the corpus callosum Ilan. Cell Rep 17:735–747. https://doi.org/10.1016/j.celrep.2016.09.033.Astroglial-mediated
62. Mallard C, Davidson JO, Tan S et al (2013) Astrocytes and microglia in acute cerebral injury underlying cerebral palsy associated with preterm birth. Pediatr Res 75:1–7. https://doi.org/10.1038/pr.2013.188
63. Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 32:638–647. https://doi.org/10.1016/j.tins.2009.08.002
64. Mestriner RG, Pagnussat S, Boisserand LSB et al (2011) Skilled reaching training promotes astroglial changes and facilitated sensorimotor recovery after collagenase-induced intracerebral hemorrhage. Exp Neurol 227:53–61. https://doi.org/10.1016/j.expneurol.2010.09.009
65. Po C, Kalthoff D, Kim YB et al (2012) White matter reorganization and functional response after focal cerebral ischemia in the rat. PLoS ONE. https://doi.org/10.1371/journal.pone.0045629
66. Segovia KN, Mcclure M, Moravec M et al (2008) Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. Ann Neurol 63:520–530. https://doi.org/10.1002/ana.21359.ARRESTED
67. Theodosis D, Poulain D, Oliet S (2008) Activity-dependent structural and functional plasticity of astrocyte-neuron interactions. Physiol Rev. https://doi.org/10.1152/physrev.00036.2007